BIOCATALYTIC ACYLATION OF CARBOHYDRATES TO PRODUCE FERULOYLATED OLIGOSACCHARIDES AND CARBOHYDRATE FATTY ACID ESTERS AS POTENTIAL FUNCTIONAL INGREDIENTS: NOVEL APPROACHES FOR EFFICIENT ENZYMATIC SYNTHESES BY CARBOXYLIC ESTER HYDROLASES

by

Juan Tamayo-Cabezas

Department of Food Science and Agricultural Chemistry

Macdonald Campus, McGill University

Quebec, Canada

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BIOCATALYTIC ACYLATION OF CARBOHYDRATES TO PRODUCE FERULOYLATED OLIGOSACCHARIDES AND CARBOHYDRATE FATTY ACID ESTERS

ABSTRACT

Acylation of mono- and oligosaccharides catalyzed by carboxylic ester hydrolases in non-conventional media was explored to synthesize compounds with potential bioactive properties. Multienzymatic Depol 740L from *Humicola insolens* and a pure feruloyl esterase (FAE) preparation from the same source were evaluated for their specificity and activity using synthetic and isolated feruloylated saccharides from wheat bran and sugar beet, with the FAE preparation showing higher activity and broader specificity among the substrates tested. It was then selected to evaluate the feruloylation of xylobiose, raffinose and xylo-oligosaccharides in 6 different surfactantless microemulsions as reaction media. The microemulsion composed by n-hexane/2-butanone/water (51:46:3, v/v/v) showed consistently higher bioconversions with all the acyl acceptors evaluated. MS analysis revealed simple and multiple feruloylation of the glycoside substrates. Heterofunctional supports bearing metal-chelate and epoxy groups achieved highest FAE immobilization yield and highest activity per gram of support. More specifically, FAE immobilized onto a porous metal-chelate-epoxy support modified retained 35% of the feruloylation capacity of the free FAE when xylobiose was present as the acyl acceptor.

Optimization of the FAE immobilization onto mesoporous metal-chelate-epoxy supports by response surface methodology (RSM) was performed, where optimal immobilization time (27.7 h), pH of the immobilization buffer (5.0) and interaction between the ratio of enzyme to immobilization support (22.75 mg/g) with the immobilization buffer molarity (0.86 mol/L) were determined. Enzymatic activity yield and retention of specific activity yield were increased by 33% and 47%, respectively, when compared to unoptimized conditions. Enzyme thermostability of the immobilized FAE was enhanced following a post-immobilization incubation at pH 10.0, while an increase of support pore size improved hydrolytic activity and esterifying efficiency of the immobilized biocatalyst. It was determined that optimally immobilized and stabilized FAE retained up to 92.9% of the feruloylation activity of the free enzyme.

Carbohydrate fatty acid ester synthesis reactions were performed in a mixture of lauric acid/*tert*-butanol (90/10; v/v) at 55 °C using Novozym® 435 as the biocatalyst, where bioconversion yield and productivity of 19.7% and 9.45 μmol/L min, respectively, were obtained with a ball-milled fructose concentration of 0.2 mol/L. An increase in reaction media fatty acid content adversely affected esterification. The enhancement of polar substrate solubility and an increase in acylation

reaction productivity were significant benefits of the combined low-solvent and mechanical milling of reaction substrates for lipase-catalyzed esterification approach.

The immobilized lipase-catalyzed esterification of myristic acid with fructose and fructooligosaccharides in low solvent reaction media (10%, v/v) was also studied. The acyl acceptor chain length was determined to be inversely proportional to the reaction yield. A 1:1 ratio of oleic to lauric/myristic acid in the reaction decreased the viscosity and the melting point of the media but also decreased the esterification yield by half. Maximum fructose myristate yield (22%) was obtained using 0.2 mol/L of fructose and 5% (w/v) of Lipozyme® RM IM in a reaction media composed of 10% tert-butanol, 10% oleic acid, and 80 % myristic acid, using RSM based on a 5-level and 3-factor central composite design. Without addition of oleic acid, maximum yield (25.5%) was obtained with 0.2 mol/L of fructose and 6% (w/v) of enzyme. At these latter conditions, 6 consecutive reactions were catalyzed before a reduction in synthetic activity was observed. ESI-MS analysis confirmed the production of mono- and diesters, and the selectivity of this enzyme for saturated fatty acids over unsaturated fatty acids.

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RÉSUMÉ

L'acylation de mono- et oligosaccharides catalysée par des hydrolases d'esters carboxyliques dans des milieux non conventionnels a été explorée, et ce dans le but de synthétiser des composés ayant de potentielles propriétés bioactives complémentaires. Deux préparations de FAE (EC 3.1.1.73) de *Humicola insolens* ont été évaluées pour leur spécificité vis-à-vis le substrat et leur activité enzymatique, en utilisant des saccharides feruloylés soit synthétiques soit isolés à partir de son de blé et de betterave sucrière, l'un d'entre eux présentant une activité plus élevée et une spécificité plus large parmi les substrats testés. Cet enzyme a ensuite été sélectionné pour évaluer la feruloylation du xylobiose, du raffinose et de xylo-oligosaccharides (XOS) dans six systèmes de microémulsions sans tensioactif, comme milieux réactionnels. La bioconversion la plus élevée (57%) avec l'enzyme libre a été obtenue dans le cas du xylobiose dans la microémulsion composée de n-hexane / 1,4-dioxane / eau (51: 46: 3, v / v / v), ce système générant des résultats supérieurs pour tous les acyl-accepteurs évalués. L'estérification de FAE a été confirmée par spectrométrie de masse révélant une feruloylation simple et multiple du substrat glycosidique. Les supports mésoporeux hétérofonctionnels portant des groupements métal-chélaté et époxy ont donné à la fois le rendement d'immobilisation FAE le plus élevé et l'activité FAE la plus élevée, et ce par gramme de support. Plus spécifiquement, le FAE immobilisé sur support époxy mésoporeux modifié avec acide iminodiacétique et sulfate cuivrique a conservé 35% de la capacité de féruloylation du FAE libre lorsque le xylobiose était présent comme accepteur d'acyle.

L'optimisation de l'immobilisation de FAE sur des supports époxydes mésoporeux métal-chélaté a été réalisée via la méthodologie RSM, permettant de déterminer le temps d'immobilisation optimal (27,7 h), le pH du tampon d'immobilisation (5.0) et interaction entre l'enzyme et le support d'immobilisation (22,75 mg) / g) ainsi que la molarité du tampon d'immobilisation (0,86 mol / L). Le rendement d'activité enzymatique et la rétention du rendement d'activité spécifique ont été augmentés de 33% et 47% respectivement par rapport aux conditions non optimisées. La thermo stabilité enzymatique du FAE immobilisé a été augmentée après incubation post-immobilisation à pH 10,0, tandis qu'une augmentation de la taille des pores de support a amélioré l'activité hydrolytique et l'efficacité d'estérification du biocatalyseur immobilisé. Il a été déterminé que l'époxy-FAE macroporeux immobilisé et stabilisé de manière optimale conservait jusqu'à 92,9% de l'activité de féruloylation de l'enzyme libre.

Des réactions de synthèse d'esters acides gras / saccharides ont été effectuées dans un mélange acide laurique / tert-butanol (90/10; v / v) à 55 ° C en utilisant Novozym® 435 comme biocatalyseur, avec un rendement de bioconversion et une productivité de 19,7% et 9,45 µmol. / L min, respectivement, avec une concentration en fructose (broyé par boulets) de 0,2 mol / L. Une augmentation de la teneur en acides gras du milieu réactionnel a eu un effet négatif sur l'estérification. L'amélioration de la solubilité du substrat polaire et une augmentation de la productivité de la réaction d'acylation se sont avérés des avantages significatifs de la combinaison broyage mécanique et faible teneur en solvant des substrats réactionnels, pour l'approche d'estérification catalysée par une lipase.

L'estérification de l'acide myristique catalysée par une lipase immobilisée avec substrats fructose et fructo-oligosaccharides dans des milieux réactionnels à faible teneur en solvant (10%, v / v) a également été étudiée. On a déterminé que la longueur de la chaîne d'accepteur d'acyle était inversement proportionnelle au rendement de la réaction. Un rapport 1: 1 entre acide oléique et acide laurique / myristique dans la réaction diminuait la viscosité et le point de fusion des milieux, mais diminuait également de moitié le rendement d'estérification. Le rendement maximal en myristate de fructose (22%) a été obtenu en utilisant 0,2 mol / L de fructose et 5% (p / v) de Lipozyme® RM IM dans un milieu réactionnel composé de 10% tert-butanol, 10% acide oléique et 80% acide myristique, en utilisant la méthodologie RSM basée sur un modèle composite central à 5 niveaux et 3 facteurs. Sans addition d'acide oléique, le rendement maximal (25,5%) a été obtenu avec 0,2 mol / L de fructose et 6% (p / v) d'enzyme. Sous ces dernières conditions, 6 réactions consécutives ont été catalysées avant qu'une réduction de l'activité de synthèse ne soit observée. L'analyse ESI-MS a confirmé la production de mono- et diesters et la sélectivité de cet enzyme pour les acides gras saturés par rapport aux acides gras insaturés.

STATEMENT FROM THE THESIS OFFICE

According to the regulation of the Faculty of Graduate Studies and Research of McGill University, Guidelines for Thesis Preparation include:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

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As manuscripts for publication are frequently very concise documents, where appropriate additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reports in the thesis.

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When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

CONTRIBUTION OF AUTHORS

This thesis consists of the eight following chapters:

Chapter I provides a short introduction and describes the research rational as well as the main and specific objectives of the study.

Chapter II presents a literature review divided in two parts. The first part of the literature review includes a description of the feruloylated oligosaccharides structures and their availabilities in nature, their production routes, and their bioactive and functional properties. Followed by a briefing on the enzyme of choice and its mechanism of action. Then, a summary of immobilization strategies and applications. To finish with a more detailed report on the enzymatic and hydrolase-catalyzed feruloylation. The second part includes a brief explanation of carbohydrate fatty acid ester definition and structures. It also contains a detailed summary of the health promoting properties of this type of compounds along with their food-industry potential applications followed by a succinct presentation of the biocatalyst. To end up with a detailed report on the ester synthetic routes.

Chapter III to V are presented in the form of manuscripts and have been or will be submitted for publication. The connecting statements provide the rationale linking the different parts of this study. Chapter III describes the immobilization of the hydrolase and its use on the ferulic acid acylation of oligosaccharides in surfactantless microemulsions. Chapter IV studies the optimization of the immobilization procedure and stabilization of the covalent attachment between hydrolase and support for its use in the feruloylation of oligosaccharides in surfactantless microemulsions. Chapter V reports a one-parameter-at-a-time optimization of the hydrolase-catalyzed approach for the fatty acid acylation of fructose in a low solvent reaction media. Finally, Chapter VI focuses on the multiple parameter optimization of the low solvent biosynthetic approach and its effect on the reusability of the biocatalyst.

Chapter VII provides a general conclusion to the thesis with a summary of major findings.

Chapter VIII outlines the contribution of this study to the field and provides recommendations regarding future research on the hydrolase-catalyzed acylation of potential prebiotic carbohydrates to complementary bioactive moieties in non-conventional reaction media.

Juan Tamayo, the author, was responsible for the experimental work and the preparation of the first draft of the manuscripts for publication and dissertation.

Dr. Salwa Karboune, the PhD student's supervisor, guided and supervised all research and critically revised the manuscripts prior to their submission.

Dr. Juan Pablo Carrillo-Montes, the second author of manuscripts 3 and 4 (Chapters V and VI), contributed to the research and experimental work related to the hydrolase-catalyzed fatty acid acylation of fructose in low solvent media.

PUBLICATIONS

1. **Tamayo-Cabezas, J**., & Karboune, S. (2018). Immobilized feruloyl esterase from Humicola insolens catalyzes the synthesis of feruloylated oligosaccharides. Process Biochemistry (forthcoming) doi: 10.1016/J.PROCBIO.2018.12.013.
2. **Tamayo-Cabezas, J.**, & Karboune, S. (2018). Optimum conditions for the immobilization and stabilization of feruloyl esterase from *Humicola insolens* on a heterofunctional metal chelate-epoxy support and its application for the feruloylation of oligosaccharides (*To be submitted*).
3. **Tamayo-Cabezas, J.**, Carrillo-Montes, J.P., & Karboune, S. (2018). Direct esterification of fructose with medium and long fatty acids catalyzed by immobilized lipases in a low-solvent reaction media. (Submitted).
4. **Tamayo-Cabezas, J.**, Carrillo-Montes, J.P., & Karboune, S. (2018). Lipase-catalyzed synthesis of myristic acid esters in a low solvent reaction media: effect of acyl acceptor, optimization of the bioconversion and reusability of the biocatalyst (*To be submitted*).

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LIST OF ABBREVIATIONS

2M2B	2-methylbutan-2-ol
AAPH	2,2'-azobis-(2-methylpropionami-dine) dihydrochloride
APCI-MS	Atmospheric-pressure chemical ionization mass spectrometry
AXOS	Arabinoxylooligosaccharides
BSA	Bovine serum albumin
CALB	*Candida Antarctica* lipase B
CCRD	Central composite rotatable design
CFAE	Carbohydrate fatty acid ester
CLEA	Cross-linked enzyme aggregate
CMC	Critical micelle concentration
CTAB	Cetltrimethylammonium bromide
Cu	Copper symbol
DE	Degree of esterification
DMF	Dimethyl formamide
DMP	Dimethylpyrolidone
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DPPH	2,2-diphenyl-1- picrylhydrazyl
EC	Enzyme commission
EDA	Ethylene diamine
EMK	Ethyl methylketone
ELSD	Evaporative light scattering detector
ESI MS/MS	Electrospray ionization mass spectrometry
FAE	Feruloyl esterase
FDA	Unites States Food & Drug Administration
FO	Feruloylated oligosaccharide
FOFAE	Feruloyl esterase from *Fusarium oxysporum*
FOS	Fructooligosaccharides
H NMR	Proton nuclear magnetic resonance

HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
h	Hour
IDA	Iminodiacetic acid
JECFA	The Joint Expert Committee on Food Additives
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein
MCA	Multipoint covalent attachment
MES	3-N-morpholino-ethanesulfonic acid
MOPS	4-Morpholinepropanesulfonic acid sodium salt
NaOH	Sodium hydroxide
NDO	Non-digestible oligosaccharides
PEG	Polyethylene glycol
PTFE	Polytetrafluoroethylene
RP-HPLC-UV	Reverse phase high performance liquid chromatography/ultraviolet
rpm	Revolutions per minute
RSM	Response surface methodology
s	Seconds
StFAEC	*Sporotrichum thermophile* type C feruloyl esterase
TLC	Thin-layer chromatography
TFA	Trifluoroacetic acid
XOS	Xylo-oligosaccharides

CHAPTER I. GENERAL INTRODUCTION

Acylation of saccharides with bioactive moieties has attracted attention in recent times as potential routes to produce value-added products from low cost renewable sources. The coupling of diverse acyl groups with additional functionalities to mono- and oligosaccharides has found wide application in the pharmaceutical, food and cosmetic industries. The abundance, low cost and natural origin of the substrates heightens the potential of saccharide acylation. However, the acylation of sugars is a challenge due to the different attachment possibilities of the acyl donor to the saccharide unit, with each potential coupling leading to the synthesis of a specific compound with unique physicochemical properties (Brask, 2009).

Sugar acylation can be achieved either through chemical or enzymatic pathways. Since the glycosidic bond is very labile under acidic conditions, traditional chemical synthesis is constrained to alkaline media (Drummond et al., 2003). Additionally, intramolecular acyl migration requires the application of intermediate protective sequences and energy-demanding conditions to achieve selective chemical acylation (Chauvin et al., 1993). On the contrary, enzymatic acylation is a simpler process that uses milder reaction conditions. As enzymes are highly selective, the tailored enzymatic acylation of saccharides is of high interest (Cao et al., 1996; Van Kempen et al., 2013). However, crucial aspects of enzymatic acylation of saccharides still need to be studied, such as selection of appropriate reaction media, effect of reaction substrate chain length, reusability of the biocatalyst, and optimization of the process conditions.

Carboxylic ester hydrolases encompass an extensive group of enzymes ubiquitous in all domains of cellular life with wide substrate acceptance and activity in non-aqueous environments. These unique features favor their use in industrial and biomass refining applications (Levisson et al., 2009). *In vivo* they catalyze the hydrolysis of ester bonds, producing an alcohol and a carboxylic acid. However, in near anhydrous media they catalyze reverse esterification and trans-esterification reactions. Generally, the latter reaction is preferred, since the use of activated esters shifts the reaction equilibrium towards the synthesis of the acylated compounds. Two of the most well-known carboxyl ester hydrolases subclasses are lipases and esterases.

Among the acylated saccharides, feruloylated oligosaccharides are water-soluble and thermally stable hydroxycinnamic derivates. These compounds show potential in nutraceutical applications due to the physiological functions of their substrates, as well as their functional advantages due to the amphiphilicity of the molecule. Additionally, they have shown inhibition against glycation and

immunomodulatory activity over inflammation agents (Fang et al., 2012; Wang et al., 2009). Currently, commercially available products containing feruloylated oligosaccharides are obtained through the enzymatic hydrolysis of the water insoluble fraction of arabinoxylan from wheat bran, resulting in a product with low bound ferulic acid (EAS Consulting Group, 2010). Enzymatic acylation in non-conventional media arises as an alternative pathway for the feruloylation of oligosaccharides, which may improve the low yield and inconsistent structures obtained by enzymatic hydrolysis of plant cell wall material. Although lipases and proteases have been used to catalyze the transesterification of vinyl esters of ferulic acid to saccharides, feruloyl esterases (FAE) (EC 3.1.1.73) have emerged as a potential biocatalyst option for the feruloylation of mono- and oligosaccharides as they show greater substrate affinity and regioselectivity. However, FAE are less resistant to the denaturing effects of organic solvents compared to lipases (Zeuner et al., 2012). Therefore, surfactantless microemulsions with a low water content have been successfully developed as reaction media for acylation reactions (Couto et al., 2011, 2010). FAE-catalyzed feruloylation shows dependency on the acyl acceptor and the composition of the reaction media (Couto et al., 2010).

Modulating the composition of the reaction media and selecting an appropriate acyl acceptor will improve the yield and the selectivity of the feruloylation reaction and enhance the stability of the biocatalyst. Immobilization is a commonly used approach for enzyme stabilization. Indeed, immobilization into polymeric supports produces immobilized biocatalysts with physicochemical, mechanical and kinetic properties defined by the choice of carrier. Confinement of the enzyme in a support favors its recovery and reutilization, and provides functional advantages over the use of free enzymes, such as lack of aggregation, higher productivity and improved resistance to mechanical damage and thermal inactivation (Brena and Batista-Viera, 2006). Enzymes are immobilized into resin supports either by physical adsorption or covalent attachment of reactive groups on the enzyme surface with functional groups on the carrier surface. In addition, a pre-immobilization step that involves the introduction of a chemical moiety able to rapidly adsorb the targeted enzyme in a specific orientation into the support may improve the stability of the immobilized enzyme. To date, limited studies have investigated the immobilization of FAE and none have employed the immobilized FAE to catalyze the direct esterification of oligosaccharides (Matsuo et al., 2008; Thörn et al., 2011). The absence of an efficient and robust immobilization method for FAE as a biocatalyst for acylation reactions in non-conventional media hinders

escalation of the enzymatic feruloylation process to a preparative level. Immobilization of FAE into monofunctional and heterofunctional supports at the micro- and nano scale will be applied to evaluate its effectiveness as a stabilization strategy.

Carbohydrate fatty acid esters (CFAE) being amphiphilic surfactants are another group of acylated saccharides of interest as functional ingredients. Indeed, CFAE have shown both techno-functional properties and antimicrobial activity against five bacteria commonly present in the food industry (Zhao et al., 2015). In addition, their surface activity make them excellent emulsifiers and wetting agents with applications in agrochemistry, bioremediation, cosmetic production, and the food industry (Hill, 2010). Sorbitan esters, sucrose esters and alkyl polyglycosides are the main categories of commercially available CFAE produced by via chemical pathway (Neta et al., 2015). However, purification challenges and the multi-ester species obtained through chemical synthesis has incited research into considering selective enzymatic synthesis as a feasible alternate CFAE synthesis. Lipases are the biocatalyst of choice due to their stability in non-conventional media and activation in the presence of a water-oil interface (Khaled et al., 1991; Oguntimein et al., 1993; Scheckermann et al., 1995). Low-water or anhydrous conditions required to shift the enzymatic reaction equilibrium towards synthesis are not suitable for the dissolution of polar substrates, while hydrophilic organic solvents such as dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and pyridine cause enzymatic deactivation. Therefore, low solvent reaction media formed by an excess of the non-polar substrate and a cosolvent at a concentration unable to solvate the polar substrate may overcome these issues (Cao et al., 1999; Šabeder et al., 2006). Additionally, reduction of the particle size of the saccharide substrates may increase their surface area-to-mass ratio, improving wettability and increasing their dissolution rate.

Lipase-catalyzed direct esterification of mono- and polysaccharides with medium and long chain fatty acids in a low solvent media will be studied. Assessing the effect of the reaction media, the nature of the cosolvent, the acyl donor chain length, and the particle size of the substrates on the reaction yield is essential to achieving a better understanding of the low solvent media approach. Reduced presence of organic solvent in the reaction media has been shown to increase the number of cycles of reutilization of the enzyme (Ye and Hayes, 2012). However, to keep the reaction at a liquid state when using saturated fatty acids of length C_{14} or longer, enzymatic acylation of saccharides must take place at temperatures higher than the optimum range for most lipases. The addition of an unsaturated fatty acid with a lower melting point may avoid mass transfer limitations

by reducing the melting point of the reaction media. However, if mixtures of saturated and unsaturated fatty acids are used it is important to select a chemoselective lipase with a preference towards saturated acyl donors to avoid unwanted side reactions. Combinations of fatty acids in different proportions will be evaluated for their effect on the yield and productivity of the esterification reaction. The optimum combination will be chosen to assess its impact on the reusability of the selected enzyme.

The main objective of this research work was the development of novel biosynthetic approaches to produce acylated mono- and oligosaccharides catalyzed by immobilized carboxyl ester hydrolases. It is divided into the following specific objectives:

1) Development of a biosynthetic approach to produce feruloylated oligosaccharides catalyzed by FAE from *Humicola insolens* in surfactantless microemulsions.
2) Identification of the most adequate support for the immobilization of FAE from *H. insolens* and optimization of the immobilization of this FAE into a heterofunctional support.
3) Assessment of the effect of the immobilization support pore size and the promotion of multipoint immobilization on the feruloylation ability of the FAE.
4) Development of an enzymatic approach to produce fatty acid esters of fructose in a low solvent reaction media (>10%, v/v) catalyzed by immobilized lipases.
5) Evaluation of the effects of reaction system, acyl donor, cosolvent selection, substrate particle size, and biocatalyst type on the yield and the productivity of the lipase-catalyzed fatty acid acylation of fructose.
6) Investigation of the lipase-catalyzed acylation of fructose and fructooligosaccharides (FOS) with myristic acid in low solvent reaction media, and the effect of the acyl acceptor in the reaction yield and productivity.
7) Optimization of the biosynthesis of myristic acid esters of fructose in a low solvent reaction media using RSM and characterization of the esterified reaction products.

CHAPTER II. LITERATURE REVIEW

2.1. Feruloylated glycosides

Feruloylated glycosides are a subgroup of phenolated carbohydrates formed by the association via an ester-linkage between ferulic acid and glycosides (mono, oligo and polysaccharides). The presence of polysaccharide-bound hydroxycinammic acids (coumaric, sinapic and ferulic acid) in the cell walls of different plant species has long been known. Ferulic acid is distributed throughout the primary and secondary cell wall in plants, and is particularly abundant in the epidermis, bundle sheaths and sclerenchyma (Faulds and Williamson, 1999). Ferulic acid constitutes about 0.66% (w/w, dry weight) in wheat, 1.24% in wheat straw (Benoit et al., 2006), 3.1% in maize bran (Saulnier et al., 1995), 0.9% in rice endosperm cell wall (Shibuya, 1984), 0.14% in barley grains (Nordkvist et al., 1984), 0.32% in barley spent grain (Bartolome et al., 1997), 2.2% to 3.8% in oat hulls (Yu et al., 2002), 1.4% in barley hulls (Tenkanen et al., 1991), and 0.87% in sugar beet pulp (Kroon and Williamson, 1996).

Feruloylated glycosides play important defence and structural roles in the growing and mature of the plant cell wall (Faulds and Williamson, 1999). They impart structural rigidity and strengthen cell wall architecture by cross-linking pentosan chains, arabinoxylans, and hemicelluloses, rendering these components less available to hydrolytic enzymes during germination (Graf, 1992), and are involved in the regulation of the cell wall extensibility and cessation of elongation processes (Hossain et al., 2007). In addition, feruloylated glycosides can connect cell wall polymers, especially polysaccharides to each other but also to lignin and to proteins (Ralph et al., 1995). It has been determined that cross-linking of plant cell wall polymers contributes to the defence mechanisms against pathogens. However, the process and extent of cell wall feruloylation remain uncertain (Buanafina, 2009). Feruloylated glycosides also possess a range of functional properties: they are involved in dough and baked good properties (Hartmann et al., 2005), formation of gels (Robertson et al., 2008), crispness and softening of fruits and vegetables (Waldron et al., 2003), post-harvest changes of asparagus accompanied by sensory toughness (Rodriguez-Arcos et al., 2004), and physicochemical properties of dietary fibers (Berlanga-Reyes et al., 2014; Snelders et al., 2013). For example, Ou et al. (2001) chemically-synthesized corn starch ferulate and determined its food and biological properties. It showed lower viscosity, higher water-holding capacity, and much less retrogradation during low temperature storage than native starch, opening another pathway to deliver health components, such as ferulic acid, safely to the colon. The pasting properties using rapid viscosity analysis of commercial corn starch and starches

from sorghum cultivars esterified with ferulic acid were studied, to investigate the model-system interactions between ferulic acid and starch. The significance of these interactions is important, especially in food matrices where phenolics are added as functional ingredients (Beta and Corke, 2004).

2.1.1. Structure of naturally-occurring feruloylated glycosides

The linkage of ferulic acid to wall polysaccharides has been studied by determining the structure of water-soluble fractions released from plant cell walls. In flowering plants with one seed inside the seed soat (monocots), ferulic acid is attached to cell-wall polymers by ester bonds through its carboxylic acid group with the C-5-hydroxyl of α-L-arabinosyl side chains of xylans (Hartley and Ford, 1989). Ferulic acid in dicots (flowering plants with two seeds inside the seed soat) is associated with pectic polysaccharides via ester linkages to the C-2 hydroxyl group of arabinofuranose, to the position C-4 of α-D-xylopyranosyl residues in xyloglucans (Ishii and Hiroi, 1990), or the C-6 hydroxyl group of galactopyranose residues. Such linkages have been found in spinach (Fry, 1982) and sugar beet (Colquhoun et al., 1994). It has been estimated that 45% to 50% of ferulic acid is linked to O-6 of galactose residues and 50% to 55% to O-2 arabinose residues in sugar beet pulp (Ralet et al., 1994).

The four most frequent feruloyl ester linkages in plant polysaccharides are: 5'-feruloyl α-1,3-L-arabinofuranosyl-D-xylopyranose occurring in plant arabinoxylans; 2'-feruloyl α-1,5-L-arabinofuranosyl-L-arabinose occurring in arabinans and pectic compounds; 6'-feruloyl-β-D-galactopyranose in β-galactans; and 4'-feruloyl-α-1,6-D-xylopyranosyl-D-glucopyranose occurring in xyloglucans (Figure 2.1) (Biely and Côté, 2005). Arabinose has been administered into spinach cultured cells and traced its incorporation into units of the major wall polysaccharides. The research showed that arabinosylation and feruloylation occurred co-synthetically and intracellularly (Fry and Miller, 1989). Yet, there is not a complete scientific agreement either on the site of feruloylation of plant cell wall polysaccharides or the origin of the feruloyl donor. Evidence suggests that feruloylation in gramineous is an esterification reaction with elevated structural specificity. Hydroxycinnamic groups are attached to cell wall polysaccharides in a precise manner suggesting that a specific mechanism is involved (Buanafina, 2009).

The formation of covalent cross-linkages between wall polysaccharides involving ferulic acid derivatives was first demonstrated in 1971 by Geissmann and Neukom (Geissmann and Neukom, 1971). They demonstrated that the feruloyl residues on arabinoxylan oxidatively coupled to form diferulates, and it was subsequently suggested that this reaction is a possible polysaccharide cross-linking mechanism in growing cells (Fry, 1986). Coupling of the diferulate at position 5-5 of the aromatic ring was identified in Italian ryegrass (Hartley and Jones, 1976) and rice endosperm (Shibuya, 1984). The existence of the diferuloyl diester cross-link was later identified, when Ishii (1991) isolated and characterized a diferuloyl arabinoxylan polyshaccharide from an enzymatic hydrolyzate of bamboo shoot arabinoxylan. Corn and sugar beet pulp contain 2.5% and 0.17% diferulates, respectively (Saulnier et al., 1999). The linking of diferulates to pectic arabinans and galactans was demonstrated by a liquid chromatography-mass spectrometry (LC-MS) study (Ralet et al., 2005). It has been shown that wall-bound ferulic acid can form higher order oligomers such as trimers and tetramers (Bunzel et al., 2005). In addition, ferulic acid also radically couple to monolignols of the growing lignin polymer. This results in a highly cross-linked matrix in the plant cell wall involving both carbohydrates and lignin (Grabber et al., 2000).

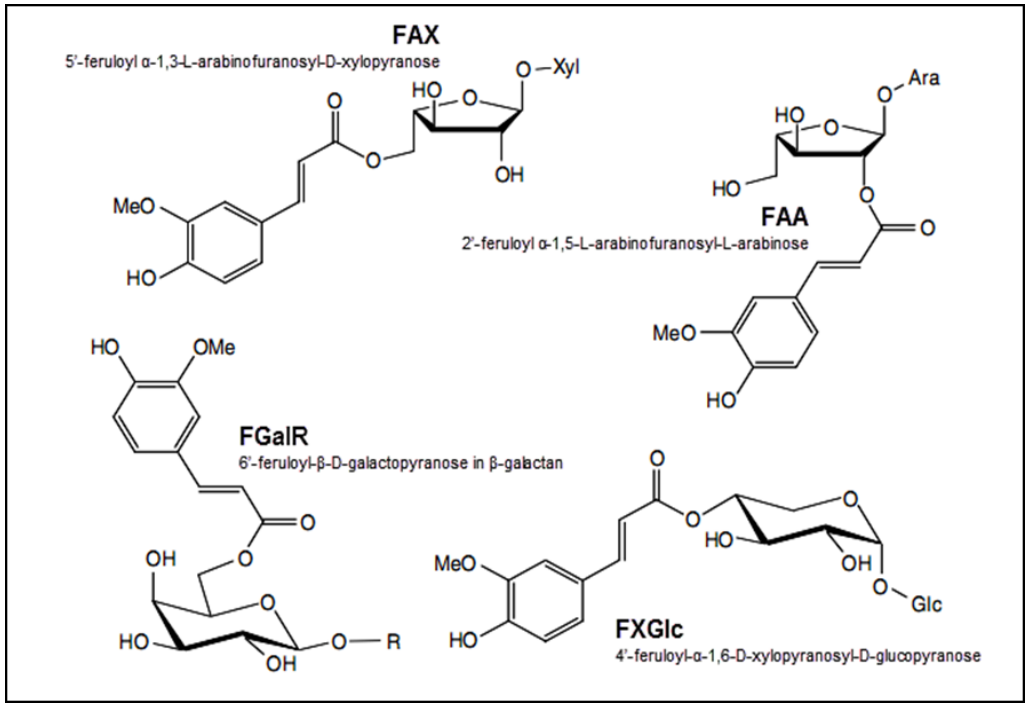

Figure 2.1 Structures of four most frequent feruloyl ester linkages in plant polysaccharides (Biely and Côté, 2005).

2.1.2. Health-promoting properties of feruloylated oligosaccharides

It has been reported that feruloylated oligosaccharides stimulate the growth of beneficial bacteria in the hindgut giving prebiotic characteristics to the molecule (Hutkins et al., 2014; Ou et al., 2016). Moreover, these molecules display antioxidant activities due to the phenolic acid moiety esterified to the carbohydrate chain (Snelders et al., 2013). The glycosides 5-O-feruloyl-L-arabinofuranose, feruloyl-arabinoxylan, and O-(5-O-feruloyl-α-L-arabino-furanosyl)-(1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose were reported to possess similar or stronger antioxidant activities than ferulic acid in the microsomal lipid peroxidation system and in the low-density lipoprotein (LDL) oxidation system (Ohta et al., 1997, 1994). A feruloylated oligosaccharide from wheat flour showed a high antioxidant activity in the 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging assay and inhibited the copper-mediated oxidation of human LDL (Katapodis et al., 2003). Hopkins et al. (2003) investigated the degradation of cross-linked and non-cross-linked arabinoxylans by the intestinal microbiota in children and adults; they found that ferulic acid cross-linking reduced the rate of arabinoxylan fermentation.

Feruloylated oligosaccharides released from wheat bran were found to promote the *in vitro* growth of *Bifidobacterium bifidum* F-35 and display antioxidant activity as measured by the inhibition of oxidative erythrocyte hemolysis mediated by peroxyl radicals generated from 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) (Yuan et al., 2005a, 2005b). Indeed, the feruloylated oligosaccharides inhibited hemolysis of erythrocytes in a dose-dependent manner with 91.7% inhibition of erythrocytes hemolysis at 4 mg/mL. The main mechanism of action of phenolic antioxidants could be the scavenging of peroxyl radicals, thereby protecting the polyunsaturated fatty acid-rich membranes of erythrocytes, which are very susceptible to free radical- mediated peroxidation (Yuan et al., 2005b).

The protective effect of feruloyl oligosaccharides from wheat bran against oxidative DNA damage in normal human peripheral blood lymphocytes induced by hydrogen peroxide was investigated by Wang et al. (2008). DNA damage in human lymphocytes induced by 100 μmol/L H_2O_2 was inhibited by feruloylated oligosaccharides in a concentration dependent manner, reaching a 91.1% inhibition at a concentration of 500 μmol/L of feruloylated oligosaccharides compared to the control trial. These results suggest that feruloylated oligosaccharides may enhance the ability of human lymphocytes to resist H_2O_2-induced oxidative damage (Wang et al., 2008). Kylli et al,

(2008) have used the DPPH radical scavenging assay as well as emulsion and liposome model systems to assess the antioxidant activity of feruloyl glycosides. The esterification in the primary hydroxyl group of the glycoside showed improved radical scavenging activity of feruloyl and sinapoyl glycosides compared to conjugation to the secondary hydroxyl group. This study showed that DPPH radical scavenging activity of free ferulic acid (46%) was lower than feruloyl glucoside esters (86%).

2.1.3. Production of feruloylated oligosaccharides

2.1.3.1. Enzymatic hydrolysis

The most common method of isolating feruloylated oligosaccharides is by enzymatic hydrolysis. Many studies have investigated the isolation of feruloyl oligosaccharides by treatment with a mixture of polysaccharide-hydrolyzing enzymes without esterase activities (Ishii, 1997). A commercial preparation, Driselase from *Basidiomycetes* sp., contains various glycosyl hydrolases and glycosidases, but lacks ferulic acid esterase activity (Micard et al., 1994). Driselase can be used to release most of the feruloyl groups from plant cell walls as small, soluble feruloylated oligosaccharides. While sugar-beet pulp was readily degraded (80%) using enzymatic hydrolysis with Driselase, only feruloylated oligosaccharides – and not monosaccharides – were obtained. Approximately 6%, 10%, and 13% of the ferulic acid initially present in the sugar-beet pulp was recovered in the galactose disaccharides, arabinose disaccharides, and feruloylated arabinose trisaccharides, respectively (Ralet et al., 1994).

The feruloylated oligosaccharides, 5-O-feruloyl-L-arabinofuranose and O-(5- O-feruloyl-α-L-arabinofuranosyl)-(1→3)-O-β-D-xylopyranosyl- (1→4)-D-xylopyranose were obtained from the enzymatic hydrolysate of refined corn bran insoluble dietary fibers (Ohta et al., 1994). Hydrolysis of wheat bran by endoxylanase from a thermophilic *Bacillus* sp. released seven feruloylated oligosaccharides (Lequart et al., 1999). The digestion of wheat aleurone walls with Driselase resulted in at least five feruloyl oligosaccharides (Rhodes et al., 2002). The reaction conditions for the optimum feruloylated oligosaccharide production from wheat bran insoluble dietary fiber by enzymatic hydrolysis with xylanases from *Bacillus subtilis* were obtained using RSM (Yuan et al., 2006). This study reported a yield of 1.55 mmol/L of feruloylated oligosaccharides from 1 g of wheat bran insoluble dietary fiber upon the hydrolysis by xylanases for 35 h. Katapodis and Christakopoulos (2008) reported and improvement on the isolation of feruloylated xylo-

oligosaccharides from corn cobs by combining enzymatic hydrolysis with a xylanase from *Thermoascus aurantiacus* with thermal treatment. Lately, direct fermentation of wheat bran with fungus has been evaluated as a one-step alternative for the isolation of feruloylated saccharides (Xie et al., 2014; Yu and Gu, 2014).

2.1.3.2. Acid hydrolysis

Feruloyl oligosaccharides can also be obtained by acid hydrolysis. Acid treatment breaks glycosidic bonds and solubilizes sugars while leaving ester bonds generally intact. The use of trifluoroacetic acid (TFA) hydrolysis on wheat bran (McCallum et al., 1991), sugar beet pulp (Ralet et al., 1994), corn hulls (Hosny and Rosazza, 1997), corn bran (Allerdings et al., 2006), and rice bran (Li et al., 2008) has been documented. Isolation of feruloylated saccharides from corn bran insoluble fiber reveals that the structures of feruloylated side-chains from heteroxylans are more complex than expected and may limit the enzymatic degradation of fibre (Allerdings et al., 2006). Li et al. (2008) have investigated the optimum conditions for preparing feruloylated oligosaccharides from rice bran using TFA. The optimum hydrolysis conditions for maximizing feruloylated oligosaccharides recovery were 193 mmol/L of TFA and 1.36 h of hydrolysis time. The amount of feruloylated oligosaccharides collected was quantified as 0.022 g per g of rice bran, and the acyl ferulic group of the partially purified feruloylated oligosaccharides was quantified as 916.14 µg per g of rice bran, equivalent to 54.08 % of the total concentration of acyl ferulic group present in the rice bran. Overall, acid hydrolysis treatments may lead to modifications of other components of the plant cell wall and/or to unwanted chemical changes.

2.1.3.3. Chemical synthesis

Synthesis reactions are also used as a means of producing feruloylated oligosaccharides. It has been determined that the esterification of glycosides with hydroxycinnamic acids at the primary position could be obtained using methyl glucopyranoside, methyl galactopyranoside and acetates of *p*-coumaroyl and feruloyl chlorides in pyridine as principal reactants (Helm et al., 1992). To shorten the prolonged protecting sequences usually associated with the chemical synthesis, another study has reported a chemoenzymatic approach using lipases or esterases for the regioselective acylation or deacylation of saccharide hydroxyl groups (Mastihubová et al., 2003). The same authors have reported the regioselective preparation of feruloylated p-nitrophenyl monosaccharides using a more efficient and eco-friendly catalyst such as zinc oxide instead of pyridine and a different chemoselective de-O-acetylation procedure by dibutyltin oxide. The

obtained yield of monoferuloylated monosaccharides was in the range of 65% to 85% (Mastihubová and Biely, 2010).

The chemical synthesis of 1-O-β-feruloyl and 1-O-β-sinapoyl glucopyranoses has also been documented (Zhu and Ralph, 2011). This chemical synthesis involved the stereoselective glycosylation of a chloroacetyl-protected sugar donor and 4-O-chloroacetylated hydroxycinnamic acids followed by removal of the chloroacetyl groups under mild conditions. Using this procedure, a yield of 78% of 1-O-β-feruloyl glucopyranose was obtained. Chemical synthesis gives high yields and several methods are available. However, the poor selectivity over reaction products and the necessity of lengthy protecting/deprotecting sequences limit its application.

2.1.3.4. Enzymatic synthesis

Enzymatic esterification of glycosides to ferulic acid and its derivatives in non-aqueous media have emerged as an alternative to the poor selectivity of chemical synthesis and the tedious isolation of naturally occurring feruloylated oligosaccharides. To date, most of the glycoside acylations published in the literature were largely oriented to the incorporation of hydrophobic alkyl chains into saccharides. Lipases are known to be active at low water activity conditions and are widely available in immobilized form. Indeed, lipases were initially the catalyst of choice for the enzymatic feruloylation of saccharides. Gao et al., (2000) investigated the enzymatic acylation of the flavonoid glycosides isoquercitin and naringine with vinyl ferulate, catalyzed by lipases (Novozyme® 435 and Lipozyme® RM IM) in a reaction media composed of *tert*-butanol and pyridine (9:1, v/v). Novozyme® 435 was also used for the investigation of the esterification of cinnamic and *p*-hydroxyphenylacetic acid with glucose, fructose and n-octyl glycoside in 100% *tert*-butanol (Stamatis et al., 2001). However, esterification was shown to take place only if the aromatic cycle is not *p*-hydroxylated and the lateral chain is saturated (Otto et al., 2000).

Feruloyl esterases (EC 3.1.1.73) may provide advantages over lipases, such as higher substrate affinity and regioselectivity in the synthesis of hydroxycinnamate saccharide esters. (E. Topakas et al., 2005) were the first to report the transesterification of methyl ferulate to L-arabinose catalyzed by a FAE from *Sporotrichum thermophile* (StFAEC) in the reaction media composed of n-hexane/*t*-butanol/water (47.2:50.8:2.0, v/v/v). StFAEC also catalyzed the feruloylation of arabinobiose in the microemulsion medium consisting of n-hexane/2-methyl-2-propanol/water (47.2:50.8:2.0, v/v/v) (Vafiadi et al., 2006), and the esterification of four linear arabino-

oligosaccharides, containing three to six arabinofuranose units in the reaction media composed by n-hexane, t*ert*-butanol and 50 mmol/L buffer piperazine-HCl pH 6.0 (53.4:43.4:3.2 v/v/v) (Vafiadi et al., 2007b). Commercial enzyme preparations from *H. insolens*, *Thermomyces lanuginosus* and *Aspergillus niger* showing FAE activity were studied for transesterifications of vinyl ferulate to various glycosides at their primary hydroxyl group in pure selected organic solvents (Mastihubová et al., 2006). Couto et al., (2010) tested the ability of commercial enzyme preparations containing FAE activity for the regioselective feruloylation of selected monosaccharides using two reaction mixtures: n-hexane/1-butanol/3-N-morpholino-ethanesulfonic acid (MES)-NaOH buffer, and n-hexane/2-butanone/MES-NaOH buffer, both at ratios of 51/46/3 v/v/v respectively. Couto et al., (2011) optimized the esterification of selected di- and oligosaccharides catalyzed by the FAEs present in enriched Depol 740L from *Humicola* sp., in the reaction media formed by n-hexane/2-butanone/MES-NaOH buffer (51/46/3, v/v/v). These authors also evaluated the application of ionic liquids as co-solvents of the reaction microemulsion replacing the ketone in the microemulsion or in a reaction medium constituted by ionic liquid and MES-NaOH buffer (97/3, v/v). The enzymatic acylation of saccharides opens the possibility for the development of tailored processes for the preparation of phenolated glycosides with potential bioactive functionalities

2.2. Feruloyl esterase

2.2.1. Feruloyl esterase-biocatalyst types

FAE are a subclass of the carboxylic ester hydrolases which liberate hydroxycinnamic acids and their dimers from naturally occurring hemicelluloses and pectins. FAE comprise a very diverse set of enzymes, with few sequence and physical characteristics in common. Since the first reports on the occurrence and purification of *Streptomyces olivochromogenes* FAE 25 years ago, these enzymes have been recognized as common components of hemicellulolytic enzyme systems of many microorganisms. To date, many FAE have been purified and characterized showing different variations in physical characteristics and a diverse specificity.

These enzymes play a key physiological role in the degradation of the structure of plant cell wall by hydrolyzing ferulate ester groups involved in the cross-linking between hemicelluloses and between hemicellulose and lignin (Fazary and Ju, 2008). These enzymes have the ability not only to deconstruct plant biomass but also to synthesize novel bioactive components for use in health and pharmaceutical industries. Specific FAE could be employed in the custom-made synthesis of

esters based on unsaturated arylaliphatic hydroxycinnamic acids such as ferulic acid and its derivatives, which are known to display antioxidant activity. The potential use of FAE for the synthesis of feruloylated compounds instigates the need for modified biopolymers with new properties and bioactivities (Topakas et al., 2007). They are also involved in colonic fermentation, where their extracellular and intracellular activities in the microbiota improve the breakdown of polysaccharides and increase microbial production of short chain fatty acids. Their specificity can also be employed to synthesize bioactive compounds for cosmetic and health applications (Faulds, 2010).

There has been considerable interest in a large number of potential applications of FAE due to their roles in various biotechnological processes and industries, and their potential applications in obtaining ferulic acid from agro-industrial waste materials obtained from milling, brewing, and sugar industries (Fazary and Ju, 2008). For instance, FAE have been used to release ferulic acid from agro industrial by-products such as wheat bran (Ralet et al., 1994) maize bran (Faulds et al., 1995), maize fiber (Shin et al., 2006), barley spent grain (Faulds et al., 2004), sugar beet pulp (Ferreira et al., 1999), coastal Bermuda grass (Borneman et al., 1990), oat hulls (Yu et al., 2002), jojoba meal (Laszlo et al., 2006), wheat straw, coffee pulp, and apple marc (Benoit et al., 2006). The involvement of FAE in the improved enzymatic and microbial saccharification of cereal-derived material demonstrates a high importance for these enzymes in animal feed preparation and bioalcohol production.

FAE might enhance the paper pulping process by removing substitutions and linkages between polymers during pulping, easing the solubilization of lignin–carbohydrate complexes, replacing chemicals and significantly reducing water pollution and the associated cleanup costs (Williamson et al., 1998). Other potential applications of FAE include biomass degradation for biofuel processing, improvement of bread quality, and juice clarification (Koseki et al., 2009; Tabka et al., 2006). However, regardless of these possible applications, according to Fazary and Ju (2008), there is a lack of response to the demand for commercially-produced purified FAE, with only one commercial preparation available at present time (Megazyme, 2017).

2.2.2. Mechanism of action

The FAE produced from *A. niger* is one of the most carefully studied hydrolases (Fazary and Ju, 2007) and its catalytic triad is comprised of three specific amino acids: serine, histidine and aspartic

acid (McAuley et al., 2004). The protein with the closest sequence to the type A FAE is a lipase from *R. miehei*, which has an overall sequence identity of 32% (Aliwan et al., 1999). The ping-pong bi-bi mechanism, defined as a non-sequential mechanism, is the most generally accepted mechanism for esterase-mediated esterification reactions. Catalysis commences with the first substrate, the phenolic acid, binding to the enzyme to form a binary complex. The first product, water, is then released to form an acyl-enzyme. Subsequently, glycoside, the second substrate, binds to the acyl-enzyme to yield the second product, the phenolated glycoside, leaving the enzyme intact in its original form (Flores and Halling, 2002). When both products are present, the basic mechanism leads to determining the kinetic parameters.

Microbial FAE have a broad range of pH and temperature dependence, with optimal activities occurring between pH values of 4.0 to 8.0 and a temperature range of 30 °C to 65 °C respectively. Hydrolytic activity of FAE is limited predominantly to the location of the feruloyl groups on the polymer, the conformation of feruloylated arabinoxylan, and the interactions between the feruloylated polysaccharides and other plant cell wall components (Topakas et al., 2007). It is interesting to note that most of the FAE examined to date as tools for the synthesis of "bioactive compounds" show a similar specificity for the substitutions around the phenolic ring for synthetic reactions as they display for hydrolytic reactions (Faulds, 2010).

2.2.3. FAE specificity

FAE exhibit different specificities toward the aromatic moiety of hydrocinnamates, unique selectivity for the ester linkage to the primary sugar in feruloylated oligosaccharides and variation in their ability to release dehydrodimeric forms of ferulic acid from plant cell wall material (Topakas and Christakopoulos, 2007). FAE were originally classified into two groups (type A and type B) based on their induction and substrate specificity (Kroon et al., 1996). The use of multiple sequence alignments demonstrating FAE activity and related sequences helped to construct a phylogenic tree. The result of this genetic comparison, also supported by substrate specificity data, allows FAE to be sub-classified into 4 types: A, B, C and D (Crepin et al., 2004a).

Type A FAE show a preference for the phenolic moiety of the substrate that contains methoxy substitutions, especially at meta- position(s) as occurs in ferulic and sinapic acids. Type B show preference for substrates containing one or two hydroxyl substitutions, as found in *p*-coumaric or caffeic acid. Moreover, only type A and D are also able to release small amounts of dehydrodimeric

ferulic acid. Type C and D show broad specificity against synthetic hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic and sinapic acid), only differing in their ability to release 5-5' dehydroferulic acid (Crepin et al., 2004a, 2004b).

The sequences of type C enzymes such as *A. niger* FAE type B (De Vries et al., 2002), *T. stipitatus* FAE type C (Garcia-Conesa et al., 2004), *F. oxysporum* FAE type C (Moukouli et al., 2008), and *A. oryzae* FAE type C (Koseki et al., 2009) are like that of fungal tannases. These FAE, except for *A. niger* FAE type B, exhibit a broad substrate specificity and are active toward ferulic, p-coumaric, caffeic, and sinapic acids. It has been reported that the *A. niger* FAE type B and *A. oryzae* FAE type B and C also exhibit hydrolytic activity toward chrologenic acid (Levasseur et al., 2004). The xylanase type D esterase from *Pseudomonas fluorescens* subsp. *cellulosa*, which is a type D FAE, exhibits broad substrate specificity and is active toward ferulic, *p*-coumaric, caffeic, and sinapic acids. This enzyme particularly exhibits highest activity toward substrates containing acetyl residues such as 4-nitrophenyl acetate (Ferreira et al., 1993).

2.2.4. Enzymatic feruloylation using feruloyl esterases

It has been observed that the synthetic activity pattern of FAE was parallel to their hydrolytic action toward various methyl esters of cinnamic acids (Topakas et al., 2005a). These authors have reported that the type C FAE from *S. thermophile* demonstrated a maximum hydrolytic activity against methyl ferulate among model substrates tested, indicating that it may be the most promising type of biocatalyst for the enzymatic feruloylation of aliphatic alcohols, oligo-and polysaccharides. Indeed, this enzyme was proven to catalyze the transfer of the feruloyl group to L-arabinose. The obtained structures were similar to those published for 5-O-(trans-feruloyl)-L-arabinofuranose isolated from wild rice, resulting in the first example of enzymatic feruloylation of a carbohydrate (Topakas et al., 2005b).

Vafiadi et al. (2006) have performed a regioselective enzymatic feruloylation of arabinobiose. Using proton nuclear magnetic resonance and ion-trap electrospray ionization mass spectrometry spectral data, the structure of the reaction product was identified as O-[5-O-(trans-feruloyl)- α-L-arabinofuranosyl]-(1→5)-L-arabinofuranose. Approximately 24% of the disaccharide was converted into feruloylated arabinobiose. The conversion yield is comparable for the production of feruloylated L-arabinose catalyzed by StFAEC in a slightly different composition of the detergent-less microemulsion system (53.4:43.4:3.2, v/v/ v) (Topakas et al., 2005b). As

demonstrated before, FAE show strong preference for short chain length alkyl ferulates and especially for methyl ester ferulate. The feruloylation reaction follows Michaelis–Menten kinetic (Vafiadi et al., 2006).

Commercial enzyme preparations from *H. insolens*, *T. lanuginosus* and *A. niger* exhibiting FAE activity catalyzed the transesterification of vinyl ferulate and 2,2,2-trifluoroethyl ester of ferulic acid, with various glycosides at their primary hydroxyl group using pure organic solvents as reaction medium. Results showed that higher conversion was achieved in polar aprotic solvents such as acetonitrile (Mastihubová et al., 2006). The enzymatic feruloylation of four linear arabino-oligosaccharides, containing from three to six arabinofuranose units was performed. The full mass spectrometry of products revealed the presence of a monoferuloylated oligosaccharide in each case and did not show any ions corresponding to products with higher than mono- feruloylation degree. The ring fragmentation showed that the feruloylation took place on the non-reducing ring of each L- arabino-oligosaccharide studied (Vafiadi et al., 2007b).

Couto et al. (2010) assessed the hydrolytic activity and the substrate specificity of FAE expressed in 20 multi-enzymatic preparations from different microbial sources using methyl ferulate and isolated feruloylated non-digestible oligosaccharides from sugar-beet pulp and wheat bran as substrates (Table 2.1). The efficiency of FAE expressed in the six best multi-enzymatic preparations from *Bacillus* spp. (Ceremix), *H. insolens* (Depol 740), *A. oryzae* (Flavourzyme), *Bacillus amyloliquefaciens* (Multifect P3000), *B. subtilis* (RP-1) and *Trichoderma reesei* (Depol 670) to catalyze the esterification of ferulic acid with various monosaccharides was investigated using a surfactant-less organic microemulsion system as reaction medium. Enzymatic feruloylation of D-arabinose catalyzed by Multifect P3000 in the n-hexane/1-butanol/MES–NaOH buffer reaction mixture (51:46:3 v/v/v) achieved a bioconversion yield of 37% while bioconversion yield for D-galactose in the same reaction media but catalyzed by Depol 670 was 61%. Depol 670 also catalyzed the feruloylation of D-xylose in the n-hexane/2-butanone/MES–NaOH buffer reaction mixture (51:46:3 v/v/v), with a bioconversion yield of 37%.

Table 2.1 Bioconversion yield of selected feruloylated glycosides obtained through FAE-catalyzed esterification reaction in surfactant-less microemulsions composed of n-hexane, 1-butanol/2-butanone and MES at a ratio of 51:46:3 (v/v/v) (Couto et al., 2010).

Enzyme preparation	Bioconversion yield[a]	
	Butanone	**Butanol**
	D-Arabinose	
Ceremix from *Bacillus* spp.	15.3	32.5
Depol 740 from *Humicola insolens*	7.2	17.7
Flavourzyme from *Aspergillus oryzae*	21.9	32.9
Multifect P 3000 *from Bacillus amyloliquefaciens*	11.3	36.7
RP-1 from *Bacillus subtilis*	<0.3	<0.3
Depol 670 from *Trichoderma reesei*	9.6	<0.3
	D-Galactose	
Ceremix from *Bacillus* spp.	<0.3	<0.3
Depol 740 from *H. insolens*	15.8	3.4
Flavourzyme from *A.s oryzae*	36.2	41.9
Multifect P 3000 *from B. amyloliquefaciens*	8.6	5.6
RP-1 from *B. subtilis*	19.8	10.2
Depol 670 from *T. reesei*	25.4	61.5
	D-Xylose	
Ceremix from *Bacillus* spp.	<0.3	<0.3
Depol 740 from *H. insolens*	20.9	26.5
Flavourzyme from *A. oryzae*	20.1	21.7
Multifect P 3000 *from B. amyloliquefaciens*	12.1	30.8
RP-1 from *B. subtilis*	4.4	16.3
Depol 670 from *T. reesei*	37.3	30.3

The bioconversion yield values obtained by Couto et al. for the esterification of ferulic acid with monosaccharides were in the same range as those obtained by Topakas et al. (2005a) for the transesterification of ferulic acid with L-arabinose by StFAEC. Atmospheric-pressure chemical ionization- mass spectrometry (APCI-MS) analysis of the end products confirmed the formation of various feruloylated glycosides by FAE-catalyzed esterification of ferulic acid with the selected glycosides. The ability of the synthesized feruloylated glycosides, as well as of their corresponding ferulic acid, to act as free radical scavengers was investigated using the DPPH radical scavenging assay. Results showed that feruloylated galactose was found to be the most potent scavenger, with a radical scavenging activity and yield of 6.3 Ab at 517 nm min^{-1} mL^{-1} and 93%, respectively.

Although the scavenging activity of the feruloylated xylose (2.1 Ab at 517 nm min^{-1} mL^{-1}) was two times lower than that of the ferulic acid, both esterified and free ferulic acid led to similar maximum radical scavenging yield of 93-91% at steady state. On the other hand, lower scavenging activity (2.3 Ab at 517 nm min^{-1} mL^{-1}) and maximum yield of 70% were obtained with feruloylated arabinose compared to free ferulic acid. The results indicate that the acylation of ferulic acid with hexose (galactose) did not affect its antiradical potency, whereas that with pentose (arabinose/xylose) resulted in a decrease of scavenging activity (Couto et al., 2010).

Couto et al. (2011) have reported the optimization of the feruloylation of selected di- and oligosaccharides in a surfactant-less microemulsion medium using the FAEs expressed in Depol 740L from *Humicola* sp. The highest bioconversion yields were obtained in n-hexane/2-butanone/MES-NaOH buffer mixture using arabinobiose (8%), xylobiose (9%) and raffinose (11%) as glycose donors. However, when galactobiose was the glycoside substrate, the highest bioconversion yield (27%) was obtained in the n-hexane/1,4-dioxane/ MES-NaOH buffer mixture. These results reveal that the bioconversion yield of feruloylated glycosides is dependent on the structural characteristics of di- and oligosaccharides, and in particular the type of hexose/pentose moiety, the length and the glycosidic linkages.

The free radical scavenging activity of the synthesized feruloylated di- and oligosaccharides as well as that of their corresponding ferulic acid were investigated using DPPH scavenging activity assay (Table 2.2). As compared to their corresponding ferulic acid, the synthesized feruloylated oligosaccharides demonstrated similar or higher potential radical scavenging properties. Feruloylated fructooligosaccharides required a higher time (750 s) to reach the steady state

compared to the free ferulic acid and other ferulates (405-585 s). The feruloylated raffinose showed highest affinity toward the scavenging of the free radical with an activity of 4.3 Ab at 517 nm/min mL. In contrast, Katapodis et al. (2003) reported that isolated feruloylated arabinoxylo-oligosaccharides displayed a reduced specificity towards DPPH free radical as compared to ferulic acid.

Response surface methodology, based on a 5-level and 4-factor central composite rotatable design, revealed that substrate molar ratio and the enzyme amount were the most significant model linear terms, affecting the feruloylation of raffinose by FAE (Couto et al., 2011). Conversely, water content and temperature had no significant effect on the investigated feruloylation reaction. The optimal conditions for the enzymatic esterification of feruloylated raffinose were: temperature of 35°C; ferulic acid to raffinose molar ratio of 3:1; water content of 3%, v/v; and enzyme amount of 345 enzymatic FAE units. Under the optimum conditions, the predicted concentration and bioconversion yield of feruloylated raffinose was 119.7 μM and 12%, respectively. The results showed that the quadratic model was statistically more suitable for the description of the FAE-catalyzed feruloylation reaction of raffinose (Couto et al., 2011).

Table 2.2 Scavenging activity of the ferulic acid and the feruloylated di- and oligosaccharides using DPPH* as stable free radical (Couto et al., 2011).

Components	**Scavenging activity**	**T_{sd}[a]**	**Scavenging yield**
Ferulic acid	3.4	405	92.1
Feruloylated arabinobiose	3.5	570	83.7
Feruloylated xylobiose	3.6	525	87.7
Feruloylated galactobiose	3.8	555	85.6
Feruloylated sucrose	3.8	450	93.3
Feruloylated lactose	4.0	540	86.5
Feruloylated raffinose	4.3	540	87.0
Feruloylated XOS	3.1	585	94.4
Feruloylated FOS	3.7	750	93.5

[a]Time (in seconds) to reach the steady state

2.3. Modulation of the enzyme's macro- and microenvironment

2.3.1. Enzyme immobilization

Enzyme immobilization is an approach for stabilizing enzymes, focused on retaining an enzyme on a surface or support, and directed at being able to reuse the enzyme or constrain the enzyme to a particular area. It is a well-established set of techniques that offers many advantages including (i) the development of economically feasible bioprocesses due to the possibility of biocatalyst recycling, continuous operation, and product purification, (ii) the enhancement of enzyme properties, such as stability and activity under extreme conditions of temperature, pH, or in the presence of organic solvents and (iii) the gain in the enzyme/substrate contact by using various reactor configurations.

Immobilization can be achieved by adsorption, covalent linking, crosslinking, and entrapment/encapsulation (Moehlenbrock and Minteer, 2011). There are many factors to consider when selecting an enzyme immobilization strategy, including enzyme tolerance to immobilization, chemical and physical environment, surface functional groups on the enzyme protein, size of enzyme, protein charge and polarity, its hydrophobic or hydrophilic regions, and substrate/product transport needs (Hanefeld et al., 2009). Enzyme immobilization can lead to many improvements including stability, selectivity and activity. Immobilized enzymes may perform better than the native enzymes if the immobilization method is correctly selected. Activation by immobilization is, however, often regarded as an extra benefit rather than a rational goal of enzyme immobilization. Although enzyme immobilization and improvement of enzyme performance by immobilization share the same principles, the emphasis is different, with the latter focused mainly on utilization of available immobilization techniques to improve enzyme performance for a specific application (Cao, 2005a).

The enhanced stability resulting from immobilization can be attributed to the intrinsic features of individual immobilization processes, for example: molecular confinement, favorable microenvironment, chemical modification effect in covalent bonding, and rigidification of conformation as a result of multipoint attachment (Bismuto et al., 2002). Although the best method of immobilization might differ depending on the peculiarities of each specific application, criteria for assessing the robustness of the immobilized enzymes remain the same, in that they must be cost-effective, safe, selective and very active (Cao, 2005b).

2.3.1.1. Methods of Enzyme Immobilization

2.3.1.1.1. Adsorption

Adsorption is the process of intermolecular forces resulting in the accumulation of protein on a solid surface. Adsorption is very dependent on the intermolecular interactions between the support surface and the enzyme and they might not be so strong to prevent enzyme from detachment (Cantone et al., 2013). Therefore, properties such as enzyme charge and polarity are crucially important to ensure high and reproducible coverage of enzyme on the support (Moehlenbrock and Minteer, 2011). At high enzyme loading, especially, diffusion limitation might occur because of the unequal distribution of the enzyme within a porous carrier, leading to a reduction of apparent activity.

2.3.1.1.2. Covalent binding/Crosslinking

Covalent binding and cross-linking are techniques that allow for the covalent binding of enzyme to surfaces or to other enzymes. These surfaces could be inner walls of a bioreactor or supports like glass or polymeric beads for a packed-bed reactor for industrial bioprocessing. Covalent binding or cross-linking of enzyme to enzyme to form aggregates is commonly referred to as CLEA (cross-linked enzyme aggregates) (Moehlenbrock and Minteer, 2011). In recent years, CLEA have attracted increasing attention, due to their simplicity, broad applicability, high stability and high volume activity (Cao et al., 2003). Free CLEA (CLEA without the use of any external carrier) are usually prepared by chemically cross-linking enzymes aggregates, with the use of appropriate aggregation agents, such as those widely used in non-denaturing protein separation.

The crosslinking of protein to protein or protein to surface typically results in decreasing the enzyme activity of the protein, but nevertheless provides a great deal of stability (Moehlenbrock and Minteer, 2011). One of the intrinsic drawbacks of these first generation CLEA is that their particle size is usually below 10 mm. Thus, difficulties arise when they are used in heterogeneous reaction systems, where the substrate particles and the CLEA particles might be in the same size range. This can result in serious problems for the continuous use of these immobilised enzymes (Cao et al., 2003). Although development of carrier-free enzymes can eliminate the use of the extra non-catalytic mass-carrier, the intrinsic drawbacks associated with the carrier-free immobilized enzymes, narrow reactor configuration, laborious screening of conditions for aggregation,

crystallization and cross-linking, have not yet made it the primary choice for bioprocess engineers (Cao, 2005a).

2.3.1.1.3. Entrapment/Encapsulation

Entrapment is the procedure of polymerizing a monomer or low molecular weight polymer around the protein to trap the protein on a surface. This is frequently done with sol-gels and hydrogels and is quite successful at immobilizing proteins on surfaces (Lim et al., 2007). Issues to consider with entrapment techniques are the chemical environment of the polymerization solution and whether it will denature the protein. Furthermore, pore size and interconnectivity of the pores in the polymer determine if substrate and product can diffuse in and out of the polymer, but also to ensure that the protein cannot diffuse out of the polymers. Encapsulation is similar to entrapment in that the protein is constrained within the polymeric matrix, but the polymer matrix has "pockets" or "pores" for immobilizing the protein (Moehlenbrock and Minteer, 2011). Micellar polymers are an example of polymers that can encapsulate an enzyme. Once the polymer micelles have swelled, the enzyme intercalates into the micellar pockets and the solvent is evaporated. This results in a polymer membrane that offers a microenvironment similar to a cellular microenvironment that can provide temperature, pH, and organic solvent stability to the protein (Moore et al., 2004).

Entrapment of the enzyme in hard sol–gel matrix can often be used to stabilize the overall molecule in a spatially restricted three-dimensional matrix (Cao, 2005a). It was recently found that the presence of some additives might be crucial for sol-gel encapsulated enzymes with regards to the activity retention or selectivity. Enzymes can be stabilized to a surprising extent in the inorganic sol-gel matrix. However, the particle size of these resulting inorganic sol-gel entrapped enzymes is usually below the limit of a carrier-bound immobilised enzyme (100 mm). This limits their application as robust immobilised enzymes in many processes, where the particle size might be crucial for the separation of the enzyme from the heterogeneous reaction mixture (Cao, 2005b).

2.4. Enzyme immobilization: pre- and post-treatments

Immobilization alone may not always be enough to stabilize the enzyme. Research has been focused on the development of stabilization strategies before the enzyme is bound to the carriers. The aim is often to strengthen binding of the enzyme to the carrier, control of the mode of binding, or improve enzyme performance. Pre-immobilization treatments are oriented to improve the subsequent immobilization procedure. In general, stabilization by pre-immobilization

modification can be classified into two types: alteration of the microenvironment and rigidification of the enzyme conformation. One example of the former approach is the preparation of hetero-functional epoxy supports, where the inclusion of another functionality promotes a rapid physical or chemical fixation of protein on the support surface. This is followed by an intramolecular multipoint attachment between the epoxy groups of the support, and the placing of nucleophiles near the region of the protein participating in the first immobilization step. A more specific case of this strategy is the chemical modification of epoxy-activated supports with ethylene diamine (EDA) or iminodiacetic acid (IDA), leading to the formation of a heterofunctional support bearing some charged arms but also unreacted epoxide moieties (Mateo et al., 2006). The IDA-functionalized supports can be further modified with salts such as cupric sulphate, inducing the formation of some metal-chelate reactive groups (Figure 2.2) (Mateo et al., 2007).

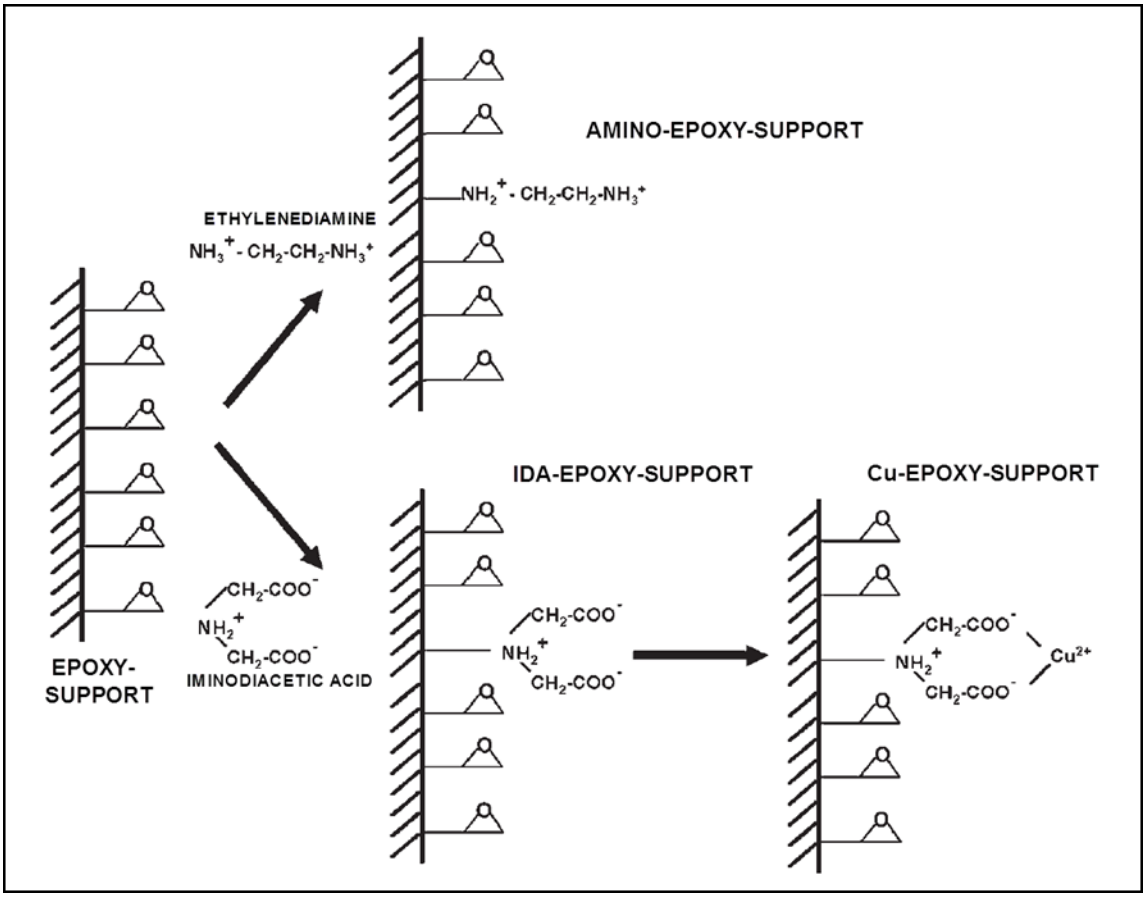

Figure 2.2 Examples of chemical modifications producing heterofunctional supports for enzyme immobilization (Mateo et al., 2006).

Often, the immobilized enzyme must be subjected to a variety of physical and chemical treatments, with the aim of further improving its activity and stability. These techniques, which are used to improve ready-made immobilized enzymes, are referred to as post-immobilization techniques and can be classified into two broad groups, physical and chemical post-treatments. The former includes the pH-imprinting technique, solvent washing (Yang et al., 1998). Chemical post-treatment refers to any chemical means subsequently used to modify the already prepared immobilized enzyme, which includes chemical modification of the enzyme, enhancement of multipoint attachment by increasing the pH of the immobilization medium, neutralization of excess active binding functionality or alteration of the microenvironment of the immobilized enzymes (Cao, 2005a). With the aim of improving the micro-environment of the immobilized enzymes, quenching of excess reactive groups in the supports is often required. The blockage of these groups fulfills a double objective: eliminating the reactivity of the support and altering its physical properties. Most of the commercial supports recommend the use of 2-mercaptoethanol as a blocking agent; however, there are numerous compounds that are able to react with the epoxy groups, such as glycine or ethanolamine (Knežević-Jugović et al., 2011).

2.5. Enzyme immobilization in nanoparticles

Enzymes are ubiquitous natural biocatalysts of nanometer scale. In the early stages of nanobiocatalysis, enzymes were immobilized on various nanomaterials using conventional approaches, such as simple adsorption and covalent attachment. Development has focused on immobilizing enzymes onto the larger surface area that nanostructured materials provide. Larger area led to a higher protein loading in these type of supports, increasing the enzyme activity per g of support compared to traditional supports (Homaei et al., 2013). One of the particularly advantageous features of nanostructured materials is the control over size at the nanometer-scale, such as the pore size in nanopores, thickness of nanofibers or nanotubes and the particle size or reactive group of nanoparticles. The uniform size distribution of nanomaterials and their similarity in size with enzyme molecules, together with other advantageous nanomaterial properties such as conductivity and magnetism, have revolutionized nanobiocatalytic approaches in various areas of enzyme technology and led to improved enzyme properties in nanobiocatalytic systems, particularly regarding enzyme stability and activity (Kim et al., 2008).

Nanoparticles are desirable from several perspectives. However, their dispersion in reaction solutions and subsequent recovery for reuse can be difficult to achieve. Enzyme aggregate coating

combines covalent enzyme attachment on various nanomaterials with enzyme cross-linking, leading to an increase in enzyme loading, overall enzyme activity and enzyme stability. Other advantageous properties of the resulting nanobiocatalytic systems are resistance to proteolytic digestion, the possibility of magnetic separation and improved rate of electron transfer (Kim et al., 2008). It is therefore anticipated that nanobiocatalytic approaches for enzyme immobilization might enable further practical applications of enzymes in that they will require smaller amounts of enzyme, prolong the lifetime of enzyme reactors and increase the potential for recycled uses of enzymes. For the eventual successes of these approaches, interactions between enzymes and nanostructured materials at this scale need to be further elucidated.

2.6. FAE immobilization

Only a few studies have been reported the immobilization of FAEs and their use in synthetic reactions. (Vafiadi et al., 2008b) have investigated CLEA of commercial multicomponent enzyme preparations with FAE activity for the transesterification of methyl ferulate to 1-butyl ferulate, using a reaction system consisting of n-hexane, 1-butanol and water. After 6 days of incubation, conversion yields of 97%, 87% and 5%, were obtained by CLEA prepared from Ultraflo L, Depol 740L (from *H. insolens*) and Depol 670L (from *T. resei*), respectively. The synthetic performance of free enzymes was too low compared to the immobilized forms (Vafiadi et al., 2008b). In particular, 3.6% and 2.6% conversion was achieved by Ultraflo L and Depol 740L, respectively, while traces of product were detected when Depol 670L was employed (Vafiadi et al., 2008b). The observed low reaction yields using 'free' enzyme was attributed to the precipitation which intervened with the addition of the free enzymes in the microemulsion reaction medium (Vafiadi et al., 2008b). FAE activity of the aggregates exceeded that of the free enzyme activities, exhibiting in the preparations before cross-linking (Vafiadi et al., 2008b). This activation is thought to find its origin in conformational changes of the protein induced by the aggregated state. Finally, CLEA were recycled by removing them from the reaction medium after 6 days of incubation and tested for their residual FAE activity (Vafiadi et al., 2008b). The recovered enzymatic activity for Ultraflo L, Depol 740L and Depol 670L was 48%, 36% and 35%, respectively, allowing the reuse of the enzymes for a second synthetic round (Vafiadi et al., 2008b). However, the performance of the recovered CLEA during the second synthetic round was lower comparing to the first round (33% for Ultraflo L, 25% Depol 740L, traces for Depol 670L) (Vafiadi et al., 2008b). After the end of the second there was no remaining FAE activity in the aggregates (Vafiadi et al., 2008b).

Vafiadi et al. (2008a) have also reported the immobilization of a FAE type A from *Pichia Pastoris* using the same immobilization technique previously applied (formation of CLEA) and used the immobilized enzyme to catalyze the transesterification reaction between methyl sinapate and 1-butanol. The reaction using CLEA reached 56% conversion after 5 days of incubation, which is lower than the conversion achieved using the free enzyme (78%). After the first reaction, CLEA retained the 52% of its initial activity and the conversion yield of the second batch was only 14% (Vafiadi et al., 2008a). Matsuo et al., (2008) have investigated the immobilization of the FAE expressed in a commercial enzyme preparation from *A. niger* (Pectinase PL) by covalent attachment to glutaraldehyde-activated chitin-based gels. The initial reaction rate of the synthesis of glyceryl ferulate by the immobilized FAE (6.4 mmol/h) was higher as compared to that of the free enzyme (3.9 mM/h). Although lower bioconversion was obtained with the immobilized enzyme, the immobilized FAE could be reused at least five times without a significant loss in activity (Vafiadi et al., 2008a).

A commercial enzyme preparation from *H. insolens* (Depol 740L) was immobilized by physical adsorption into two mesoporous silica materials of different pore size (Thörn et al., 2011). The effect of the immobilization on the efficiency of Depol 740L to catalyze the transesterification of methyl ferulate with 1-butanol was studied. Enzyme immobilization on mesoporous silica with larger pore size showed higher protein loading (68%) compared to the support with smaller pore size (31%). In addition, approximately 45% of the FAE hydrolytic activity present in Depol 740L was retained in the mesoporous silica with smaller pores while 94% of the FAE activity was retained in the mesoporous silica with larger pore size; the immobilized FAE on the mesoporous silica with larger pore size were also 3.5 times more active in terms of specific transesterification activity, resulting in 90% bioconversion yield. Moreover, the immobilized FAE retained 70% of its initial activity after 6 sequential reaction cycles, showing its high operational stability.

2.7. The use of surfactantless microemulsions as reaction media

The use of enzymes in non-aqueous media has received growing attention due to the many advantages of these media, including higher substrate solubility, the ability to use enzymes for synthetic purposes, and the capability to modify native selectivity by simply altering the reaction medium rather than the enzyme itself (Zeuner et al., 2012). Giuliani et al., (2001) have investigated the synthesis of pentyl-ferulate catalyzed by a FAEA from *A. niger* in a cetyltrimethylammonium bromide (CTAB)/hexane/pentanol water-in-oil microemulsion system and achieved a high yield

(60%). However, larger scale practical application of this enzyme-containing microemulsion may be considerably hindered due to the presence of high concentrations of surfactants, as well as by limited solubility of phenolic acids such as ferulic acid in this media.

Surfactantless microemulsions are thermodynamically stable ternary mixtures of a hydrocarbon, an alcohol/ketone, and water, where aqueous microdroplets are stabilized in dispersion by alcohol molecules adsorbed to their surface. The retention of the biocatalytic activity of the enzymes in the surfactantless microemulsions is due to the entrapment of enzyme molecules into the aqueous microdroplets, avoiding contact with the outer organic solvents by a water-rich layer. An important advantage of the use of surfactantless microemulsions as a reaction system is that they easily resolve the problem of separation of reaction products and enzyme reuse, while solubility of relatively polar phenolic acids is high due to the presence of a large amount of polar alcohol (Khmelnitsky et al., 1988). These reaction systems have been already identified as appropriate media for the esterification or transesterification of various phenolic acids catalyzed by FoFAE-II, FoFAE-I and StFAE-A FAE from *F. oxysporum* and *S. thermophile*, respectively (Topakas et al., 2005b; Topakas et al., 2003). Although the bioconversion yields were lower in these reactions compared to the conversions using lipases (Giuliani et al., 2001); the solubility of the hydroxycinnamic acid and its derivatives was about 100 times higher than the values obtained in microemulsions stabilized by surfactants.

Couto et al. (2010) have studied the effect of a surfactantless organic microemulsion medium composed of n-hexane, 1-butanol or 2-butanone and MES–NaOH buffer at selected ratios (51:46:3, 30:67:3 and 67:30:3 (v/v/v)), on the feruloylation efficiency of FAE present in selected commercial multienzymatic preparations using D-arabinose, D-xylose and D-galactose as glycosides. Following this investigation, the surfactant-less organic microemulsion mixture composed of n-hexane, 2-butanone and MES–NaOH buffer with a ratio of 51:46:3 (v/v/v) was determined to be the most appropriate reaction medium for the enzymatic feruloylation of monosaccharides. Couto and colleagues (2011) used the same reaction media for the optimization of the feruloylation of raffinose catalyzed by the FAEs from *Humicola* sp. (Depol 740).

2.8. Enzymatic synthesis of carbohydrate fatty acid esters

2.8.1. Introduction

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. They contain a hydrophobic portion with little affinity for the bulk medium and a hydrophilic group that is attracted to the bulk medium (Mulligan, 2005). Surfactants are ingredients critical to improve the emulsifying, gelling, film-forming and foaming characteristics of food products used on different segments of the industry. Surfactants synthesized through a chemical route such as sorbitan esters and their etoxylates are widely used on food matrices but potential toxic traces, larger carbon footprint production and non-selective synthesis have raised awareness among consumers and the scientific community. Bioprocessing approaches using microorganism metabolites or enzymatic synthesis arose as 'green alternatives' for surfactant production which can lead to process simplification, waste reduction and decreased toxicity of the obtained compounds.

Biosurfactant molecules have a huge structural diversity and are surface-active compounds released produced by bacteria (Rendell et al., 1990), yeasts (Mulligan, 2005), fungi (Zavala-Moreno et al., 2014) and in the last decades has been produced in-vitro using enzymes as Novozyme® 435 (Lipase B from *Candida antarctica*) (Chaiyaso et al., 2006; Chang and Shaw, 2009; Fukuoka et al., 2011) and Lipozyme® RM IM (*Rhizomucor miehei*) (Abdulmalek et al., 2012; Coulon et al., 1996; Khaled et al., 1991). Due to their low toxicity, biodegradability, and environmental compatibility they are of increasing interest and offer some advantages compared with chemically products. Biosurfactant production by microorganisms depends on the fermentation production, environmental factors and nutrient availability (Rahman and Gakpe, 2008; Zavala-Moreno et al., 2014). Enzymatic production depends on the solubility of substrates (Alissandratos and Halling, 2012; Chang and Shaw, 2009; van den Broek and Boeriu, 2013), substrate molar ratio (Abdulmalek et al., 2012), solvent (Karam et al., 2009), type of enzyme and its immobilization (Karboune et al., 2010), water activity (Chamouleau et al., 2001; Giacometti et al., 2001), and temperature (Gandhi et al., 1995; Gumel et al., 2011).

Carbohydrate fatty acid ester (CFAE) are a major class of biosurfactants consisting of low-molecular-mass substances (<1500 g/mol) with a hydrophilic mono-, di-, or oligosaccharide unit linked ester-like or glycosidically to hydrophobic long-chain hydrocarbon units. The most common saccharide are glucose, fructose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate (Rahman and Gakpe, 2008). The advantage of oligo- and polysaccharides fatty acid esters as surfactants is that sugars are more soluble in water due to the increased hydrophilicity of their sugar groups (Plou et al., 2002) Table 2.3 shows a compendium of various glycolipids produced by microbial or enzymatic route.

2.9. Health-promoting properties of carbohydrate fatty acid esters

Ustilagic acids produced by *Pseudozyma fusiformata* display antimycotic activity against many yeasts and filamentous fungi, including phytopathogenic and medically important species of the genera *Filobasidiella, Malassezia, Taphrina* and *Ustilago* (Kulakovskaya et al., 2005). Sophorolipids inhibit the growth of *B. subtilis, Staphylococcus epidermidis* and *Streptococcus faecium* at concentrations of 6–29 mg/l. Sophorolipids-2 also inhibits the germination of conidia of the fungus *Glomerella cingulata* at a concentration of 50 mg/l. Different sophorolipids (lactone forms) produced by *Candida apicola* IMET 43733 inhibit the growth of not only gram-positive bacteria but also gram-negative bacteria such as *Escherichia coli* and *Serratia marcescens* (Kitamoto et al., 2009).

The antimicrobial activities of sucrose monolaurate and lactose monolaurate, were tested. Gram-positive bacteria were more susceptible than Gram-negative bacteria to both esters. The minimal bactericidal concentrations of lactose monolaurate were 5 to 9.5 mmol/L for *Listeria monocytogenes* isolates and 0.2 to 2 mmol/L for *Mycobacterium* isolate (Wagh et al., 2012). Rhamnolipids can also be used as biological control products; they exhibit zoosporicidal activity on species of three representative genera of zoosporic phytopathogens: *Pythium aphanidermatum, Phytophtora capsici* and *Plasmopara lactucaeradicis*, and on the harmful algal bloom species (Kitamoto et al., 2009).Sugars esters are promising candidates as antitumor agents. Two fatty acids esters of maltotriose were synthesized and showed inhibitory effects towards two different tumour cell lines (Hep-G2 and HeLa). The trisaccharides were more promising inhibitors as their mono- and disaccharides counterparts (Ferrer et al., 2005). Dextran-based hydrogels were prepared and showed superior mechanical properties above the chemically prepared dextran-based hydrogels.

These hydrogels are biodegradable and have potential in biomedical applications like tissue engineering and controlled drug delivery (Ferreira et al., 2005).

2.10. Food applications of carbohydrate fatty acid esters

Most sugar fatty acid esters might find potential use in food due to their tasteless and odourless characteristics along with their intrinsic emulsifying properties and surface activity. Due to the difference in the structure of each of its counterparts, every sugar ester has a different critical micelle concentration (CMC) and hydrophilic-lipophilic balance (HLB). In the food industry, the most useful property of a CFAE is its ability to form stable emulsions, which improves the texture and creaminess of diverse products. Sucrose fatty acid esters are recognized as food additives by the US Food and Drug Administration (FDA) and by the European Commission Scientific Committee for Food and the Joint FAO/WHO Expert Committee on Food Analysis (JECFA). Due to the chemical nature of sucrose it is possible to esterify up to 8 molecules of fatty acid to one sucrose molecule and the degree of acylation greatly affects the functional properties of the esters. Therefore, food grade sucrose fatty acid esters are categorized according to their degree of fatty acid acylation and ester content. For example sucroglycerides have a content of 40% to 60% mono- to triesters and may contain residual glycerides; while sucrose esters should have a mono- to triesters content above 80% and no glyceride residual, (European Food Safety Authority, 2010).

Many applications have been reported for CFAE including their use to retard staling, solubilize flavor oils, and improve organoleptic properties in bakery and ice cream formulations, and as fat stabilizers during cooking of fats (Banat et al., 2010). Lactose ester as the best biosurfactant, with a surface tension of 38.0 N/m and an emulsification index of 54.1%, when used in a ratio of 1:10 (biosurfactant:coconut milk, v/v) (Neta et al., 2012). The main emulsifiers used by the food industry are monoglycerides. These compounds can also act as mild dough conditioners, leading to improved handling properties of the dough, enhanced slicing performance and superior bread quality (Sawa et al., 2009). The sugar ester surfactants are used to assist blending and emulsification of ingredients, to control the agglomeration of fat globules, stabilize aerated systems, modify rheological properties of wheat dough, improve consistency, and to interact with the components of the flour and other ingredients in the mix for softer crumb improving the palatability (Rajendran et al., 2009). Sugar ester surfactants may play a crucial role in the

manufacture of food-grade colloidal delivery systems, namely microemulsions and nanoemulsions. There is a growing interest within the food and beverage industries in the use of colloidal delivery systems to encapsulate functional agents, such as flavors, colors, antimicrobials, micronutrients, and nutraceuticals. One of the most important applications of these type of emulsions is to incorporate lipophilic active ingredients into aqueous-based foods or beverages that need to remain transparent, such as some fortified waters, soft drinks, sauces and dips (Velikov and Pelan, 2008).

2.1. Carbohydrate fatty acid ester production strategies

2.1.1. Chemical synthesis

Chemical esterification of carbohydrates in monophasic media with derivatives from vegetable oils has provided a wide range of commercially-available sugar-based surfactant such as sucrose fatty acid esters, sorbitan esters, and alkyl polyglycosides. The most common sources for acyl acceptors being sucrose, starch, and corn syrup while triglycerides, acyl chlorides, and methyl esters derived from vegetable oils have been evaluated as the hydrophobic preccursors of the CFAE. (Drummond et al., 2003). The traditional method for sucrose esters synthesis – called Ryoto process – uses an alkaline catalyst in DMF or DMSO at 90 °C (Hass et al., 1959). While a newer procedure for sucrose fatty acid esters synthesis use vinyl esters of fatty acids as acyl donors along with *p*-methoxyphenol as stabilizer of the reaction, finalizing with purification using supercritical CO_2 (European Food Safety Authority, 2010).

Although commercial CFAE are being mostly produced through chemical processes. CFAE chemical synthesis is an energy-demanding process requiring the use of specific base catalysts to produce complex mixtures of two to three amphiphilic species (Pantelic and Cuckovic, 2014). Additionally, chemical synthesis requires the use of multiple protecting and deprotecting steps to attain certain level of selectivity, longer procedures for separation of the end-products, and the use of high temperature (up to 190 °C) might degrade or decolorate the saccharide substrates (Panpipat and Chaijan, 2016). Eventhough solvent-free or reduced-solvent chemical procedures have been lately developed as an alternative to reduce the amount of solvent waste and reduce the number of purification steps after the chemical synthesis of sugar esters and alkyl polyglycosides. However, the poor selectivity, temperature effect over the saccharide substrates, and low customisation of sugar ester chemical synthesis are hurdles yet to be elucidated.

Table 2.3 Glycolipids produced by microorganism metabolites and enzymatic reaction.

Glycolipid	Microbial/Enzyme	Surface tension (mN/m)	CMC (mg/L)	Reference
Rhammnolipids	*Pseudomonas aeruginosa*	30-32	30	*(Dyke et al., 1993)*
Ustilagyc acid	*Ustilago maydis*			*(Zavala-Moreno et al., 2014)*
Mannosylerythritol lipid	*U. maydis*			*(Zavala-Moreno et al., 2014)*
Sophorolipids	*Turolopsis bombicola*			*(Asmer et al., 1988)*
Monoester of cellobiose	*Arthrobacter* spp.	1-5	40	*(Li et al., 1984)*
Monoester of maltose	*Arthrobacter* spp.	35	50	*(Li et al., 1984)*
Glucose–Fatty acid ester	Novozyme® 435			*(Cao et al., 1996)*
Fructose–Fatty acid ester	Novozyme® 435			*(Coulon et al., 1996)*
Galactose-Fatty acid ester	Lipozyme® RM IM			*(Abdulmalek et al., 2012)*
Mannosylerythritol lipid D	Novozyme® 435	24		*(Fukuoka et al., 2011)*

2.1.2. Enzymatic synthesis

Synthesis of carbohydrate esters and related compounds using biocatalysts and substrates obtained directly from renewable material might help to overcome the low selectivity of chemical synthesis methods but also could lead to an enhanced sustainability profile of the CFAE synthetic procedure. Because of the chemo-, regio-, and enantioselectivity of the biocatalysts, milder reaction conditions of the enzymatic process, potential fewer traces, and ease of end-product separation there is growing interest in the application of enzymes for carbohydrate fatty acid esters production. However, enzyme stability and reusability issues along with economic considerations hinder scaling up the enzymatic acylation of carbohydrates to an industrial level.

2.1.2.1. Enzymatic esterification of mono- and oligosaccharide esters of fatty acids in non-conventional media

Therisod and Klibanov (1986) pioneered the enzymatic acylation of simple sugars in non-aqueous media. They reported the synthesis of monosaccharide esters in anhydrous pyridine catalyzed by porcine pancreatic lipase using trichloroethanol activated acyl donors. This report was followed by a study on the capability of lipases to catalyze transesterification of the secondary hydroxyl groups of D-glucose, in which the C_6 hydroxyl group has been blocked. The Klibanov research team also reported for the first time the use of protease subtilisin in the regioselective transesterification of maltose, cellobiose, lactose, and sucrose with trichloroethanol activated acyl donors in anhydrous dimethylformamide (DMF) (Riva et al., 1988).

Enzymatic acylation of lipophilic donor to mono- and oligosaccharides in non-aqueous media generally produce monoesters, although traces of diesters may occur. Simple product mixture facilitates further downstream purification. It has been reported high yields of acylation catalyzed by enzymes. For instance, a conversion yield of 98% for 6-O-lauryolsucrose using immobilized *T. lanuginosus* lipase was reported for the transesterification of vinyl laurate using a mixture of 2-methylbutan-2-ol (2M2B) and DMSO (80:20, v/v) (Ferrer et al., 2005). While a yield of 76% of 6-O-palmitoyl glucose was attained for palmitic acid acylation in 100% acetone catalyzed by Novozym® 435 (Chaiyaso et al., 2006).

One of the key issues to resolve when developing a novel protocol for the biosynthesis of CFAE is the selection of an appropriate reaction media. Although sugars are relatively soluble in some polar aprotic solvents (DMF, pyridine, DMSO); most enzymes demonstrate a low activity in these

solvents due to a water-stripping effect affecting the hydration-shell of the protein. Therefore acylation with these solvents require long reaction times and induce rapid inactivation of the biocatalyst, not to mention the intrinsic toxicity of polar aprotic solvents which prohibits their use for production of food-grade ingredients (Prat et al., 2014). Therefore, solvents of intermediate polarity that can partially solubilize both acyl donor and acceptor, but also not affect the catalytic activity of the enzyme are recommended. Tertiary alcohols are particularly effective solvents in this respect providing high enzyme stability and activity. Moreover, they cannot act as a substrate to lipases due to steric hindrance at the hydroxy group and their boiling points facilitate their removal after reaction. Numerous experiments have been reported using 2-methyl-2-butanol, *tert*-butanol or methyl *tert*-butyl-ether as reaction media (Degn et al., 1999; Khaled et al., 1991; Li et al., 2015b).

Biphasic mixtures of solvents such as tert-butanol/pyridine and *tert*-butanol/DMSO have also been used (Pérez-Victoria and Morales, 2006). Oligofructose fatty acid monoesters have been synthesized by Van Kempen et al. (2013) using Novozyme® 435 in DMSO–*tert*-butanol (20:80, v/v) in the presence of molecular sieves. Reaction will occur as follows: a hydrophobic substrate, such as steroids or fats, is mostly located in the organic solvent portion and is partitioned into the aqueous phase. The substrate is converted by the enzyme, and then the product is extracted into the organic solvent phase (Degn and Zimmermann, 2001). The advantage of this system is that it enables the reaction shift towards the synthesis of esters. Then, to a certain degree, the problem of low solubility solves itself during the reaction, because the formation of glycolipids can lead to a higher solubility of the sugar after certain concentration of the product has been formed (Cauglia and Canepa, 2008).

Enzymatic catalysis of carbohydrate esters can proceed in a solvent-free mixture of substrates to overcome the problem of poor solubility of the polar substrate in organic media. (Bjorkling et al., 1989) developed an enzyme-catalyzed synthesis of fatty acid glucopyranosides utilizing immobilized lipase (*C. antarctica*) and ethyl glucosides under solvent-free conditions at 70 °C attaining conversions in the range of 85 % to 90 %. Other methods that have been developed to increase the solubility of the substrates are: substrate immobilization (Sharma and Chattopadhyay, 1993); derivatization of sugars (Fregapane et al., 1991); complexation of sugar with phenylboronic acid (Scheckermann et al., 1995); and alkylation of the acyl acceptor (Adelhorst et al., 1990). Hydrophobization of the polar substrate is very helpful for di - and oligosaccharides difficult to

solubilize in relatively polar solvents (Drummond et al., 2003). A different solvent-free reaction approach, namely solid-phase reaction systems, has also been reported. Where sugars are directly dissolved in the fatty acid and only few volume of solvents in a concentration unable to dissolve the polar substrate, as so called adjuvants, are added (Cao et al., 1996). This has the advantage that the use of hazardous solvents can be avoided, and that the product often crystallizes from the reaction media (Cao et al., 1997).

A key parameter to control in the enzymatic acylation of mono- and oligosaccharides with free fatty acids in non-aqueous media is the water content in the reaction media. It is generally accepted that a minimal amount of water is necessary to ensure the enzyme optimal conformation and activity. However, high water contents might shift the reaction equilibrium, cause unwanted side reactions, and prevent the contact of a lipophilic substrate with the enzyme (Watanabe et al., 2000). It is recommended to constantly remove water from the reaction media in order to constantly shift the reaction towards the ester formation (Kobayashi and Adachi, 2004). Numerous methods have been reported for the removal of water formed during the esterification reaction, such as evaporation under reduced pressure (Ducret et al., 1995; Izák et al., 2005), azeotropic distillation (Yan et al., 1999), addition of molecular sieves (Šabeder et al., 2006), addition of water controlling agents (Ye et al., 2016). However, there is still no general procedure regarding the effectiveness or the amount of desiccant required to achieve the desired conversion. It has been reported that the molecular sieve acts as a catalyst, as well as an adsorbent (ter Haar et al., 2010). There are some cases in which undesirable diester formation or degradation of the substrates occurred (Sonwalkar et al., 2003).

The use of free fatty acids as acyl donors might allow the use of cheap renewable starting materials. However, the biggest drawback of this approach is the generation of water during the esterification reaction. Which will affect the equilibrium of the reaction directly resulting in a decrement of the bioconversion yield. Using esters as acyl donors lead to a transesterification reaction instead of an esterification. Water is not generating preventing subsequent hydrolysis of the formed esters. Vinyl esters of fatty acids additionally will tautomerize into acetaldehyde, which evaporates at 20 °C (Bornscheuer and Yamane, 1995). It has however to be considered that many side products from activated acyl donors might have a negative impact on the catalytic properties of the biocatalysts (Weber et al., 1995). Furthermore, vinyl esters of fatty acids are not as commercially available as free fatty acids or their mixtures. If the objective is to develop a procedure aiming to use the

purified end-products for food grade applications then it is more desirable to use the more natural fatty acids instead of vinyl esters (Van Kempen et al., 2013).

The hydrophilic-lipophilic balance (HLB) values determine the physicochemical properties of surface active substances for specific uses. Sugar esters with low HLB values (3–6) are good water-in-oil emulsifier, with medium HLB values (7–9) are good wetting agent, and with high HLB values (10–16) are appropriate emulsifier for oil-in-water emulsion. Although most studies on sugar ester synthesis focus on bioconversion or selectivity of the enzyme, it is also pertinent to examine the degree of esterification (DE) of sugar to acyl donors. Higher DE of the reaction end-products results in a lower HLB value. Prediction and determination of exact HLB values may assist to ensure a proper application for the reaction end-products (Pérez et al., 2017). In the determination of DE of synthetic fructose ester of lauric acid catalyzed by Novozym® 435, a preferential formation of diesters in reactions with methyl ethyl ketone (EMK) was observed compared to 2M2B suggesting Novozym® 435 lipase conformation binding monoesters favored diester synthesis. (Li et al., 2015b).

2.1. Lipases

Lipases comprise an extensive group of enzymes belonging to the hydrolase group, which catalyse the hydrolysis of glyceride ester bonds. These enzymes are found widely in nature where their major role is to enable the breakdown of lipids as a source of energy. Lipases from various sources are readily commercially available. The most important lipase sources with high catalytic efficiency properties are *Bacillus subtilis, Bacillus licheniformis, Streptomyces sp., R. miehei, C. antarctica, C. cylindracea* and *Chromobacterium viscosum*. Bacterial lipases are robust enzymes since they are active over a wide range of pH and temperature. Lipases can be classified on basis of structural and physicochemical properties of the scissile fatty acid binding sites to understand the substrate specificity of lipases (Patkar et al., 1998). They may be regiospecific or nonspecific towards triacylglycerols, possess fatty acid-specificity regarding the alkyl chain or degree of unsaturation, and display enantioselectivity (Gupta et al., 2004). The lipases from group B have large acyl binding cleft but narrow alcohol binding cleft, makes them the most promising biocatalysts for acylation in non-aqueous media. These lipases show preference for long unbranched fatty acids (Soultani et al., 2001).

Table 2.4 Enzymatic synthesis of biosurfactants using coated enzymes

Alcohol group	Acyl Group	Surfactant	Enzyme	Solvent	Yield	References
Glycerol	Palmitic acid	Span 60	Lipase from *Rhizopus arrhizus*	Isooctane	72%	(Sasi et al., 2006)
Lauryl alcohol	Lauric acid	Glutamic acid didodecyl ester	Lipase from *Candida rugosa*	Isooactane	95%	(Wu et al., 2002)
Geraniol	Acetic acid	Span 85	Lipase from *Candida cylindrecea*	Isooctane, n-hexane, n-heptane, toluene, benzene, chloroform, decane, n-butanol, acetonitrile	97%	(Huang et al., 1998)
Chinese tallow kernel oil from *Sapium sebiferum* (L.) Roxb.	C16:0, C18:0, C18:1, C18:2, and C18:3	Non-ion surfactant	Lipase AY®	n-hexane	--	(Gao et al., 2009)

Lipases are insoluble in organic solvents in their native form. Two types of methods, namely covalent and noncovalent modifications are in common use for lipase solubilisation. Covalent modifications are achieved by using chemical modifiers like polyethylene glycol (PEG), poly N-vinylpyrrolidone, polystyrene and polymethyl methacrylate. Immobilization confers better homogeneity of the enzyme for reaction and improves thermostability of the protein. Such chemical modifications greatly affect the activity, stability and selectivity of the enzyme. Often immobilized lipases like Novozym ® 435 or the lipase from M. miehei immobilized on macroporous ion-exchange resin (Lipozyme® RM IM) are employed for reactions in organic solvents due to their enhanced stability (Neta et al., 2015). Non-covalent modification of lipases has essentially been restricted to coating the lipase molecule with different surfactants (Table 2.4). Dissolution of the lipase and the surfactant in aqueous solution are the most widely applied techniques. This enzyme-surfactant complex, which precipitates from the aqueous solution, can be dissolved in a variety of organic solvents. The solubilized lipase has shown greater catalytic activities than dry lipase preparations.

CONNECTING STATEMENT 1

Literature review, presented in Chapter II, covers the structure identification, the bioactive properties, and the preparation of feruloylated oligosaccharides along with a short description of the enzyme immobilization techniques and treatments. The synthesis of feruloylated oligosaccharides in non-conventional media catalyzed by pure FAE in its free and immobilized form is reported in Chapter III. Selectivity of the pure enzyme preparations was assessed using synthetic and naturally occurring feruloylated oligosaccharides as substrates for hydrolysis. This chapter also includes an evaluation of the effects of surfactantless microemulsion composition and glycoside structure on the feruloylation yield of the reaction along with a comparative study of the immobilization efficiency of FAE on selected commercial immobilization supports. The immobilization supports retaining greater FAE hydrolytic activity and yielding higher protein content after immobilization were selected to undergo pre- and post-immobilization treatments such as partial chemical modification or neutralization of their functional groups. Finally, effect of FAE immobilization on their feruloylating capability was studied using both modified and unmodified supports.

The results from this study were presented at Biotrans 2013 – 9th International Symposium on Biocatalysis & Biotransformations and submitted to a scientific journal.

Tamayo-Cabezas, J., & Karboune, S. (2018). Immobilized feruloyl esterase from Humicola insolens catalyzes the synthesis of feruloylated oligosaccharides. Process Biochemistry (forthcoming) doi: 10.1016/J.PROCBIO.2018.12.013.

CHAPTER III. IMMOBILIZED FERULOYL ESTERASE FROM HUMICOLA INSOLENS CATALYZED THE SYNTHESIS OF FERULOYLATED OLIGOSACCHARIDES

3.1. Abstract

Ferulic acid acylation of oligosaccharides catalyzed by feruloyl esterases (FAE) is a promising route to produce feruloylated oligosaccharides. However, modulation of FAE synthetic properties is a key step to improve the acylation. The efficiency of *H. insolens* FAE to catalyze the feruloylation in six different surfactantless microemulsions reaction systems was evaluated. The highest yield (57 %) was obtained with xylobiose in n-Hexane/1,4Dioxane/water; however, no significant feruloylation of raffinose and XOS could be achieved in the 1,4 Dioxane-based microemulsions. In contrast, the 2-Butanone-based microemulsions led to broader substrate specificity of FAE-catalysed feruloylation. The use of magnetic non-porous SiMAG-PGL microparticles led to the highest immobilization yield but the retained activity was low; while non-porous FluidMAG PEA nanoparticles resulted in a lower immobilization yield and a higher retention of activity. A good compromise between the immobilization yield and the retained activity was achieved with the porous epoxy-activated supports. Additional modification of the epoxy supports with iminodiacetic acid (IDA), with/without the inclusion of metal-chelate groups, led to higher immobilized FAE activity/gram of support. The feruloylation capacity of immobilized FAE was found to be dependent on the immobilization support.

3.2. Introduction

Feruloylated oligosaccharides are a subgroup of hydroxycinnamic acid derivatives formed by an oligosaccharide ester-linked to one or multiple ferulic acid units. These molecules exist naturally in the plant cell wall and have gained widespread interest due to their potential complementary antioxidant and prebiotic properties (Malunga and Beta, 2015; Ou et al., 2016; Zhang et al., 2016). Additionally, feruloylated oligosaccharides showed *in vitro* immunomodulatory activity over inflammatory mediators (Fang et al., 2012). Feruloylated oligosaccharides as functional ingredients might open a pathway for the safe deliver of antioxidant compounds in the lower gastrointestinal tract, reducing the risk of chronic inflammatory diseases and improving the host's immunity.

The isolation of feruloylated carbohydrates from plant cell walls are limited by the very low yield, the inconsistency of the isolated structures and by the predominant presence of ferulic acid as phenolated moiety. Enzymatic acylation of phenolic acids with carbohydrates in non-conventional media via carboxylic ester hydrolase-catalyzed esterification reaction is a promising route to

produce phenolated glycosides. Most reported studies in the literature are focused on the acylation of phenolic acids with alkyl fatty chains, where lipases were identified as appropriate biocatalysts (Karboune et al., 2005b; Sorour et al., 2012a, 2012b; Sun et al., 2013; Zhang et al., 2018). However, the use of lipases to catalyze the enzymatic feruloylation of glycosides is hindered due to the steric effect caused by the reduced flexibility of carbohydrates and by the lack of interfacial tension (Otto et al., 2000). Finding the most appropriate biocatalyst for the acylation of phenolic acids with carbohydrates is a key step. A regioselective enzymatic synthesis of feruloylated oligosaccharides using feruloyl esterases (FAE) expressed in a commercial multi-enzymatic preparation has been described as an alternative to the lengthy isolation of naturally occurring feruloylated oligosaccharides (Couto et al., 2011, 2010). FAE (EC 3.1.1.73), are a subclass of the carboxylic ester hydrolases, which catalyze the release of hydroxycinnamic acids from the plant cell wall. To reverse the thermodynamically favorable hydrolytic reaction towards a synthetic one, non-conventional media, surfactantless and ionic liquid emulsions have been used (Topakas et al., 2003; Vafiadi et al., 2009). The bioconversion yield of the enzymatic feruloylation was found to be dependent on the structural characteristics of the carbohydrate substrate and on the type of media (Couto et al., 2011). Large scale production of feruloylated oligosaccharides is limited by the poor availability of robust FAE specifically designed for this synthetic purpose. Enzyme immobilization is a promising approach for modulating the micro-environment of biocatalysts and for stabilizing their structures by retaining them on a support, a surface, or by forming aggregates within themselves. In addition, immobilization of enzymes offers the advantages of catalyst reusability, continuous operation, and simpler product purification (Cao, 2005a). Only few publications have investigated the immobilization of FAE and all of them were oriented to the subsequent use of the immobilized enzyme in the synthesis/hydrolysis of alkyl ferulates or the esterification of ferulic acid with polyols (Bonzom et al., 2018; He et al., 2015; Matsuo et al., 2008; Thörn et al., 2011; Vafiadi et al., 2008b; Zerva et al., 2018). The present research work aimed at the development of a novel biosynthetic approach to produce feruloylated oligosaccharides via FAE-catalyzed esterification reaction. First, the effects of the reaction media and glycoside substrate on the bioconversion yield of the feruloylation reaction catalyzed by the most efficient FAE preparation was evaluated. Then, the stabilization and the modulation of the catalyst's microenvironment was studied through the immobilization of the enzyme and the application of

pre- and post-immobilization-treatments. Subsequently, the immobilized FAE was used to catalyze the feruloylation reaction in surfactantless microemulsions.

3.3. Materials and methods

3.3.1. Materials

One purified FAE preparation from Humicola insolens and the commercial Depol 740L multienzymatic preparation produced by the same fungal source were kindly donated by Biocatalysts Limited (Wales, UK). Sugar-beet pulp was a gift from Lantic Inc. (Taber, AB, Canada). Wheat bran was acquired from a local grocery store. 1,4 β-D-Xylobiose was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Xylooligosaccharides (XOS) were kindly donated by Shandong Longlive Bio-Technology Co., Ltd. (Shandong, China). Sepabeads® supports were a gift from Resindion S.R.L. (Binasco, Italy). Magnetic silica microparticles (SiMAG-PGL) and nanoparticles (fluidMAG-PEA) were purchased from Chemicell GmbH (Berlin, Germany). Ethanol was obtained from Commercial Alcohols (Brampton, ON). Glycine, bovine serum albumin, iminodiacetic acid (IDA), sodium hydroxide, high performance liquid chromatography (HPLC) grade acetonitrile, 2-butanone, 2-heptanone, 1,4-dioxane, *n*-hexane, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Eupergit C, Eupergit C250L, D (+) raffinose pentahydrate, ferulic acid, ethanolamine, ethylenediamine (EDA), 4-Morpholinepropanesulfonic acid sodium salt (MOPS), 2-(N-morpholino)ethanesulfonic acid (MES), amylase and glucoamylase from *A. niger*, Driselase, Folin Ciocalteu's Phenol Reagent, sulfuric acid and all other chemicals were purchased from Sigma Chemical Co. (St-Louis, MO, USA). Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

3.3.2. Preparation of feruloylated oligosaccharides

The isolation of feruloylated non-digestible oligosaccharides (NDO) from sugar-beet pulp and wheat bran was carried out according to the procedure described by Couto et al. (2010). This method is a modification of the procedures applied by Ralet et al. (1994) for the extraction of feruloylated NDO in sugar-beet pulp, and by Bunzel et al. (2001) for the isolation of these compounds in wheat bran. After autoclaving at 121 °C for 45 min, wheat bran (10 g) was destarched using two succeeding treatments with α-amylase (1 mL, pH 6.0, 100 °C, 40 min) and amyloglucosidase (4 mL, pH 4.5, 60 °C, 60 min). Both sugar-beet pulp and destarched wheat bran

were ground to reduce their particle size to pass a 1.18 mm ground mesh screen. Sugar-beet pulp particles between 600 and 1180 μm were boiled in an aqueous ethanol solution (70 %, v/v) for 5 min and then extensively rinsed with the same ethanol solution at room temperature. While destarched wheat bran was, successively, washed two times with hot water, ethanol (95 %, v/v) and acetone. Both sugar-beet-pulp and destarched wheat bran residues were recovered by filtration on a G-4 sintered glass and then air-dried overnight at 40-60°C in a vacuum oven. The isolation of feruloylated NDO from these selected plant cell walls (0.8 g) was carried out using the multi-enzymatic Driselase (0.01 g mL^{-1}). Hydrolysis was carried out at 37°C with continuous shaking at 150 rpm for 24 h in an orbital incubator shaker (Forma Scientific, Inc.; Marjetta, OH), followed by addition of absolute ethanol (4x10 mL) to precipitate the polymeric fragments. The ethanol soluble fractions containing the phenolated NDO were recovered by centrifugation at 8000 *g* for 20 min and concentrated under vacuum to a final volume of 8 mL using an Automatic Environmental Speed Vac system (Savant Instruments Inc, Holbrook, NY).

3.3.3. FAE activity assay

The hydrolytic activity of solutions containing free FAE from *H. insolens* was assayed using methyl ferulate and feruloylated NDO, extracted from sugar-beet pulp and wheat bran as substrates, following the method described by Couto et al. (2010). The FAE activity assay was initiated by the addition of 0.1 mL of enzyme suspension at different dilutions to 0.7 mL of substrate solutions in MOPS buffer (0.1 mol/L, pH 6.0, 25 °C) (0.33-4 mmol/L of methyl ferulate and feruloylated NDO). The concentration of the hydrolyzed feruloylated substrate was monitored spectrophotometrically at 335 nm over a reaction period of 5 to 20 min using a Beckman Coulter DU-800 spectrophotometer (Beckman Instruments Inc.; San Raman, CA). Control trials without the enzyme or the substrate were carried out along with the enzymatic reactions to account for potential side reactions. All assays were run in triplicate. One unit of FAE activity (1 U) was defined as the amount of enzyme hydrolyzing 1 nmol of the feruloylated substrate/min under the experimental conditions previously described. The specific FAE activity was defined as nmol of hydrolyzed feruloylated substrate · mg of $protein^{-1}$ · min^{-1}. Immobilized FAE activity was defined as nmol of hydrolyzed feruloylated substrate · g of $support^{-1}$ · min^{-1}.

3.3.4. FAE immobilization

Modification of epoxy-activated supports. Partially modified epoxy-supports were prepared as previously described by Mateo et al. (2000). For the preparation of aminated epoxy-support, one gram of wet epoxy-activated support (Eupergit C, Eupergit C250L or Sepabeads EP-S-R) was suspended at room temperature in 10 mL of 5 % (v/v) EDA solution at pH 8.5 for 15 min under gentle stirring. For the preparation of iminodiacetic acid (IDA) modified supports, 1 g of wet epoxy-activated support was incubated for 5 h at room temperature in 5 mL of an IDA aqueous solution (1.8 mol/L, pH 9.0) under gentle stirring. To obtain chelate-epoxy supports, 1 g of IDA-epoxy supports is suspended in 6 mL of deionized water with 0.2 g of anhydrous cupric sulfate for 2 h under very gentle stirring. All the modified supports were washed 8 times with 10 mL of deionized water and 5 times with 10 mL of MOPS buffer (0.02 mol/L) before storage at 4 °C.

Enzyme immobilization. FAE was immobilized by ionic interaction on Sepabeads HA-S and fluidMAG-PEA supports, and by covalent attachment on Sepabeads HA-RS and SiMAG-PGL supports. Multipoint covalent attachment between the FAE and the support was promoted onto selected modified and unmodified epoxy-activated polymethacrylate beads (Eupergit C, Eupergit C250L, and Sepabeads EP-RS supports) by increasing the pH of the immobilization buffer. Prior to immobilization, all the prepared supports were washed 5 times with MOPS (0.2 mol/L, pH 6.0) or potassium phosphate buffer (0.2 mol/L, pH 8.0 and 1 mol/L, pH 6.0). Immobilization of FAE was carried out by suspending the support in the FAE solution to achieve an initial enzyme loading of 10 mg protein/g of support, except when using the magnetic nanoparticles (fluidMAG-PEA) where a ratio of 5 mg of protein/g of support was used. After incubation for 14 h or 36 h under gentle stirring at 6 ºC, the suspensions were centrifuged at 2400 x *g* for 5 min, and the recovered supernatants were analyzed for protein content and FAE activity. The supports containing the immobilized enzyme were washed with MOPS buffer (0.02 mol/L, pH 6.0) and resuspended in the same buffer before storage at 4 °C. Washing solutions protein content and FAE activity were also measured. Aliquots of the enzyme dilution were taken at the beginning of the immobilization procedure to measure the protein content (Bradford assay) and enzyme activity. A blank of FAE dilution without support was incubated along with the immobilization trials to account for any enzymatic inactivation that may occur during the incubation time. Immobilization yield (%) was calculated subtracting the FAE activity of the supernatant solution and the washing solution from the FAE activity of the blank FAE dilution divided by the initial activity of the blank FAE dilution,

multiplied by 100. Retention of FAE activity (%) was calculated by dividing the specific activity of the free FAE dilution by the specific activity of the immobilized FAE, multiplied by 100.

Effect of blocking agents on the immobilization efficiency. The blockage of the excess of epoxide moieties of the epoxy supports and its impact on the activity of the immobilized enzyme was studied. The supports containing the immobilized enzyme were incubated with a 3 mol/L glycine solution according to the procedure by Mateo et al., (2007) or 0.2 mol/L ethanolamine solution in 0.2 mol/L phosphate buffer, pH 8.0 under smooth stirring (Knežević-Jugović et al., 2011). After 8 h of incubation the samples were centrifuged, and supports were recovered and washed with 0.02 mol/L potassium phosphate buffer. FAE activity and protein content in the supernatant solution recovered after the blocking step were measured following the same procedure previously described.

3.3.5. Esterification reaction of di- and oligosaccharides with ferulic acid

Enzymatic esterification of ferulic acid with selected di- and oligosaccharides using free and immobilized FAE was carried out using three surfactantless organic microemulsion mixtures as reaction media at a ratio of 51:46:3 (v/v/v), according to a modification of the method reported by Topakas et al. (2005). Before esterification reaction, the selected di- and oligosaccharides (0.1 mol/L) were dissolved in deionized water or MES-NaOH buffer (0.02 mol/L, pH 6.0), while a ferulic acid stock solution (0.03 mol/L) was prepared in non-polar cosolvent. Substrate stock solutions were diluted to reach a final substrate molar ratio of ferulic acid to di- or oligosaccharide of 3:1 in the microemulsion media. The enzymatic esterification reaction was carried out in 25 mL Erlenmeyer flasks and initiated by the addition of 1000 U of enzyme. All flasks were vacuum sealed and incubated at 35 °C with continuous shaking at 70 rpm in a Forma Scientific orbital incubator shaker. Control trials without enzyme and glycoside substrate were carried out along the enzymatic reactions. All the reactions were run in duplicate. The esterified products were quantified by HPLC after 3 and 6 days of reaction period.

3.3.6. Analysis of the di- and oligosaccharide feruloylation

Quantitative analysis of reaction mixtures of di- and oligosaccharide feruloylation was carried out, according to a modification of the method of Couto et al. (2010). A Waters Breeze 2 HPLC system (Waters Corp., Milford, MA) equipped with a binary pump (Model 1525), and a photodiode array detector (Model 2998) was used for the quantitative analysis of the synthetic reaction. Feruloylated

compounds were retained in a Zorbax SB-C18 reversed-phase column (5 µm, 250×4.6 mm, Agilent Technologies Canada Inc.; Mississauga, ON), using a linear gradient elution that switched from 100% water/formic acid (8.5:0.5, v/v)/acetonitrile (80:20, v/v) to 60 % pure acetonitrile in a period of 30 min before reverting back to the initial eluent composition at a constant flow rate of 0.7 mL/min. Injection volume was 20 µL and detection of reaction components was performed in the UV spectra at 320 nm wavelength. Standard curves were obtained using feruloylated oligosaccharides isolated from wheat bran and ferulic acid. Bioconversion yield (%) was calculated from the concentration of the synthesized feruloylated glycosides divided by the initial concentration of the glycoside substrate, multiplied by 100.

Characterization of the feruloylated molecular structures was performed using an HPLC interfaced to an APCI-MS system (ThermoFinnigan, San Jose, CA, USA) equipped with a Surveyor LC pump, an autosampler coupled to an LCQ Advantage mass spectrometer (ion trap) and with Xcalibur® software (Version 1.3) to control the system acquisition and data processing. The mass spectrometer was operated in positive-ion mode with full scan detection in the m/z range of 200-1500, where the source of fragmentation was turned on (collision energy of 15 V). The ion spray and capillary voltage were set at 4.0 kV and 15.6 V, respectively.

3.3.7. Effect of reaction media on the bioconversion yield

Selected surfactantless organic microemulsions at a ratio of 51:46:3 (v/v/v) were used to assess the effect of reaction media composition on the bioconversion yield of the esterification reaction. The composition of the selected microemulsions was n-Hexane/2-Butanone/MES (pH 6.0), n-Hexane/2-Butanone/Water, n-Hexane/1,4 Dioxane/MES (pH 6.0), n-Hexane/1,4 Dioxane/Water, n-Hexane/2-Heptanone/MES (pH 6.0), and n-Hexane/2-Heptanone/Water.

3.3.8. Effect of glycoside structure on the bioconversion yield

The effects of glycoside structure and length on the bioconversion yield of the feruloylated glycosides were evaluated using selected di- and oligosaccharides as glycoside substrates; including D-xylobiose, D-raffinose, and XOS as glycoside substrates. Both FAE in free and immobilized forms were used to catalyze the feruloylation reaction.

3.4. Results and discussion

3.4.1. Specific activity of FAE

The hydrolytic activities of the FAE expressed in the multi-enzymatic Depol 740L preparation and in the pure FAE preparation from the same microbial source were investigated using three substrates: methyl ferulate, feruloylated arabino/galactooligosaccharides extracted from sugar-beet pulp, and feruloylated arabino(xylo)oligosaccharides isolated from wheat bran.

Table 3.1 Enzymatic activity of the FAE expressed in the multi-enzymatic preparation Depol 740L from *H. insolens* and in the pure preparation from the same fungal source on selected synthetic and naturally-occurring feruloylated substrates.

Enzyme	Specific Activity (nmol mg of protein^{-1} · min^{-1})		
	Methyl ferulate	Sugar-beet pulp feruloylated NDO	Wheat bran feruloylated NDO
Depol 740L	17.1	4.7	14.7
Pure FAE Preparation	4934.8	5177.1	5562.6

The results (Table 1) show that the substrate specificity of the FAE activity varied depending on the enzymatic preparation. As expected, the pure FAE preparation exhibited higher specific activity on methyl ferulate (4934.8 nmol · mg of protein^{-1} min^{-1}) than that of the multi-enzymatic Depol 740L product (17.1 nmol mg of protein^{-1} min^{-1}). The results also reveal that as compared to methyl ferulate substrate, the pure FAE preparation showed a similar hydrolytic catalytic efficiency on the feruloylated arabino(xylo)oligosaccharides isolated from wheat bran (5,562.6 nmol mg of protein^{-1} min^{-1}) and the feruloylated arabino/galactooligosaccharides obtained from sugar-beet pulp (5,177.1 nmol · mg of protein^{-1} · min^{-1}). These results reveal the large substrate specificity of this FAE variant for which its catalytic efficiency is independent on the type and the position of the sugar esterifying the ferulic group. On the contrary, the specific activity of the multi-enzymatic Depol 740L preparation and the other purified FAE variant was lower towards feruloylated NDO as compared to methyl ferulate. While higher substrate specificity towards the feruloylated arabino(xylo)oligosaccharides from wheat bran than arabino/galactooligosaccharides from sugar-beet pulp was observed with Depol 740L. These results may be attributed to the interaction of FAE with other glycosyl hydrolytic activities present in the multi-enzymatic Depol

740L preparation. Based on the specific activity values obtained with all the evaluated feruloylated substrates, the pure FAE preparation was selected for further study.

3.4.2. Effect of glycoside structure and reaction media on the bioconversion yield

The feruloylation of xylobiose, XOS, and raffinose by FAE-catalyzed esterification reaction was investigated in selected surfactantless microemulsions at a ratio of 51:46:3 (v/v/v). Table 3.2 shows that using the n-Hexane/2-Butanone/MES microemulsion medium, the highest bioconversion yield was obtained with xylobiose (27.4 %), while the bioconversion yield for the feruloylation of XOS (10.6 %) was lower than that obtained with raffinose (16.2 %). These results reveal the effect of the degree of polymerization of the saccharide donor on the feruloylation efficiency of FAE from *H. insolens*, which was not observed for hydrolytic reactions catalyzed by the same FAE. Similarly, Couto et al. (2011) reported that the FAEs expressed in Depol 740L exhibited more synthetic specificity towards monosaccharides than their corresponding di- and oligosaccharides using the same reaction medium. Compared to the feruloylation yield previously reported for enriched Depol 740 using n-Hexane/2-Butanone/MES microemulsion, yields obtained for the feruloylation of the xylobiose and XOSs by the FAE variant from *H. insolens* were 2.9-fold and 6.3-fold, respectively. (Couto et al., 2010). The substitution of MES buffer with water in the 2-Butanone-based microemulsion medium changes the pH of the microenvironment of the enzyme, which can explain the alteration of the saccharide substrate specificity of the FAE. Indeed, contrary to n-Hexane/2-Butanone/MES microemulsion, XOS led to a higher feruloylation yield of 17.8 % than those obtained with xylobiose (12.8 %) and raffinose (12.2 %) as acyl acceptors. Additionally, it has been shown through the molecular docking of the binding pocket of an immobilized FAE from *Fusarium oxysporum* that MOPS buffer molecules interact with amino acid residues in the active site of the enzyme; these interactions promote protein movement and alter the product selectivity of the enzyme (Thörn et al., 2013a). MES molecules may have caused a similar effect by interacting with amino acid residues on or near the active site of the FAE that might have led to protein structure changes, affecting the binding affinity of the saccharide substrate.

Table 3.2 Effect of media on the bioconversion yield of selected feruloylated di- and oligosaccharides obtained through FAE-catalyzed esterification reaction using 'free' FAE from *H. insolens*.

Media[c]	log P [d]	Xylobiose		XOSs		Raffinose	
		A[a]	B[b]	A[a]	B[b]	A[a]	B[b]
n-Hexane/2-Butanone/MES	1.80	27.4	273.7	10.6	106.1	16.2	161.8
n-Hexane/2-Butanone/ H_2O	1.81	12.8	128.4	17.8	178.3	12.2	121.6
n-Hexane/1,4 Dioxane/MES	1.31	0.7	6.7	< 0.1	< 1.0	< 0.1	< 1.0
n-Hexane/1,4 Dioxane/H_2O	1.31	57.0	570.0	0.4	3.8	0.3	2.7
n-Hexane/2-Heptanone/MES	2.78	< 0.1	< 1.0	24.6	246.1	< 0.1	< 1.0
n-Hexane/2-Heptanone/H_2O	2.79	8.1	81.4	41.6	416.1	< 0.1	< 1.0

[a] Maximum bioconversion yield (%) after 144 h. The bioconversion yield was calculated as the concentration of consumed glycosides over the initial concentration, multiplied by 100.

[b] Concentration of esterified glycosides (µmol/L)

[c] Reaction media at a ratio of 51:46:3 (v/v/v)

[d] Log P value is defined as the partition coefficient of the reaction media between water and 1-octanol. This value is obtained according to the formula – Log P mixture = X_1 Log P_1 + X_2 Log P_2 + X_3Log P_3 – in which X_1,X_2 and X_3 are the molar fractions of the components of the reaction media and Log P_1, Log P_2 and Log P_3 their Log P value.

The ketone in the microemulsion (2-butanone) was substituted with 1,4 dioxane or 2-heptanone. The use of 1,4 dioxane as co-solvent in the microemulsion medium resulted in a decrease of its log P value to 1.31. As a result, bioconversion yields of feruloylated carbohydrates were very low (< 0.4%) in this media, except for feruloylated xylobiose (57 %). The high bioconversion yield obtained with xylobiose might be attributed to the stripping water effect of 1,4 dioxane, which in this case may have favored the binding of the xylobiose at the FAE active site (Couto et al., 2011). Indeed, the hydrophilic/hydrophobic nature of the reaction system might have an effect on the ability of these media to strip the water from the enzyme microenvironment, affecting the interaction between the catalyst and water, which is critical for the activity of the enzyme (Halling, 2008). It has been reported that media with a high log P can improve the synthetic ability of hydrolases and maintain the enzyme active confirmation (Laane et al., 1987). This statement does not apply for all the FAE esterification reactions we evaluated. The use of n-hexane/2-heptanone/water or MES microemulsion media with the highest log P value (2.79) led to low feruloylation of xylobiose and raffinose or even none. In contrast, the esterification reaction of XOS with ferulic acid by FAE was efficient in the n-hexane/2-heptanone/water or MES microemulsion media with a yield of 24.6 % to 41.6 %, respectively.

The obtained bioconversion yields for xylobiose are in the same range than those reported for the feruloylation of arabinobiose with methyl ferulate as acyl acceptor, where a FAE type C from *Sporotrichum thermophile* converted 24 % of the disaccharide in a reaction media consisting of n-hexane/2-methyl-2-propanol/water 47.2:50.8:2.0 (v/v/v) (Vafiadi et al., 2006). The same authors reported the successful feruloylation of arabinotriose, arabinotetraose, arabinopentaose, and arabinohexaose although attained bioconversion yields were not disclosed.

3.4.3. Structural characterization of selected feruloylated di- and oligosaccharides

The end-products of the feruloylation reaction of xylobiose and XOS were characterized using HPLC/APCI-MS. The spectra reveal the simple and the multiple feruloylation of xylobiose and XOS catalyzed by FAE from *H. insolens*. Multiple feruloylation of the glycoside substrate was previously reported in the FAE-catalyzed feruloylation of fructooligosaccharides (Couto et al., 2011). Fragmentation pattern in Figure 1(a) corresponding to the reaction using xylobiose as glycoside donor shows abundant molecular ions at *m/z* 458.5 $[M+H]^+$ next to a smaller adduct at

441 $[M+H-H_2O]^+$. Two fragment peaks can be seen at *m/z* 282.2 and 264.1, which correspond to xylobiose $[M]^+$ and xylobiose with loss of a water molecule $[M-H_2O]^+$.

Figures 3.1(b) to 3.1(d) show fragmentation patterns of the products obtained with XOSs as acyl acceptor. Mass spectra of feruloylated xylotriose (Figure 3.1b) displays a molecular adduct at *m/z* 590.7 $[M+H]^+$ accompanied by another adduct at *m/z* 570.3 representing cationized xylotetraose $[M+Na]^+$. Fragment ions were observed at *m/z* 194.1 $[M]^+$ and 414.3 $[M]^+$ corresponding to ferulic acid and xylotriose, respectively. Fragmentation of the less polar peak (Figure 3.1c) shows abundant molecular ions at *m/z* 326.6 corresponding to feruloylated xylose, with the most prominent fragment ions observed at *m/z* 194.1 $[M]^+$ and 150.9 $[M]^+$ representing ferulic acid and xylose, respectively. While APCI-MS analysis of the most polar reaction product (Figure 3.1d) show multiple feruloylation of the oligosaccharide, which can be seen with the molecular adduct at *m/z* 913.2 corresponding to triferuloylated xylotriose $[M+2H-2H_2O]^+$. Other molecular ions found in the aforementioned mass spectra are feruloylated xylotetraose at *m/z* 723.6 $[M+H]^+$ and feruloylated xylohexaose at *m/z* 949.3 $[M+H-2H_2O]^+$. While fragment ions were observed at *m/z* 570.3 corresponding to cationized xylotetraose $[M+Na]^+$, and ions at *m/z* 414.3; and 194.1 correspond to xylotriose $[M]^+$ and ferulic acid $[M]^+$, respectively. The exact positions where the acylation of the glycosides took place are unknown and structures depicted in Figure 3.1 are for illustrative purposes only.

3.4.4. Immobilization of FAE on selected supports

FAE from *H. insolens* was immobilized on selected epoxy-activated and amino functionalized porous matrixes as well as on magnetic non-porous micro- and nanoparticles. The selected amino supports were an anionic exchanger (Sepabeads HA-S) and a glutaraldehyde-functionalized exchanger with a formyl group for covalent binding (Sepabeads HA-S-R), in which both amino supports exhibit moderate hydrophilic behavior. The epoxy-activated supports (Eupergit C, Eupergit C250L, Sepabeads EP-S-R) were more hydrophobic. The SiMAG-PGL magnetic non-porous microparticles were functionalized with glutaraldehyde to promote covalent linkage; FluidMAG PEA non-porous nanoparticles possess a long amino functional group that acts as a spacer and an anion exchanger for the immobilization of enzymes though ionic interaction.

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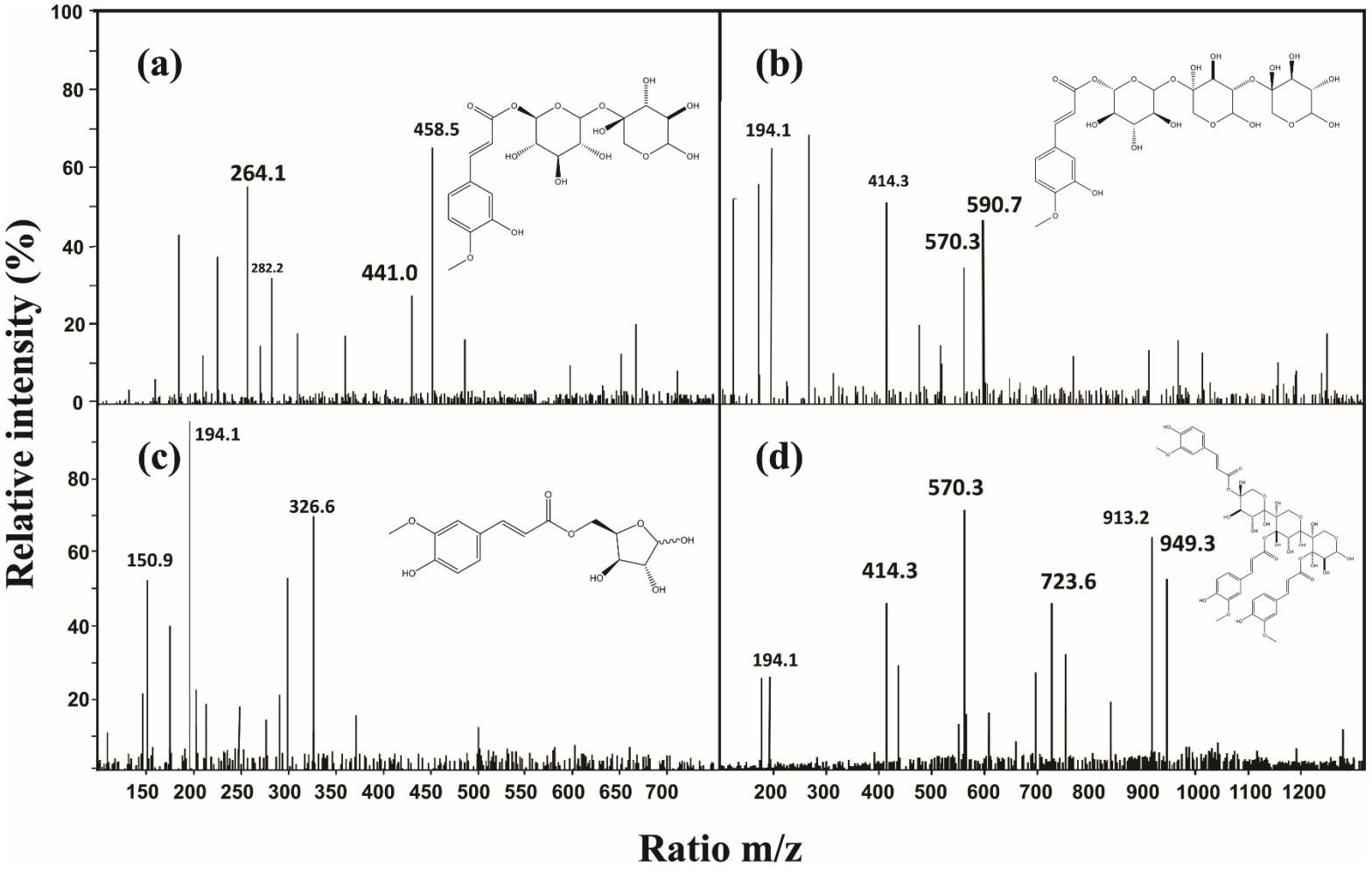

Figure 3. 1 APCI-MS spectrum of the fragmentation pattern of the feruloylated glycosides obtained by FAE from *H. insolens* feruloylation of xylobiose and XOS using a surfactantless microemulsion as reaction media: (a) feruloylated xylobiose, (b) feruloylated xylotriose (c) feruloylated xylose (d) triferuloylated xylotriose.

Table 3.3 Immobilization of FAE from *H. insolens* into selected commercial supports.

Support	pH	Immobilization Yield (%)	Activity (nmol/g support · min)	Retention of Activity[d] (%)
Eupergit C	6[a]	16.5 ± 0.8	2441.6 ± 163.6	56.1 ± 7.2
Eupergit C	8[b]	5.9 ± 0.3	1512.9 ± 84.7	129.9 ± 1.8
Eupergit C - 36 hours	8[b]	20.1 ± 0.2	1648.3 ± 21.4	31.1 ± 0.3
Eupergit C250L	6[a]	9.7 ± 0.7	1493.5 ± 44.5	122.4 ± 8.8
Eupergit C250L	8[b]	12.4 ± 1.2	2401.5 ± 105.2	73.3 ± 6.8
Sepabeads EP-S-R	6[c]	29.9 ± 2.2	4549.6 ± 551.4	57.8 ± 4.2
Sepabeads EP-S-R	8[b]	32.9 ± 3.0	2274.1 ± 272.9	26.3 ± 2.4
Sepabeads HA-S-R	8[b]	70.8 ± 0.7	444.6 ± 53.4	2.4 ± 0.2
Sepabeads HA-S	6[a]	36.5 ± 2.0	812 ± 36.4	8.4 ± 0.5
Sepabeads HA-S	8[b]	28.5 ± 3.1	628.1 ± 132.0	8.4 ± 0.9
SiMAG-PGL	8[b]	85.9 ± 8.3	4005.1 ± 921.2	17.7 ± 1.7
FluidMAG-PEA	8[b]	32.8 ± 2.9	3789.6 ± 299.4	276.45 ± 24.5

[a] Immobilization buffer: MOPS 0.2 mol/L.

[b] Immobilization buffer: Potassium phosphate 0.2 mol/L.

[c] Immobilization buffer: Potassium phosphate 1 mol/L.

[d] The retention of enzymatic activity is obtained dividing the specific activity of the native enzyme dilution by the specific activity of the immobilized FAE, multiplied by 100.

FAE was immobilized at two different pH values (pH 6.0 and pH 8.0) and at a constant moderate or high ionic strength (0.2 mol/L and 1 mol/L) for 14 h. According to the manufacturer, the working pH of the FAE from *H. insolens* ranges between 5.0 and 9.0, with optimum activity at pH 6.0 (Biocatalysts Ltd, 2010). Considering that a low enzyme loading reduces the effect of mass diffusional limitations, 10 mg of FAE protein/g of support was the enzyme load used for all investigated supports. The results are summarized in Table 3.3. As compared to the porous supports, Sepabeads HA-S-R support led to the he highest immobilization yield (70.2 %). This support possesses a linker functionalized with an amino group that has been previously activated with glutaraldehyde. With this type of carrier, immobilization occurs through covalent linkage between the aldehyde functional group and a primary amine group ($-NH_2$) of the enzyme's surface, creating an imino bond. Despite the high immobilization yield obtained with the Sepabeads HA-S-R support, the retention of activity was significantly low (2.4 %). This low retention of FAE activity upon immobilization may be explained by the high reactivity of glutaraldehyde with the primary amine groups of the enzymes, affecting the active tridimensional conformation of the enzyme and causing a steric hindrance of substrates at its active sites. Significant loss of enzyme activity was also observed when D-amino acid oxidase was immobilized in agarose previously activated with glutaraldehyde (Betancor et al., 2006).

Among the epoxy-activated supports, higher immobilization yields (29.9 - 32.9 %) were obtained with Sepabeads EP-S-R. Irreversible covalent immobilization of enzymes on epoxy supports occurs upon the reaction of the oxirane functional groups with amine and thiol groups on the protein surface, forming strong covalent bonds with minimal chemical modification of the protein. The oxirane groups loaded on the epoxy supports can react with different nucleophile groups of enzymes in a wide range of pH values (3.0 to 11.0). Incubation at higher pH values may be required to improve the activity of lysine residues in the enzyme surface and hence, promote multipoint attachment (Mateo et al., 2000). The results show an increase in the immobilization yields upon the increase of the immobilization pH from 6 to 8 using the epoxy-activated Sepabeads EP-S-R and Eupergit C250L. In contrast, the epoxy-activated Eupergit C led to lower immobilization yield at pH 8 than at pH 6. Nonetheless, it is important to mention that increasing the incubation time to 36 h augmented the immobilization yield at pH 8 from 5.9 to 20.1 % for Eupergit C support. Sepabeads EP-S-R support resembles Eupergit C in terms of functional groups, particle size and pore diameter. The main differences between them are the polymeric matrix of the support and its

internal morphology, giving Sepabeads supports a more hydrophobic behavior than that of Eupergit supports. It has been reported that the increase in hydrophobicity of the support might improve the preliminary adsorption of enzymes but it diminishes the retention and the long-term stabilization of the protein on the support (Mateo et al., 2002). This is in good agreement with our results, in which highly hydrophobic Sepabeads EP-S-R (26.3-57.8%) resulted in a lower retention of FAE activity than epoxy-activated Eupergit C (56.1-129.9%). However, the FAE activity retained upon the immobilization on epoxy-activated Sepabeads EP-S-R (26.3-57.8 %) was considerably higher than that obtained with glutaraldehyde-activated Sepabeads HA-S-R (2.4%). The results also indicate that the retention of FAE activity (73.3- 122.4%) was higher at both pH 6 and 8 upon immobilization on Eupergit C250L, revealing the ability of this support to preserve the active confirmation of the FAE. These findings may be due to the geometrical characteristics of Eupergit C250L, which has larger pores compared to the other epoxy supports evaluated, offering a larger plane surface to the FAE (Boller et al., 2002).

Table 3 shows that the immobilization yield of FAE on the ionic Sepabeads HA-S support (28.5 % - 36.5 %) was close to those obtained by covalent attachment on Sepabeads EP-S-R supports. Indeed, Sepabeads HA-S support possess spacers each one with a positively charged amino functional group, and the adsorption of the enzyme on this support involves ionic interaction with negatively charged regions on the enzyme surface. Although slightly higher immobilization yield was obtained at pH 6.0 than at pH 8.0, the FAE activity retained upon ionic immobilization on Sepabeads HA-S was similar and low at both pH values (8.4 %).

Comparable to the results obtained with Sepabeads HA-S-R support, 86 % of FAE was immobilized onto the SiMAG-PGL magnetic silica-coated microparticles, this high immobilization yield is due to the high reactivity between glutaraldehyde and reactive amino groups with low pK value present on the enzyme's surface. The main difference between SiMAG-PGL and Sepabeads HA-S-R supports is that the former support is non-porous and holds various glutaraldehyde functional groups attached to each of the particles. However, a major drawback in using supports pretreated with glutaraldehyde is potential rigidification of the enzyme onto the support, blocking the access of the substrates to the active sites of the protein. An evidence of this effect is the low retention of FAE activity (17.7 %) obtained upon immobilization on the SiMAG-PGL microparticles. FluidMAG PEA are magnetite nanoparticles covered by a hydrophilic polymer with an amine terminal functional group with strong positive charge. The immobilization

of FAE on the FluidMAG PEA support was achieved with a yield of 32.8 %. Immobilization in this support resulted in the highest retention of FAE activity (276.5 %) amongst the tested supports. This activation effect might be due to: (a) the presence of a spacer arm that provides separation between the enzyme and the support, or (b) to the use of particles at the nanoscale, believed to reduce the diffusional limitations commonly found in conventional heterogeneous immobilization systems.

3.4.5. Effect of pre-immobilization treatments

The immobilization on epoxy-activated supports (Eupergit C, Eupergit C250L, and Sepabeads EP-S-R) is first achieved through an adsorption of the protein followed by covalent immobilization on the support (Mateo et al., 2002). Post-immobilization treatments, aiming at the modification of some functional oxirane groups of epoxy-activated supports with EDA, IDA, or copper (Cu)-IDA, were evaluated to improve the immobilization of FAE from *H. insolens*. This modification generates additional functional groups that might be able to physically adsorb proteins prior to the covalent immobilization. Table 3.4 summarizes the results of FAE immobilization on modified epoxy-activated supports.

Pre-treatment with EDA introduces relatively long spacers bearing a positive charged amino group on the epoxy support. The modification of Eupergit C250L and Eupergit C by EDA increased the adsorption affinity on these supports attaining an immobilization yield of 16.2 % using EDA-Eupergit C250L and 27.9 % with EDA-Eupergit C. However, this modification caused a sharp decrease in the retention of activity of these supports. From 122.4 % to 17.1 % for the former and from 56.1% to 9.8 % for the latter. The adsorption step promoted by the EDA functional groups, which influenced the orientation of enzyme on the surface of the support, resulted in less active FAE. IDA is a dicarboxylic acid amine, which reacts with the oxirane group present in the epoxy-activated supports forming a functional group with two carboxylate anions that allows physical adsorption of the protein through ionic exchange with enzyme regions that are rich in positive charges. Compared to the unmodified support, the modification of Eupergit C with IDA improved the FAE immobilization yield at both pH values of 6.0 and 8.0; however, this modification decreased FAE on IDA-Eupergit C at high ionic strength (1 mol/L) improved both the yield (19.8 % to 25.8 %) and the retention of activity (17.5 % to 32.9 %) as compared to immobilization at low ionic strength (0.2 mol/L). This increase reveals the multi-covalent attachments of FAE on

IDA-Eupergit C, favoring the hydrophobic interactions at high ionic strength and hence the immobilization yield. Contrary to the Eupergit C, the modification of Eupergit C250L with IDA at low ionic strength improved significantly the immobilization yield and the retention of activity by 2.7 and 2.1 ratio, respectively (pH 6.0). Retention of activity almost tripled upon immobilization into modified IDA-Eupergit C250L at pH 8.0 compared to the non-modified support. On the other hand, IDA modification of Sepabeads EP-S-R negatively affected the immobilization of FAE.

Metal chelate supports have been used extensively in protein chromatography because of the ability of histidine residues to bind to several types of metal ions. The incubation of IDA-epoxy supports with a cupric sulfate solution creates an epoxy-metal chelate support that combines the properties of epoxy supports for enzyme immobilization with the good performance of metal-chelate affinity chromatography for protein purification. Epoxy-metal chelate supports have been used before for a single step purification-immobilization-stabilization of polyhistidine-tagged enzymes (Pessela et al., 2003). The immobilization yield and the retention of activity were enhanced with the inclusion of the Cu-IDA functional group into the Eupergit C matrix. Cu-IDA Eupergit C at pH 6.0 immobilized 45.3 % of FAE and retained 39.7 % of its initial activity. When the enzyme was immobilized into the modified Cu-IDA Eupergit C at pH 8.0, the immobilization yield was 1.4-fold the yield with the unmodified support. The retention of activity was estimated at 222 %, corresponding to a 1.7-fold compared to the retention of activity with the unmodified support at the same pH.

Similarly, Cu-IDA modification of Sepabeads EP-S-R drastically enhanced the immobilization efficiency of FAE from *H. insolens*. The immobilization yield obtained with this modification at pH 6.0 was 71.7 %, which corresponds to a 2.4-fold the immobilization yield obtained with the unmodified support. The retention of activity was 81.7 %, a 1.4-fold compared to the retention obtained with unmodified Sepabeads EP-S-R (pH 6.0). The Cu-IDA Sepabeads EP-S-R achieved an immobilization yield of 66.2 % at pH 8.0, doubling the yield obtained with immobilization into the unmodified support at the same pH; while the retained enzymatic activity was 97 %, equivalent to a 3.6-fold compared to the activity retained with immobilization into unmodified support.

Table 3.4 Immobilization of FAE from *H. insolens* into chemically modified epoxy-activated supports

Support	pH	Immobilization Yield (%)	Activity (nmol/g support · min)	Retention of Activity (%)
EDA[d]-Eupergit C	6[a]	27.9 ± 0.7	717.1 ± 129.1	9.8 ± 1.2
EDA-EupergitC250L	6[a]	16.2 ± 0.5	726.7 ± 79.9	17.1 ± 1.0
IDA[e]-Eupergit C	6[a]	19.8 ± 0.6	910.3 ± 127.1	17.5 ± 1.6
IDA-Eupergit C	6[c]	25.8 ± 0.3	2234.3 ± 446.9	32.9 ± 2.3
IDA-Eupergit C	8[b]	15.2 ± 0.4	801.5 ± 24.9	28.9 ± 2.7
IDA-Eupergit C250L	6[a]	26.0 ± 0.6	3457.8 ± 411.5	257.2 ± 8.0
IDA-Eupergit C250L	8[b]	10.4 ± 0.1	5845.8 ± 460.0	214.2 ± 20.4
IDA-Sepabeads EP-S-R	6[c]	12.3 ± 0.3	1245.0 ± 85.3	38.3 ± 2.0
Cu-IDA-Eupergit C	6[c]	45.3 ± 1.9	1838.4 ± 229.8	39.7 ± 2.6
Cu-IDA-Eupergit C	8[b]	8.4 ± 0.3	919.8 ± 35.1	222.0 ± 15.0
Cu-IDA-Sepabeads EP-S-R	6[c]	71.7 ± 1.3	12121.1 ± 2424.2	81.7 ± 6.4
Cu-IDA-Sepabeads EP-S-R	8[b]	66.2 ± 3.0	14420.8 ± 1874.7	97.0 ± 6.9

[a] Immobilization buffer: MOPS 0.2 mol/L.

[b] Immobilization buffer: Potassium phosphate 0.2 mol/L.

[c] Immobilization buffer: Potassium phosphate 1 mol/L.

[d] EDA: Ethylenediamine.

[e] IDA: Iminodiacetic acid

3.4.6. Effect of post-treatment on the immobilization efficiency of FAE

Following enzyme immobilization, it is advisable that the remaining oxirane groups of the epoxy-activated supports should be neutralized by incubating the immobilized enzymes with low molecular weight compounds containing amino groups. Through this reaction a more hydrophilic matrix can be generated and undesired side reactions between the substrate and the support are prevented. The effect of glycine (3 mol/L) and ethanolamine (0.2 mol/L) as blocking agents on the immobilization parameters were assessed using unmodified commercial supports (Eupergit C, Eupergit C250L and Sepabeads EP-S-R), and the modified IDA- Eupergit C. Table 3.5 summarizes the FAE specific activity and the retention of activity measured in the immobilization experiments with and without the blocking step.

Overall, glycine as a blocking agent resulted in a higher activity and retention of activity compared to the trials with ethanolamine. However, in all cases the enzymatic activity and the retention of activity decreased compared to the results obtained without post-treatment. Eupergit C250L blocked with glycine achieved a comparable enzyme activity to that of their unblocked counterpart (2159.5 nmol · g support^{-1} · min^{-1} vs. 2401.5 nmol · g support^{-1} · min^{-1}). However, the retention of activity decreased from 73.3 % in the original support to 14.4 % with the blocking step. While with IDA-Eupergit C blocked with glycine the retention of activity decreased by 79 % and the activity in the support decreased from 801.5 nmol · g support^{-1} · min^{-1} to 451.4 nmol · g support^{-1} · min^{-1}. In the experiments with ethanolamine as a blocking agent, the best results were obtained using Eupergit C and Eupergit C250L. Nonetheless, and this was a pattern in all the experiments with ethanolamine, the retention of activity and the activity of the support showed a sharp decrease. For instance, only 3.7 % of the original specific activity was retained upon ethanolamine addition into FAE-Eupergit C, being this the highest value of retention of activity obtained using ethanolamine as blocking agent.

3.4.7. Effect of enzyme immobilization on the feruloylation yield

Using xylobiose, XOS and glycerol as substrates, the feruloylation efficiencies of FAE immobilized into unmodified supports (Sepabeads HA, Sepabeads EP-SR, Eupergit C and Eupergit C250L) and three modified supports (IDA-Eupergit C, Cu-IDA Eupergit C, and Cu-IDA Sepabeads EP-SR) were investigated and compared with the feruloylation catalyzed by the free FAE from *H. insolens*. The supports were selected due to promising results obtained in the

previous sections of this study. Incubation of the FAE with the blocking agents was omitted since it greatly affected the activity of the immobilized enzyme. Figure 3.2 shows the retention of the bioconversion yield using immobilized FAE into the different supports. The retained bioconversion yield seems to be not only dependent on the type of the support, but also on the saccharide substrates. For xylobiose and XOS, higher retention of their feruloylation bioconversion yield was obtained upon the use of FAE immobilized on Eupergit C250L followed by Cu-IDA Eupergit C and Cu-IDA Sepabeads EP-R. Highest retention of the bioconversion (70.5 %) was achieved upon the use of FAE immobilized into Eupergit C250L for the feruloylation of XOS.

Table 3.5 Effect of selected epoxy-group blocking agents on the immobilization of FAE into selected supports.

Support	With blocking procedure[c]		Without blocking procedure	
	Activity[d]	Retention of Activity (%)	Activity	Retention of Activity (%)
Eupergit C[a]	136.5 ± 4.9	1.8 ± 0.1	1512.9 ± 84.7	129.9 ± 1.8
Eupergit C[b]	134.6 ± 14.0	3.7 ± 0.1	1512.9 ± 84.7	129.9 ± 1.8
IDA-Eupergit C[a]	451.4 ± 45.1	6.2 ± 0.5	801.5 ± 24.9	28.9 ± 2.7
IDA-Eupergit C[b]	84.9 ± 5.9	1.1 ± 0.1	801.5 ± 24.9	28.9 ± 2.7
Eupergit C250L[a]	2159.5 ± 431.9	14.4 ± 1.0	2401.5 ± 105.2	73.3 ± 6.8
Eupergit C250L[b]	106.2 ± 21.2	3.6 ± 0.3	2401.5 ± 105.2	73.3 ± 6.8
Sepabeads EP-S-R[a]	246.2 ± 12.7	6.5 ± 0.52	2274.1 ± 272.9	26.3 ± 2.4
Sepabeads EP-S-R[b]	44.6 ± 8.9	1.3 ± 0.01	2274.1 ± 272.9	26.3 ± 2.4

[a] Blocking agent: Glycine 3 mol/L.

[b] Blocking agent: Ethanolamine 0.2 mol/L.

[c] Incubation with all the blocking agents was performed at pH 8.

[d] The activity of the immobilized enzyme is given in nmol/g support . min.

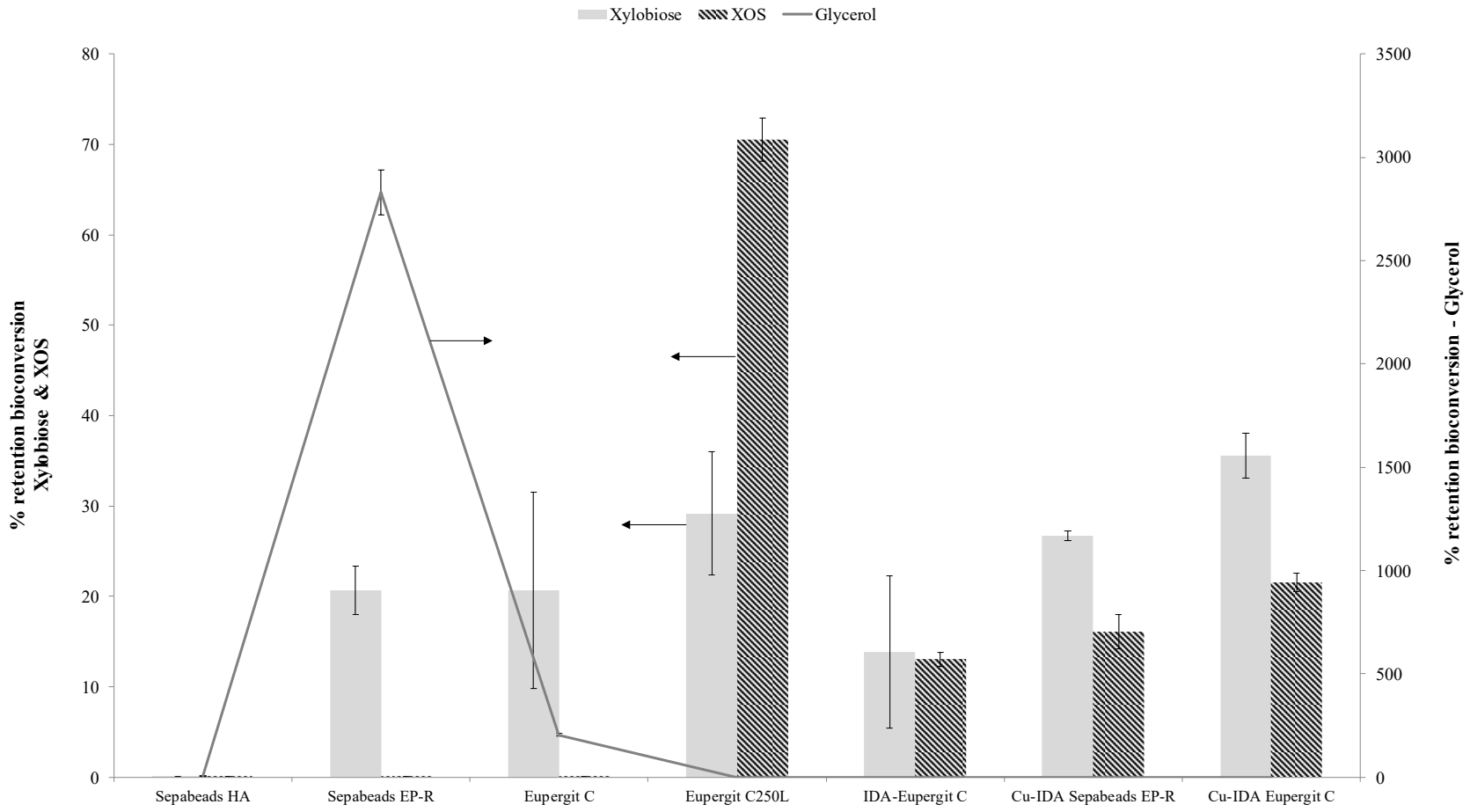

Figure 3.2 Retention of the bioconversion yield in selected feruloylation reactions catalyzed by FAE from *H. insolens* immobilized into modified and unmodified supports.

Larger substrates require larger pores and lower porosity to avoid the immobilization of the enzyme in the inner pores that would be hardly accessible by substrates with a long chain, as is the case of XOS. Thörn et al. (2011) also reported better immobilization results and transeserification yields using supports with a larger pore size. In addition, the modification of the epoxy supports with IDA and Cu-IDA functional groups is expected to increase the hydrophilicity of the enzyme's micro-environment, enhancing the availability of XOS substrate at the active site of FAE immobilized on modified supports as compared to the non-modified ones. The observed higher bioconversion yields compared to the non-modified supports confirm the presence of this micro-environment effect upon the modification of the epoxy supports. On the contrary, mesoporous epoxy supports (Eupergit C and Sepabeads EP-S-R) showed an activation effect over FAE in the reactions with glycerol as acyl acceptor. This effect might be caused by the creation of a more hydrophobic microenvironment surrounding the enzyme, which is more compatible with this substrate. As expected, the modification of the epoxy-supports decreased the binding affinity of glycerol resulting in a very low retention of its bioconversion. The accumulation of glycerol in the micro-environment of the more hydrophilic modified supports may have led to enzyme inhibition.

The bioconversion yields were generally low using the FAE immobilized on Sepabeads HA-S. Physical adsorption is generally too weak to keep the enzyme immobilized on the carrier; organic solvents may have promoted desorption or conformational changes of the enzyme. Additionally, the length of the spacer may have probably increased the exposure of the enzyme to the reaction media, which may have caused its deactivation. It has been reported that the commercial preparation Depol 740L was physically adsorbed onto mesoporous silica (Thörn et al., 2011) and used for the transesterification of methyl ferulate with 1-butanol in a reaction media composed of 92.5 % 1-butanol and 7.5 % MOPS buffer (pH 6.0). The immobilized enzyme catalyzed the conversion of 90 % of the phenolic methyl ester into butyl ferulate. Nonetheless, our results suggest that covalent binding of the FAE to the epoxy supports provides higher enzyme stability in the evaluated surfactantless microemulsion compared to the FAE physically immobilized through ionic interactions.

Vafiadi et al. (2008a) have prepared crosslinked enzyme aggregates (CLEA) of *A.s niger* type-A FAE using glutaraldehyde as the crosslinker agent and investigated its potential to catalyze the transesterification reaction between methyl sinapate and 1-butanol. These CLEA catalyzed a 78 % conversion of the hydroxycinnamate ester. In addition, FAE expressed in the commercial

preparation Pectinase PL from *A. niger* were immobilized by covalent attachment onto chitin-based gels activated with glutaraldehyde (Matsuo et al., 2008). Immobilized FAE converted 48 % of the ferulic acid to glyceryl ferulate in a reaction medium of (98:2 v/v) glycerol:acetate buffer (0.1 mol/L, pH 4.0). However, one known major drawback of the immobilization techniques that use glutaraldehyde as a crosslinker or activation agent is that it might deactivate certain enzymes. This inactivation effect of glutaraldehyde was clearly seen in our immobilization experiments using the Sepabeads EP-HA-R support.

3.4.8. Effect of Cu-IDA modification on the immobilization of FAE

Since higher immobilization yield and retention of FAE activity was achieved with immobilization into Cu-IDA Sepabeads EP-S-R, we proceeded to investigate how metal-chelate modification of epoxy supports affects the immobilization efficiency of FAE from *H. insolens*. Preparation of this heterofunctional support follows a two-step procedure. First, Sepabeads EP-S-R are incubated with a 1.8 mol/L of IDA solution that reacts with the epoxide groups over the support surface. Then, the beads are thoroughly washed and a copper sulfate solution (0.13 mol/L) is added to the support for 2 h, forming the metal-chelate functional group (Mateo et al., 2000). Modification with IDA was tested at different pH values (9.0 and 11.0) and incubation times (5, 24, and 48 h). The obtained results (Figure 3.3a) indicate that increasing the pH of the IDA solution enhanced the immobilization yield, while longer incubation times result in a higher retention of enzymatic activity upon immobilization. No major variation in the immobilization yield was observed upon increasing the incubation time. To optimize the second step of the preparation of Cu-IDA epoxy supports, the modification with copper sulfate at three different support/$CuSO_4$ solution ratios was investigated. Figure 3b displays the effect of copper modification of Sepabeads EP-S-R on the immobilization of FAE at different ratios (w/v) of support/$CuSO_4$ (0.13 mol/L) solution. The immobilization yield was higher at a support/$CuSO_4$ ratio of 1:6.

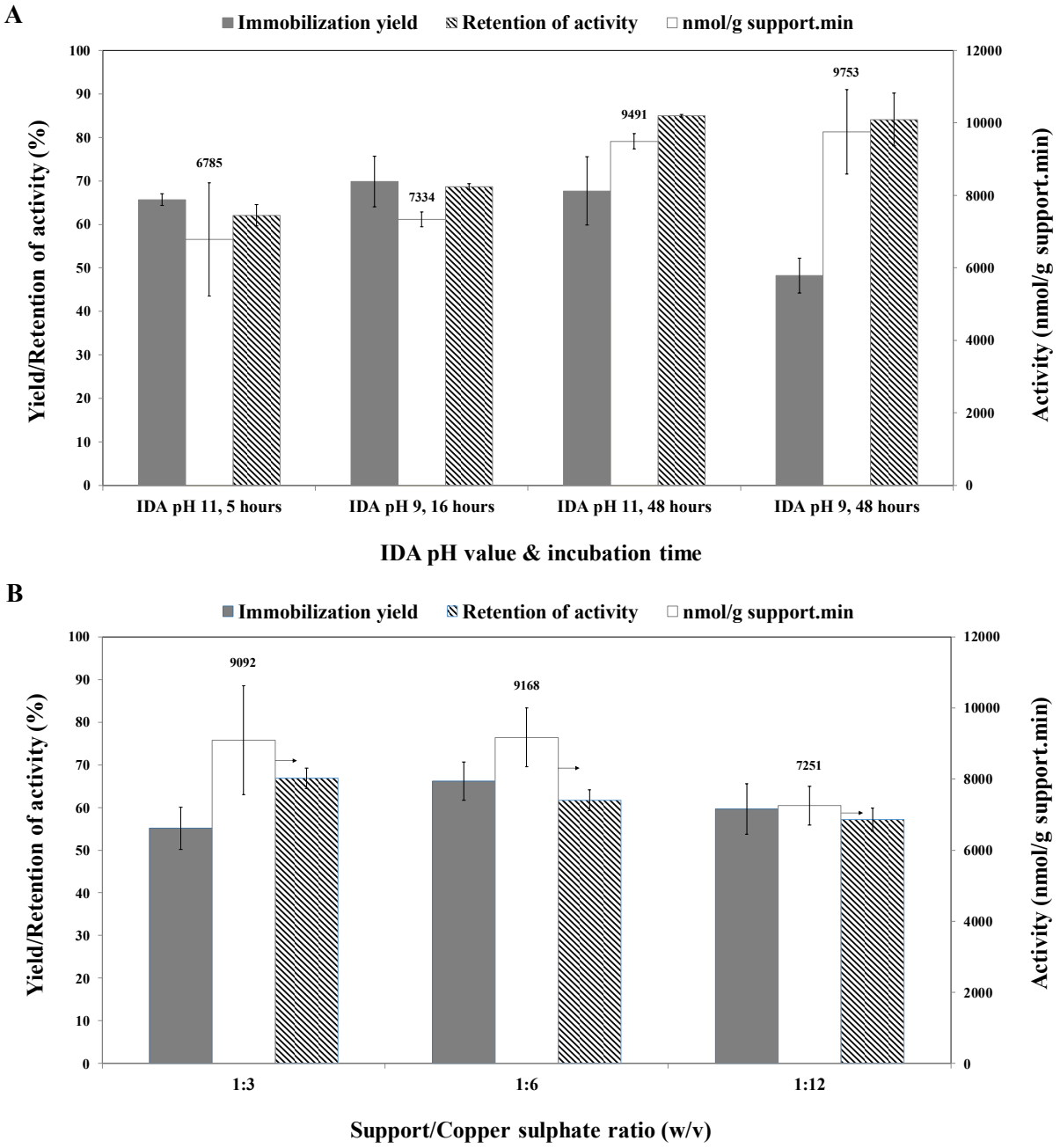

Figure 3.3 Effect of (a) iminodiacetic acid (IDA) and (b) copper sulfate modification of the epoxy support Sepabeads EP-S-R on the immobilization of a FAE from *H. insolens*.

3.5. Conclusions

The esterification of selected oligosaccharides with ferulic acid catalyzed by a FAE purified preparation from *H. insolens* was studied in different surfactantless microemulsions. Reaction medium composed of n-hexane/2-butanone/water favoured the acylation of all the evaluated oligosaccharides being the selected composition for the reaction media for the remaining of the investigation. Xylobiose was determined to be the most effective acyl acceptor compared to raffinose and XOS, confirming the direct effect of carbohydrate length on the FAE-catalyzed feruloylation of oligosaccharides. Epoxy supports were the most adequate for the immobilization of the FAE in study. Protein immobilization and the retention of enzymatic activity were enhanced through the inclusion of metal-chelate functional groups through partial chemical modification of the epoxide in the supports. Immobilized FAE onto Cu-IDA epoxy supports retained up to 35 % of the synthetic activity of its free counterpart. The development of a model biocatalytic approach based on the FAE-catalyzed esterification reaction is of great interest for the preparation of well-defined non-digestible phenolated oligosaccharides with nutraceutical functionalities. Such study is expected to lay out the scientific-based foundation for the efficient synthesis of novel bioactive molecules of a potential use as health-promoting ingredients in functional foods.

CONNECTING STATEMENT 2

The immobilization of FAE by covalent attachment and ion exchange was thoroughly investigated in chapter III. It was found that metal-chelate-epoxy support was the most adequate support for the immobilization of the selected FAE preparation and its posterior use for synthetic purposes. Chapter IV focuses on the optimization of the FAE immobilization onto a mesoporous metal-chelate-epoxy support by applying RSM at a 5-level 4-variable CCRD. The variables included in the experimental design were mg FAE/g support, immobilization buffer pH and molar concentration, and immobilization time. Statistical significance and interaction of the variables on the FAE hydrolytic activity yield, specific activity retention, and protein yield responses were determined through an analysis of variance.

After RSM optimization, the effect of pore size of the supports on the promotion of multipoint immobilization between the enzyme and the metal-chelate-epoxy support was studied. The impact of multipoint immobilization on the hydrolytic activity and thermostability of the enzyme was evaluated. Lastly, optimally immobilized and stabilized FAE was used to catalyze the ferulic acid acylation of xylobiose and XOS.

The results from this study were presented at the 2014 IFT Annual Meeting & Food Expo-The Institute of Food Technologists.

Tamayo-Cabezas, J., & Karboune, S. (2018). Optimum conditions for the immobilization and stabilization of feruloyl esterase from *Humicola insolens* on a heterofunctional metal chelate-epoxy support and its application for the feruloylation of oligosaccharides (*To be submitted*).

CHAPTER IV. OPTIMUM CONDITIONS FOR THE IMMOBILIZATION AND STABILIZATION OF FERULOYL ESTERASE FROM HUMICOLA INSOLENS ON A HETEROFUNCTIONAL METAL CHELATE-EPOXY SUPPORT AND ITS APPLICATION FOR THE FERULOYLATION OF OLIGOSACCHARIDES

4.1. Abstract

Immobilization of a feruloyl esterase (FAE) from *Humicola insolens* into a modified metal chelate-epoxy support Sepabeads® EC-EP R was optimized using response surface methodology on a central composite rotatable design of 5 levels and 4 variables. Immobilization time (27.7 h), pH of the immobilization buffer (5.0) and interaction between the ratio of enzyme to immobilization support (22.75 mg/g) with the immobilization buffer molarity (0.86 mol/L) were found to be the optimum conditions for FAE-immobilization with this modified support. Immobilization under these conditions increased the enzymatic activity yield by 33.0% and the retention of specific activity by 47.0% compared to the unoptimized immobilization experiments using the same immobilization support. Further incubation of the immobilized FAE at pH 10.0 improved the thermostability of the enzyme. Increasing the pore size of the support improved the retention of FAE hydrolytic activity and the esterifying efficiency of the immobilized biocatalyst. Optimally immobilized and stabilized FAE retained up to 92.9% of the feruloylation activity of the free enzyme. This study is expected to lay the ground for a better immobilization of FAE of great potential for the synthesis of amphiphilic compounds intended to food or pharmaceutical applications.

4.2. Introduction

Feruloylated glycosides are the main form of hydroxycinnamate derivative found in nature, playing important defense and structural roles in the growing and maturation of the plant cell wall. With a generic structure of one moiety of ferulic acid esterified to a mono- or oligosaccharide chain, feruloylated oligosaccharides (FO) have attracted scientific attention due to the prebiotic and antioxidant functions inherent to their structural features. Recent *in vitro* research showed that feruloylated arabinoxylooligosaccharides (AXOS) are fermented by cultured human colon microbiota (Snelders et al., 2014b), while FO isolated from corn bran showed higher protective effect than ferulic acid against oxidative stress in H_2O_2-injured pheochromocytoma cells (Yao et al., 2014). Moreover, FO also exhibited promising techno-functional capabilities. Studies show that FO affect the formation of gels (Robertson et al., 2008), improve the antioxidant capacity of bread (Snelders et al., 2014a), and induce less retrogradation during low temperature storage than native starch (Ou et al, 2001). They also provided an adequate matrix for the gel entrapment of

proteins (Berlanga-Reyes et al., 2009), which opens a pathway for targeted delivery of nutraceuticals to specific parts of the gastrointestinal system.

Feruloyl esterase (FAE)-catalyzed esterification reaction of ferulic acid and oligosaccharides in ternary surfactant-free reaction media has been identified as a potential route for the biosynthesis of feruloylated saccharides with consistent structures (Couto et al., 2011). However, this enzymatic approach requires proper modulation of the enzyme structure and microenvironment by immobilization to increase its stability and to allow the process to become industrially feasible. Enzyme immobilization is a set of techniques aimed at stabilizing enzymes by retaining the molecules on a surface or support, where the enzyme may ultimately be reused and/or constrained to a three-dimensional space (Cantone et al., 2013). Enhanced enzymatic stability resulting from immobilization can be attributed to specific effects such as molecular confinement, favorable microenvironment, chemical modification, or rigidification of the protein structure (Cao, 2005a; Moehlenbrock and Minteer, 2011). Previously, our team evaluated the immobilization of FAE from *Humicola insolens* into selected modified and unmodified commercial supports (Tamayo-Cabezas and Karboune, 2018). It was observed that heterofunctional supports bearing metal-chelate and epoxide functional groups were the most suitable for this enzyme. Multipoint covalent attachment is an immobilization/stabilization technique that involves the formation of numerous covalent bonds between nucleophiles on the enzyme's surface and epoxy-activated supports. Multipoint covalent attachment aims to develop a rigidification of the enzyme structure of the immobilized enzyme onto the support that may help to avoid conformational changes caused by heat or organic solvents (Mateo et al., 2007). The downside of this technique is that it requires long reaction times at alkaline pH values, which may be detrimental for most enzymes or for the immobilization itself. A two-step strategy for FAE immobilization into metal chelate-epoxy supports where immobilization first occurs through chelation at mild reaction conditions followed by stabilization of the enzyme at higher pH for a shorter time might help to overcome this potential issue.

The present study was aimed at optimizing the immobilization of a FAE from *H. insolens* into a metal-chelate-epoxy support and evaluating the effect of the support pore size and the promotion of multipoint covalent attachment between enzyme and support on the catalytic properties of the FAE. Firstly, response surface methodology (RSM) based on a 5-level 4-variable design was used to find the most significant factors that affect FAE immobilization. Then, immobilization onto

three metal chelate-epoxy supports differing only on their pore size was investigated, followed by assessing the development of multipoint covalent attachment at different pH values and incubation times. Experimental conditions that showed higher enzyme stability and activity were used to investigate the catalytic efficiency of immobilized FAE to esterify ferulic acid with selected oligosaccharides – xylobiose and xylo-oligosaccharides (XOS) – in a surfactantless microemulsion (n-hexane/2-butanone/water, 51/46/3 v/v/v). This study is expected to provide a better understanding of the variables affecting the immobilization of esterases for synthetic purposes.

4.3. Materials and methods

4.3.1. Materials

FAE from *H. insolens* was donated by Biocatalysts Limited (Wales, UK). 1,4 β-D-Xylobiose was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). XOS were donated by Shandong Longlive Bio-Technology Co., Ltd. (Shandong, China). Sepabeads® EC-EP R, Relizyme® EP403 R, and Relizyme® EP113 R supports were a gift from Resindion S.R.L. (Binasco, Italy). Ethanol was obtained from Commercial Alcohols (Brampton, ON). Bovine serum albumin (BSA), iminodiacetic acid (IDA), sodium hydroxide, HPLC grade acetonitrile, 2-butanone, *n*-hexane, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Ferulic acid, 4-morpholinepropanesulfonic acid sodium salt (MOPS), 2-(N-morpholino) ethanesulfonic acid (MES), and all other chemicals were purchased from Sigma Chemical Co. (St-Louis, MO, USA). Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

4.3.2. Preparation and modification of epoxy-activated supports

Partially modified epoxy-supports were prepared as previously described by Mateo et al., (2000). For the preparation of IDA-epoxy supports, one gram of wet epoxy-activated support was incubated for 5 h at room temperature in 5 mL of an IDA aqueous solution (1.8 mol/L, pH 11.0) under gentle stirring. Metal chelate (Cu-IDA)-epoxy support was prepared suspending 1 g of IDA-epoxy support in 6 mL of deionized water containing 0.2 g of anhydrous cupric sulphate for 2 h under very gentle stirring. Then, support beads were washed 8 times with 10 mL of deionized water and 5 times with 10 mL of MOPS buffer 0.02 mol/L before storage at 4 °C.

4.3.3. FAE immobilization

Prior to immobilization, Cu-IDA epoxy supports were washed 5 times with potassium phosphate buffer adjusting the pH value (4-8) and ionic strength of potassium phosphate buffer (0.27-1.25 mol/L), according to the experimental conditions to be performed. Immobilization of FAE was carried out by suspending the selected support in the FAE solution to yield final ratios ranging from 0.003 g of protein/g of support to 0.029 g of protein/g of support. After incubation under gentle stirring at room temperature, the suspensions were centrifuged at 2400*g* for 5 min, and the recovered supernatants were analyzed for protein content and FAE activity. The support beads containing the immobilized enzyme were thoroughly washed with MOPS buffer (20 mmol/L, pH 6.0), centrifuged, and resuspended in the same buffer before storage at 4 °C. Washing solutions protein content and FAE activity were also measured. Aliquots of the FAE solution were taken at the beginning of the immobilization procedure to measure the protein content and enzyme activity. Bradford assay was used to determine the content of the soluble protein content in the enzyme, supernatant and washing solutions (Life Science Group - Bio-Rad Laboratories, 1994). A blank of enzyme solution without support was incubated along with the immobilization trial to account for any enzyme inactivation that may have occurred during the incubation time. Protein yield (%) was calculated subtracting the protein content of the supernatant solution and the washing solution from the protein content of the native FAE dilution divided by the protein content of the FAE dilution, multiplied by 100. Activity yield (%) was calculated subtracting the FAE hydrolytic activity of the supernatant solution and the washing solution from the FAE hydrolytic activity of the blank FAE dilution divided by the hydrolytic activity of the blank FAE dilution, multiplied by 100. Retention of FAE activity (%) was calculated by dividing the specific activity of the native FAE dilution by the specific activity of the immobilized FAE, multiplied by 100.

4.3.4. FAE activity assay

The hydrolytic activity of free FAE from *H. insolens* was assayed using methyl ferulate as a substrate, following the method described by Couto et al. (2010). Methyl ferulate solution (33 μmol/L) was prepared in deionized water while FAE solutions were prepared in immobilization buffer. The FAE activity assay was carried out at 25 °C in a 1 mL cuvette. The assays were initiated by the addition of 0.1 mL of FAE sample at different dilutions to 0.7 mL of methyl ferulate solution. The concentration of the consumed feruloylated substrate was monitored in a

spectrophotometer at 335 nm over a reaction period of 5 min using a Beckman Coulter DU-800 spectrophotometer (Beckman Instruments Inc.; San Raman, CA).

FAE hydrolytic activity of the immobilized enzyme was measured as follows: first, 4 dilutions of immobilized FAE with dilution factors from 100 to 500 (w/v) were prepared in MOPS buffer (20 mmol/L, pH 6.0). Then, an aliquot of 0.1 mL of these dilutions was added to 0.7 mL of methyl ferulate solution (33 μmol/L). Samples were heated for 4 min at 37 °C to deactivate the FAE and centrifuged at 2400*g* for 3 min prior to the measurement of the absorbance at 335 nm. The absorbance values were plotted against the dilution factor of the samples.

To quantify the hydrolyzed methyl ferulate, a standard curve was constructed using a defined concentration of methyl ferulate under each condition of immobilization buffer ionic strength. Either with free or immobilized FAE, control trials without the enzyme or the substrate were analyzed along with the enzymatic reactions to account for potential side reactions. All assays were run in triplicate. One unit of FAE (1 U) was defined as the amount of enzyme hydrolyzing 1 nmol of the feruloylated substrate/min. The specific FAE activity was defined as nmol of hydrolyzed feruloylated substrate · mg of protein^{-1} · min^{-1}. Immobilized FAE activity was defined as nmol of hydrolyzed feruloylated substrate · g of immobilization support^{-1} · min^{-1}.

4.3.5. Optimization of FAE immobilization

RSM was used to optimize the levels of variables known to influence the immobilization of FAE onto metal-chelate Sepabeads® EC-EP R and to assess the interactions between them. RSM using a modified 5-level, 4-variable central composite rotatable design (CCRD) with 5 center points, 17 factorial points, and 8 star points was used as the experimental design. The variables included in the design and their levels were mg of FAE/g of support (3.25, 9.75, 16.25, 22.75, 29.25), immobilization buffer pH (4.0, 5.0, 6.0, 7.0, 8.0), immobilization buffer molarity (0.27, 0.51, 0.76, 1.0, 1.25 mol/L), and immobilization time (14, 20, 26, 32 and 38 h). Ranges were selected based on the results obtained with preliminary immobilization experiments.

4.3.6. Statistical analysis

Activity yield, specific activity retention, and protein yield were taken as responses upon completion of the optimization trials. The obtained experimental data was fitted into a first or second order polynomial equation by multiple regression analysis using the software Design-

Expert 8.0.2 (Stat-Ease, Inc., Minneapolis, MN, USA). Two-factor interaction model (1) and quadratic model (2) were expressed by the following equations:

$$Y_n = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 \quad (1)$$

$$Y_n = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 \quad (2)$$

Where Y_n is the predicted response, β_0 is the intercept; β_1, β_2, β_3, and β_4 are the linear terms; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} the interaction terms; β_{11}, β_{22}, β_{33}, and β_{44} the quadratic terms; and $X_{1\text{-}4}$ are the independent variables of the design. Model fitting accuracy was expressed by the coefficient of determination R^2 and its statistical significance was determined by Fisher's test (F-test).

4.3.7. Effect of multipoint covalent attachment on the immobilized FAE thermal stability

Immobilized FAE was gently stirred in sodium carbonate buffer solution (0.1 mol/L) to promote multipoint covalent attachment between the epoxy groups in the support and lysine-rich regions on the enzyme's surface, following the protocol of Mateo et al. (2007). Each sample was incubated for 4 h or 14 h either at pH 8.0 or pH 10.0. Then, the beads were meticulously washed with MOPS buffer (20 mmol/L, pH 6.0) and submerged in a water bath at 50 °C for 2 h. Along with the immobilized FAE, a solution of free FAE was incubated at the same conditions of temperature and time. Loss of specific activity retention was defined as the ratio of specific activity in the samples after and before thermal incubation, multiplied by 100.

4.3.8. Effect of pore size and functional group density on FAE immobilization.

FAE was immobilized onto three Cu-IDA epoxy supports with equal polymeric matrix and particle size but different porosity degrees and oxirane group density. The technical parameters of the immobilization supports evaluated in this section are given below

Table 4.1 Physicochemical parameters of meso- and macroporous epoxy immobilization supports (Resindion S.r.l., 2011).

Support	Average pore diameter (nm)	Group density (μmol/g dry)	Particle size range (μm)
Sepabeads® EC-EP R	10-20	min. 200	
Relizyme® EP113 R	20-50	min. 200	100-300
Relizyme® EP403 R	40-60	min. 100	

Immobilization conditions (temperature, time, concentration of the immobilization buffer, and pH of the immobilization buffer) were defined by specific optimization criteria using the models obtained through RSM. Then, the promotion of the multipoint covalent attachment was applied at experimental conditions where lower loss of specific activity retention occurred.

4.3.9. Esterification reaction of di- and oligosaccharides with ferulic acid

Enzymatic esterification of ferulic acid with xylobiose and XOS using immobilized FAE was carried out using a ternary surfactant-free mixture composed of *n*-hexane/2-butanone/water at a ratio of 51:46:3 (v/v/v) as reaction media, according to a modification of the method reported by Couto et al. (2010). Before esterification reaction, xylobiose and XOS (0.1 mol/L) were dissolved in aqueous solution, while ferulic acid solution (0.03 mol/L) was prepared in 2-butanone. Stock solutions were diluted to reach a final substrate molar ratio of ferulic acid to oligosaccharide of 3:1. The enzymatic esterification reaction was carried out in 4 mL amber vials and initiated by the addition of free or immobilized enzyme. All flasks were vacuum sealed and incubated at 35 °C with continuous shaking at 70 rpm in a Forma Scientific orbital incubator shaker. Control trials without enzyme and without glycoside substrate were carried out alongside the enzymatic reactions. Reactions were run in duplicate or in triplicate where possible.

4.3.10. Analysis of feruloylated di- and oligosaccharides

Quantitative analysis of reaction mixtures of the di- and oligosaccharide feruloylation was carried out, according to a modified method of Couto et al. (2010) using a Waters 2795 separation module (Waters Corp., Milford, MA) coupled to a Waters 996 photodiode array detector. Feruloylated

compounds were separated on a Zorbax SB-C18 reversed-phase column (5 µm, 250×4.6 mm, Agilent Technologies Canada Inc.; Mississauga, ON), using a linear gradient elution from 80% water/formic acid (8.5:0.5, v/v)/ 20% acetonitrile (v/v) to 100% pure acetonitrile in a period of 30 min before reverting to the initial eluent composition at a constant flow rate of 0.7 mL/min. Injection volume was 10 µL and detection of reaction components was performed at 320 nm wavelength. Standard curves were obtained using ferulic acid. Bioconversion yield (%) was calculated from the concentration of the synthesized feruloylated glycosides divided by the initial concentration of glycoside substrate in the reaction, multiplied by 100.

4.4. Results and discussion

4.4.1. Optimization of FAE immobilization

RSM employing CCRD was applied to optimize the selected variables – enzyme/support ratio, immobilization time, pH of the immobilization buffer, and immobilization buffer concentration – identified by our previous study to possess most influence on the immobilization efficiency of FAE into Cu-IDA epoxy mesoporous supports (Tamayo-Cabezas and Karboune, 2018). The central conditions of the experimental design were 16.25 mg FAE/g support, 26 h of immobilization time, potassium phosphate buffer with a pH of 6.0 and a concentration of 0.76 mol/L. Table 4.1 shows the coded and actual design values along with the predicted and experimental responses of the experimental design.

Among the various design trials, the maximum activity yield (98.4%) was obtained with trial No. 21 corresponding to 9.75 mg FAE/g support, 0.51 mol/L potassium phosphate buffer at pH 5.0, and 32 h of immobilization time. Increasing the ionic strength of the buffer (1 mol/L) and its pH value from 5.0 to 7.0 (trial No. 11) resulted in the highest protein immobilization into the immobilization support (75.6 %). Conditions applied in trial No. 6 (9.75 mg FAE/g support, potassium phosphate buffer at 1 mol/L at pH 7.0, and 20 h of immobilization time) resulted in the highest activation of the immobilized FAE, with a retention of FAE specific activity of 153.6%.

Table 4.2 Results of 5-level 4-variable central composite rotatable design for the optimization of FAE immobilization onto Cu-IDA Sepabeads EP-R® support showing observed and predicted responses.

Run[a]	Enzyme/Support Ratio (X_1)		pH (X_2)		Time (X_3)		Buffer concentration (X_4)		Activity yield (%)[b]		Specific activity retention (%)		Protein yield (%)	
	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	(-1)[c]	9.75	(1)	7.0	(-1)	20	(1)	1.00	94.5	94.8	127.6	127.4	68.6	69.8
2	(0)	16.25	(0)	6.0	(2)	38	(0)	0.76	87.7	87.5	74.0	83.7	64.9	68.4
3	(1)	22.75	(-1)	5.0	(-1)	20	(1)	1.00	80.9	84.8	91.5	87.9	53.3	57.2
4	(1)	22.75	(-1)	5.0	(-1)	20	(1)	1.00	88.7	84.8	88.3	87.9	59.6	57.2
5	(-2)	3.25	(0)	6.0	(0)	26	(0)	0.76	95.5	96.5	121.3	116.2	71.8	74.7
6	(-1)	9.75	(1)	7.0	(-1)	20	(1)	1.00	95.8	94.8	131.9	127.4	71.5	69.8
7	(1)	22.75	(-1)	5.0	(1)	32	(1)	1.00	85.6	87.7	108.9	97.8	59.8	59.4
8	(1)	22.75	(2)	8.0	(-1)	20	(1)	1.00	80.4	81.8	83.6	78.7	58.9	59.0
9	(0)	16.25	(0)	6.0	(0)	26	(0)	0.76	95.4	94.5	90.6	86.7	66.2	66.9
10	(0)	16.25	(0)	6.0	(0)	26	(0)	0.76	93.4	95.2	62.5	85.9	69.1	66.3
11	(-1)	9.75	(1)	7.0	(1)	32	(1)	1.00	95.8	96.4	112.1	103.4	75.6	73.2
12	(-1)	9.75	(-1)	5.0	(1)	32	(-1)	0.51	97.1	97.1	109.6	101.1	67.5	66.1
13	(-1)	9.75	(-1)	5.0	(-1)	20	(-1)	0.51	97.8	96.2	81.6	82.5	68.5	68.6
14	(1)	22.75	(1)	7.0	(-1)	20	(-1)	0.51	85.3	85.1	50.4	50.7	61.9	62.4
15	(2)	29.25	(0)	6.0	(0)	26	(0)	0.76	89.7	88.8	53.6	55.7	60.1	59.0
16	(1)	22.75	(1)	7.0	(-1)	20	(-1)	0.51	85.7	85.1	52.9	50.7	61.5	62.4
17	(1)	22.75	(1)	7.0	(1)	32	(-1)	0.51	91.2	91.7	43.5	37.6	68.5	66.7
18	(0)	16.25	(0)	6.0	(0)	26	(-2)	0.27	77.3	78.4	82.4	86.0	55.2	56.2
19	(0)	16.25	(0)	6.0	(0)	26	(0)	0.76	96.3	95.2	101.1	85.9	68.1	66.3
20	(0)	16.25	(-2)	4.0	(0)	26	(0)	0.76	96.6	97.1	60.8	62.9	45.8	46.7
21	(-1)	9.75	(-1)	5.0	(1)	32	(-1)	0.51	98.4	97.1	102.0	101.1	67.8	66.1
22	(0)	16.25	(2)	8.0	(0)	26	(0)	0.76	70.8	69.3	86.3	95.6	48.4	49.2
23	(-1)	9.75	(-1)	5.0	(-1)	20	(-1)	0.51	95.3	96.2	85.3	82.5	69.3	68.6
24	(0)	16.25	(0)	6.0	(-2)	14	(0)	0.76	60.6	61.0	87.7	74.8	48.1	46.3
25	(1)	22.75	(1)	7.0	(1)	32	(-1)	0.51	91.6	91.7	39.5	37.6	66.0	66.7
26	(-1)	9.75	(1)	7.0	(1)	32	(1)	1.00	96.5	96.4	86.3	103.4	74.0	73.2
27	(1)	22.75	(-1)	5.0	(1)	32	(1)	1.00	89.1	87.7	103.7	97.8	60.1	59.4
28	(0)	16.25	(0)	6.0	(0)	26	(0)	0.76	95.3	94.5	92.6	86.7	66.2	66.9
29	(0)	16.25	(0)	6.0	(0)	26	(2)	1.25	94.0	93.1	62.3	72.5	66.5	67.3
30	(0)	16.25	(0)	6.0	(0)	26	(0)	0.76	95.1	94.5	91.8	86.7	64.2	66.9

[a] Runs were performed in random order.
[b] The experimental results are an average percentages of duplicate trials within ± 10% error.
[c] Numbers in brackets represent coded values of the experimental process values.

4.4.2. Analysis of variance

The analysis of variance (ANOVA) is shown in Table 4.2. The best-fitting models were determined by multiple regression analysis using the software Design Expert version 8.0.2. The models were compared and evaluated for significance (*F*-test values, *P* values, lack of fit, and R^2 values). The high *F*-values obtained for each of the models along with low *P*-values implies that the proposed models are significant and accurately describe the selected responses. Activity and protein yields fit into a reduced quadratic model, while the specific activity retention response fits a two-factor interaction model. The "Lack of Fit" *F*-values for all models were not significant ($p > 0.05$), indicating the proposed models satisfy the design points. The coefficient of determination (R^2) was calculated to be 0.92 for the activity yield model, 0.9 for the specific activity retention model, and 0.93 for the protein yield (Table 4.2). These values indicate that the models and their coefficients could explain 92.0%, 90.0%, and 93.0% variability of the responses, respectively. However, it is important to point out that obtaining a model with high R^2 coefficient does not imply that the regression model accurately describes the evaluated response. For instance, adding variables to a model will always increase the value R^2, whether those variables are significant or not.

The adjusted R^2 value corrects the R^2 value for the sample size and number of terms in the model. If the sample size is insufficient or if the model is increased unnecessarily then the R^2 and adjusted R^2 values will differ significantly. In the present study, the values of adjusted R^2 – 0.85 for activity yield, 0.84 for specific activity retention and 0.87 for protein yield – are within a 10% difference margin with the R^2 values (Table 4.2). The adequate precision of a model measures the signal-to-noise ratio, an adequate precision value greater than four indicates that the proposed model can be used to navigate the design space. The value of adequate precision for the activity yield model was 24.93, for the specific activity retention was 16.09. While the adequate precision value of the protein yield model was 17.18.

The quadratic equations that describe FAE activity yield and protein yield responses are given below:

$$Y_1 = 84.68 - 1.90A - 2.09B + 1.57C - 1.25D - 0.45AB + 0.87AC - 2.38AD + 0.55BC + 2.49BD - 0.36CD - 0.38B^2 - 2.63C^2 + 0.25D^2$$

Table 4.3 The analysis of variance (ANOVA) for central composite rotatable design.

Parameters and interactions	Activity Yield		Specific activity retention		Protein yield	
	F-Value	P[a]	*F*-Value	P	*F*-Value	P
Model	13.01	< 0.0001	18.28	< 0.0001	15.27	< 0.0001
Enzyme/Support Ratio (X_1)	7.85	0.0134	69.81	< 0.0001	19.64	0.0005
pH (X_2)	6.85	0.0194	12.02	0.0026	33.44	< 0.0001
Time (X_3)	11.92	0.0035	1.92	0.1821	3.84	0.0688
Molarity (X_4)	2.17	0.1612	5.36	0.0319	5.05	0.0400
X_1X_2	0.21	0.6508	40.89	< 0.0001	2.38	0.1437
X_1X_3	2.71	0.1203	0.46	0.5070	1.72	0.2097
X_1X_4	6.52	0.0220	24.51	< 0.0001	5.20	0.0376
X_2X_3	1.15	0.3012	19.45	0.0003	3.93	0.0662
X_2X_4	9.39	0.0079	--	--	1.95	0.1829
X_3X_4	0.47	0.5055	2.19	0.1554	0.85	0.3709
$(X_2)^2$	0.87	0.3665	--	--	32.26	< 0.0001
$(X_3)^2$	37.67	< 0.0001	--	--	0.031	0.8622
$(X_4)^2$	0.33	0.5749	--	--	4.90	0.0428
Lack of Fit	1.82	0.1976	0.67	0.6940	3.03	0.071
R^2	0.92		0.90		0.93	
Adjusted R^2	0.85		0.84		0.87	
Adequate Precision	24.93		16.09		17.18	

[a] $P < 0.05$ indicates statistical significance.

$Y_3 = 57.81 – 3.21A + 4.91B + 0.95C – 2.04D – 1.59AB + 0.73AC + 2.27AD + 1.09BC + 1.21BD + 0.52CD – 2.49B^2 – 0.081C^2 + 1.01D^2$

Where Y_1 is activity yield; Y_3 is protein yield, A is enzyme/support ratio; B is pH, C is immobilization time; and D is the molarity of the immobilization buffer. While the equation that describes the retention of specific activity is given below:

$Y_2 = 78.95 – 16.39A + 11.82B – 2.85C – 8.22D – 25.67AB + 1.62AC + 19.92AD$

$– 10.31BC – 3.54CD$

Where Y_2 is the retention of specific activity; A is enzyme/support ratio; B is pH, C is immobilization time; and D is the molarity of the immobilization buffer.

Among the linear terms, the significant variables that affect the activity yield model are the enzyme/support ratio, pH of the immobilization buffer, and immobilization time. While the effects of linear terms of enzyme/support ratio and pH of the immobilization buffer were significant in the predictive models of protein yield and specific activity retention. Table 2 also shows that interaction between the enzyme/support ratio and the molarity of the immobilization buffer as well as that between pH and molarity of the buffer display major contributions to the activity yield response model. None of the interactions between variables was significant in the protein yield predictive model. In contrast, the interactions between enzyme/support ratio and pH of the immobilization buffer, between enzyme/support ratio and molarity of the immobilization buffer, and that between pH of the buffer and immobilization time exert the most significant effects on the specific activity retention predictive model (Table 4.2). Among the quadratic terms, only those of immobilization time and pH of the immobilization buffer contribute significantly to the activity yield and protein yield predictive models, respectively.

Figure 4.1 shows a diagnostic plot between the predicted versus the actual response values for the modeled responses. Predicted vs actual diagnostic plot is a visual tool which includes a 45-degree line that depicts an ideal scenario with equal predicted and experimental values for the selected response. This graph helps to detect a range of design space that is not accurately described by the proposed model or experimental results that may require further revision or repetition. Figure 1a and 1c show the diagnostic plots for the activity yield and the protein yield models. In both plots most points cluster along the diagonal line with R^2 values of 0.9182 and 0.9298 for the activity

yield model and the protein yield model, respectively. While figure 1b shows more dispersion across the plot resulting in a lower R^2 for the proposed specific activity retention model (0.8964). The obtained coefficients confirmed a satisfactory fit for the proposed FAE activity yield quadratic models.

4.4.3. Effects of immobilization parameters

The effects of the selected independent variables on the activity yield predictive model are better visualized in the series of contour plots shown in Figure 4.2. Contours were made by plotting the activity yield on a two-dimensional graph where the x- axis corresponds to the enzyme to support ratio and the y-axis to the immobilization time while keeping the other two independent variables at a fixed value (either -1, center point and +1 according to the coded values shown on Table 4.1). Overall, contours display that activity yield increased at immobilization times close to its center point value (26 h), while increasing or decreasing the immobilization time caused a negative effect on this response. These results may be attributed to an incomplete covalent binding of FAE onto the support at shorter immobilization times and to the inactivation of FAE activity at longer immobilization times. The positive contribution of an increment in the buffer molarity to activity yield seems to be more pronounced at lower FAE loadings; while lower molarity of the buffer allowed higher activity yields at higher FAE loadings. Indeed, molecular simulations showed that increasing the buffer molarity caused electrostatic interactions of a FAE type A from *Aspergillus niger* (AnFaeA) with a hydrophobic support to diminish, which positively affected the conformational stability of the enzyme; this was found to be instrumental at low enzyme loadings at which the enzyme/enzyme & enzyme/support interactions are limited (Liu et al., 2015). Figure 4.2 also shows that increasing the pH of the immobilization buffer had a detrimental impact on the activity yield of the immobilized FAE at low buffer molarity of 0.51 mol/l; no significant effect of pH was observed at buffer molarity higher than 0.75 mol/L. At the optimum pH of the FAE of 6.0, a wide range of activity yield was observed (60.0% to 90.0%) along the design space. Highest activity yield (93.0%) was obtained at very low enzyme loadings (3.25 mg FAE/g of support), 26 h of immobilization time and high ionic strength of the immobilization buffer. Comparable yields (> 90%) were obtained at a pH value of 5.0 and low ionic strength of the immobilization buffer (0.51 mol/L) along a broad spectrum of immobilization time (18 h to 32 h), but at significantly higher enzyme loadings (≥ 20 mg of FAE/g of support).

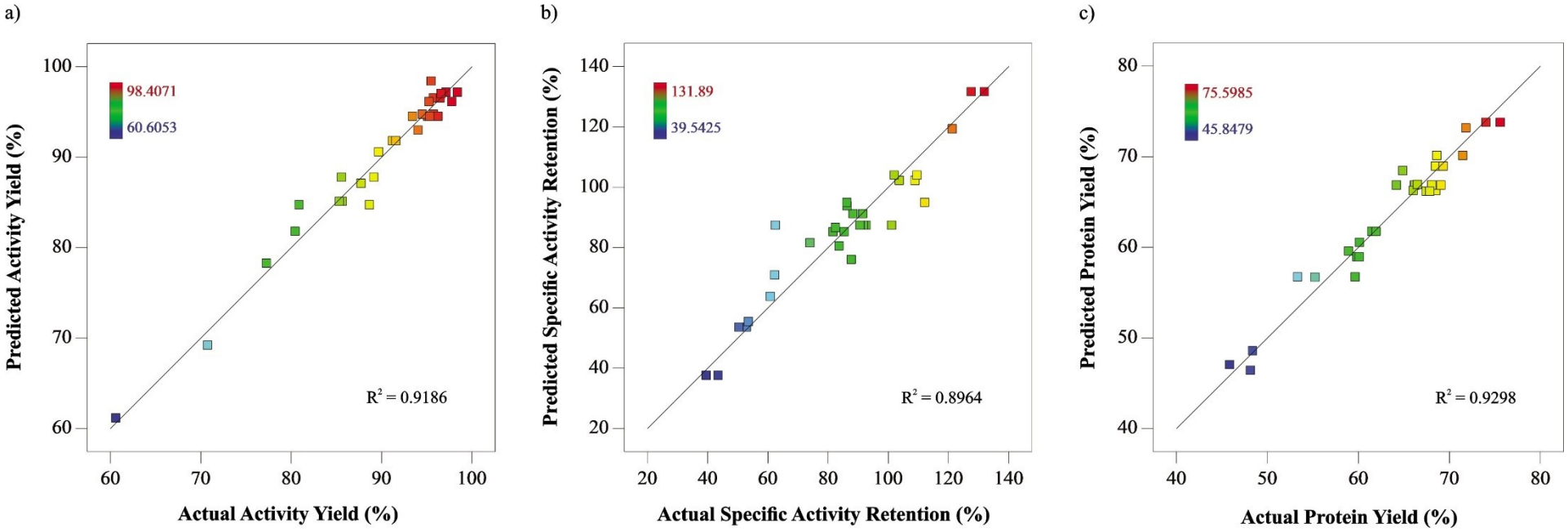

Figure 4.1 Predicted vs. actual model diagnostic plot for the dependent variables of the experimental design.

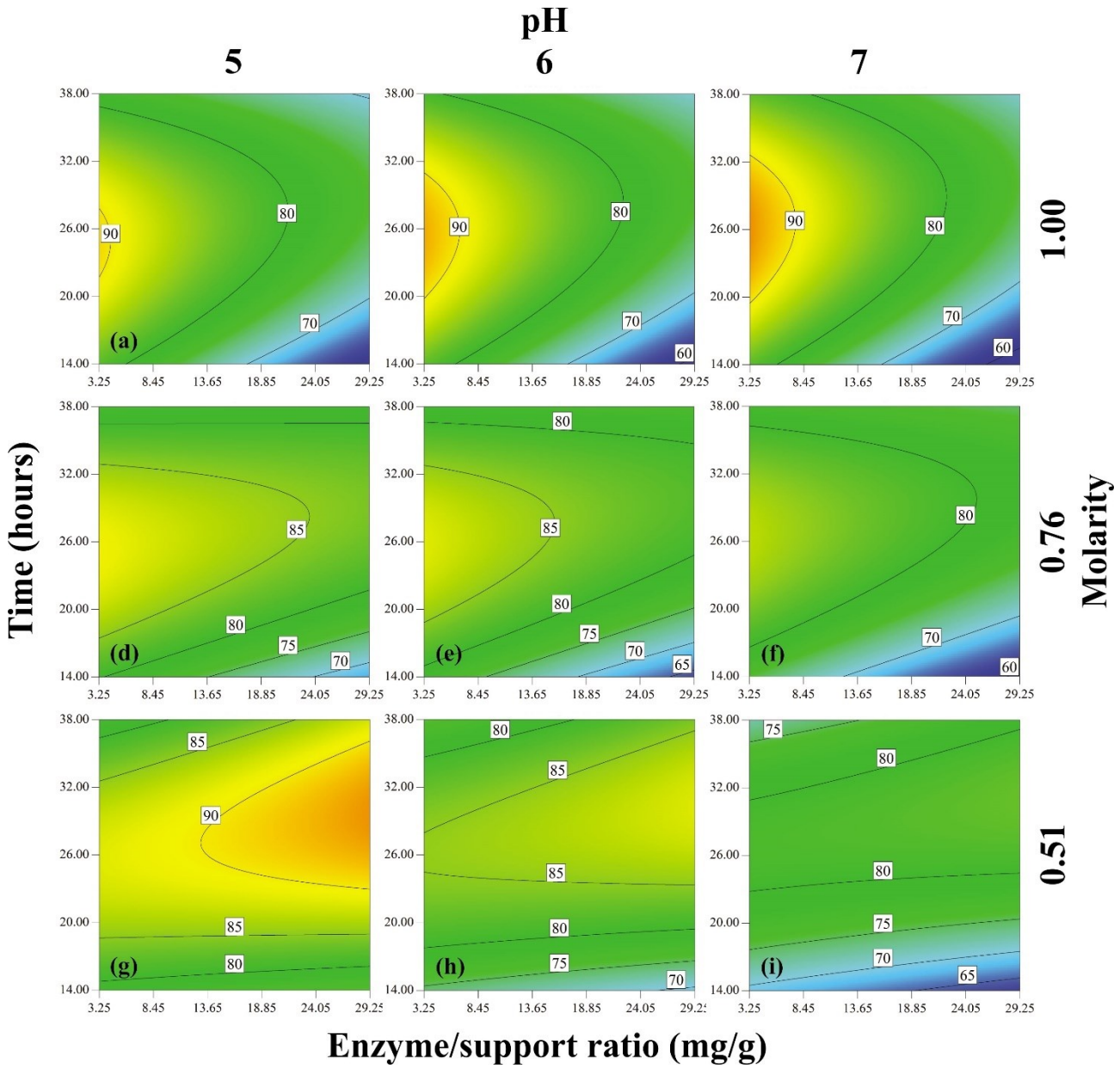

Figure 4.2 Contour plots showing the interaction of the independent variables on the activity yield quadratic model, numbers in squares indicate the hydrolytic activity yield remaining in the immobilized FAE.

The effects of two of the most significant linear terms for the protein immobilization yield and retention of FAE specific activity predictive models are illustrated in three-dimensional response surface curves shown in Figure 4.3. The curves were generated by plotting the response using the Z-axis against enzyme to support ratio (Y-axis) and molar concentration of the immobilization buffer (X-axis), while keeping the other two remaining variable levels at their center point value (26 h of immobilization time and a buffer pH of 6.0). Under these conditions, higher protein yield (66.1%) and higher FAE specific activity retention (120.9%) were obtained at a low FAE loading (9.75 mg of FAE/g of support) and immobilization buffer concentration of 0.51 mol/L. Coincidentally, the lowest values for both responses (58.0% of protein yield and 51.0% of specific activity retention) were obtained at the same molar concentration of the immobilization buffer (0.51 mol/L), but with the FAE load up to its positive factorial value (22.75 mg FAE/g of support).

These results suggest that the enzyme/support ratio is the driving parameter for both responses. From these results, it was determined that if higher ratios of enzyme/support are used, it is also desirable to use a molar concentration of immobilization buffer above its center point model value (0.76 mol/L) to avoid a sharp decrease of specific activity retention of the FAE, while keeping the protein immobilization yield above 60%. Moreover, standard enzyme immobilization protocol on epoxy supports requires the use of high ionic strength buffer (1.0 mol/L) to promote rapid initial adsorption of the enzyme via hydrophobic interactions between lipophilic pockets on the surface of the enzyme and the hydrophobic support. Hydrophobic interactions are favored at higher ionic strength of buffer due to the water-stripping effect caused by the increment of ions surrounding the enzyme.

FAE specific activity retention model shows that when high enzyme loadings (22.75 mg FAE/g of support) and medium to high buffer molar concentrations were used the specific activity retention of the evaluated FAE decreased from above 80% at pH 4.0 to 38% at pH 8.0 (results not shown). Similar pH detrimental effect was reported previously (Thörn et al., 2013b) where a FAE type C from *Fusarium oxysporum* adsorbed onto mesoporous silica at pH 8.0 showed less than half the hydrolytic specific activity of those immobilized at pH 6.0. Taking these findings into consideration and for further evaluation of the feruloylation capabilities of the immobilized enzyme, we selected immobilization conditions leading to a maximum activity yield (82.4%) and maximum FAE specific activity retention (85.5%), while aiming to maximize the enzyme/support ratio and the ionic strength of the immobilization buffer to obtain a highly active immobilized FAE

in the shortest possible incubation time. Applying this optimization criteria, optimum variable levels selected for further investigation were: 22.75 mg of FAE/g of support; immobilization buffer pH of 5.0; 27.7 h of immobilization time; and immobilization buffer molarity of 0.86 mol/L. Confirmation experiments were run in triplicate and the obtained experimental response values fell inside the prediction intervals (α risk = 0.05), thus validating the proposed models.

4.4.4. Effect of pore size and functional group density on the immobilization of FAE

The effects of pore size and functional group density of selected immobilization supports on FAE immobilization and catalytic activity were evaluated with three Cu-IDA epoxy-activated supports differing on their pore size: Sepabeads® EC-EP R (mesoporous support with smaller pore size), Relizyme® EP113 R (mesoporous support with larger pore size) and Relizyme® EP403 R (macroporous support). The oxirane group density of the supports is inversely proportional to their pore size, since supports with a smaller pore size have a larger surface area. The results (Figure 4.4) show that the highest protein yield (66.14%) was achieved using the support with the smallest pore size (Sepabeads® EC-EP R). Contradictory results were reported in the literature, in which larger pore size disfavored or benefited enzyme physical adsorption onto mesoporous silica (Takahashi et al., 2000; Thörn et al., 2011). Our results show that the highest activity yields were obtained upon immobilization on the mesoporous supports, Relizyme® EP113 R (91.73%) and Sepabeads® EC-EP R (88.23%). On the other hand, the highest specific activity retention values of 208.1 and 242.34% were achieved upon immobilization of FAE on the mesoporous Relizyme® EP113 R and the macroporous Relizyme® EP403 R.

Although immobilization on Sepabeads® EC-EP R resulted in the highest protein yield, it led to the lowest specific activity retention (143.4%). These results may be attributed to the fact that protein-protein and protein-support interactions may have been favored with the use of support with smaller pore size, leading to FAE denaturation (Karboune et al., 2005a). Similarly, Thörn et al. (2011) reported that adsorption using mesoporous supports with a larger pore size doubled the hydrolytic activity yield after immobilization of the FAE present in the enzymatic preparation Depol 740L. A simulation study showed that adsorption of AnFaeA into positively charged support surfaces permits a better post-immobilization structural conformation of the esterase (Liu et al., 2015). Higher activity yield and specific activity retention obtained upon immobilizing FAE onto Cu-IDA epoxy mesoporous supports with larger pore size may have occurred due to a nearly

complete modification of the oxirane groups in these supports, thereby allowing a modification of the net charge on the surface of the support. Additionally, the increase in pore size might reduce intraparticle diffusion limitations, facilitating diffusion of the reaction substrates through the support and inside the pores (Thörn et al., 2011).

4.4.5. Effect of multipoint covalent attachment on the immobilized FAE catalytic properties and thermal stability

Increasing the pH of the immobilization buffer has been shown to increase the reactivity of the nucleophile groups on the enzyme's surface, mainly the lysine residues. This would theoretically lead to an intense intramolecular crosslinking between the enzyme-epoxy support complex, reducing the effect of external disturbances that could affect the novel formed molecule (Blanco et al., 1989). However, in some cases the alkaline conditions required for the promotion of multipoint covalent attachment may detrimentally impact the activity of the immobilized enzyme, significantly reducing its thermostability (Cerdobbel et al., 2010). To promote multipoint covalent attachment, FAE immobilized on Cu-IDA-Sepabeads® EC-EP R were incubated in sodium carbonate buffer (0.1 mol/L) at two different pH values (8.0 and 10.0) and time (4 h and 14 h). Figure 4.5a shows that incubation at pH 8.0 and at pH 10.0 for 4 h increased Cu-IDA-Sepabeads® EC-EP R specific activity retention by 10.5% and 7.8%, respectively. An increase (16.2%) in the specific activity retention was also observed upon incubation at pH 8.0 for 14 h, while incubation of the immobilized enzyme at pH 10.0 for the longer period (14 h) led to an almost complete FAE deactivation (99.5% of specific activity retention lost). These results may be attributed to the irreversible denaturation of enzyme at high pH values. Following multipoint covalent attachment promotion procedure, the thermal stability of the immobilized FAE was assessed by incubation at 50 °C for 2 h. Immobilized FAE incubated at pH 10.0 for 4 h only lost 25% of its specific activity retention after thermal treatment. While incubation of the FAE for 14 h at pH 10.0 showed a decrement of 36.0% of its specific activity retention. Immobilized FAE incubated at pH 8.0 lost 55% of their pre-thermal treatment specific activity retention.

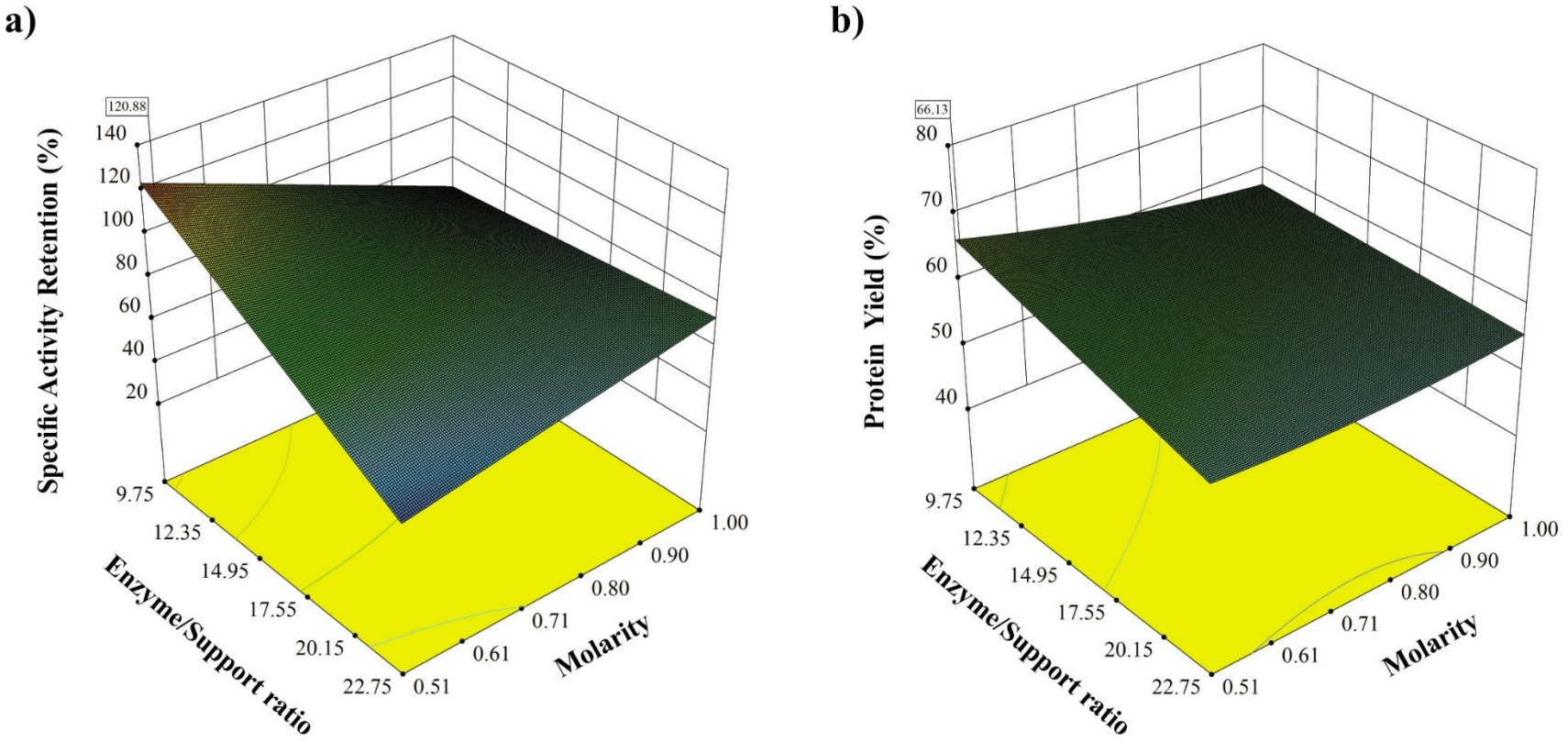

Figure 4.3 Response surface 3-dimensional plots showing the effect of the independent variables over the retention of (a) specific activity retention model and (b) protein yield model. Numbers in squares indicate the maximum yield attained for each response across the design space.

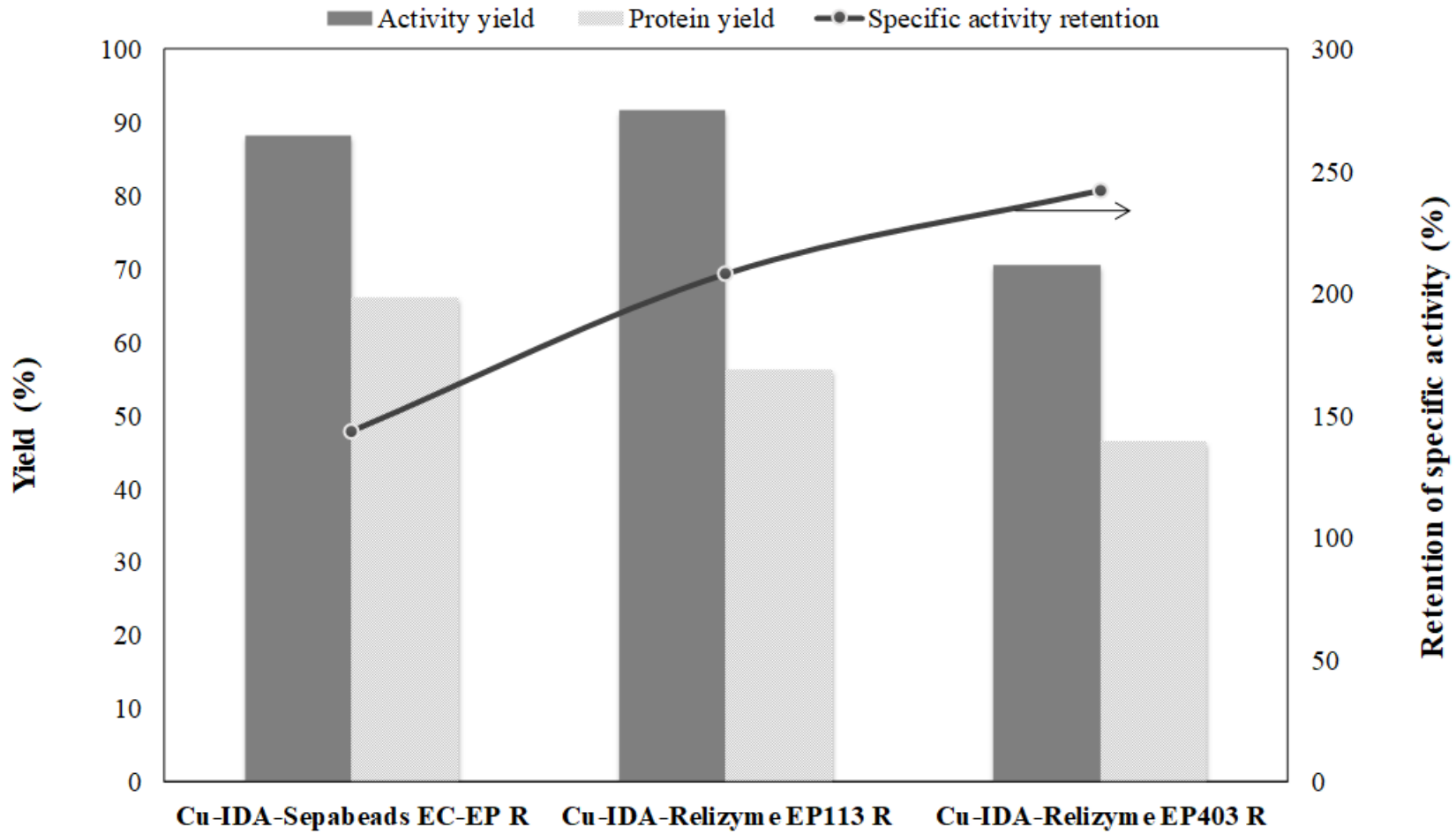

Figure 4.4 Effect of the porosity degree of Cu-IDA epoxy supports on the immobilization of pure FAE from *H. insolens*. Immobilization conditions: 22.75 mg of FAE/g of support; potassium phosphate buffer pH of 5.0; 27.7 h of immobilization time; and immobilization buffer molarity of 0.86 mol/L

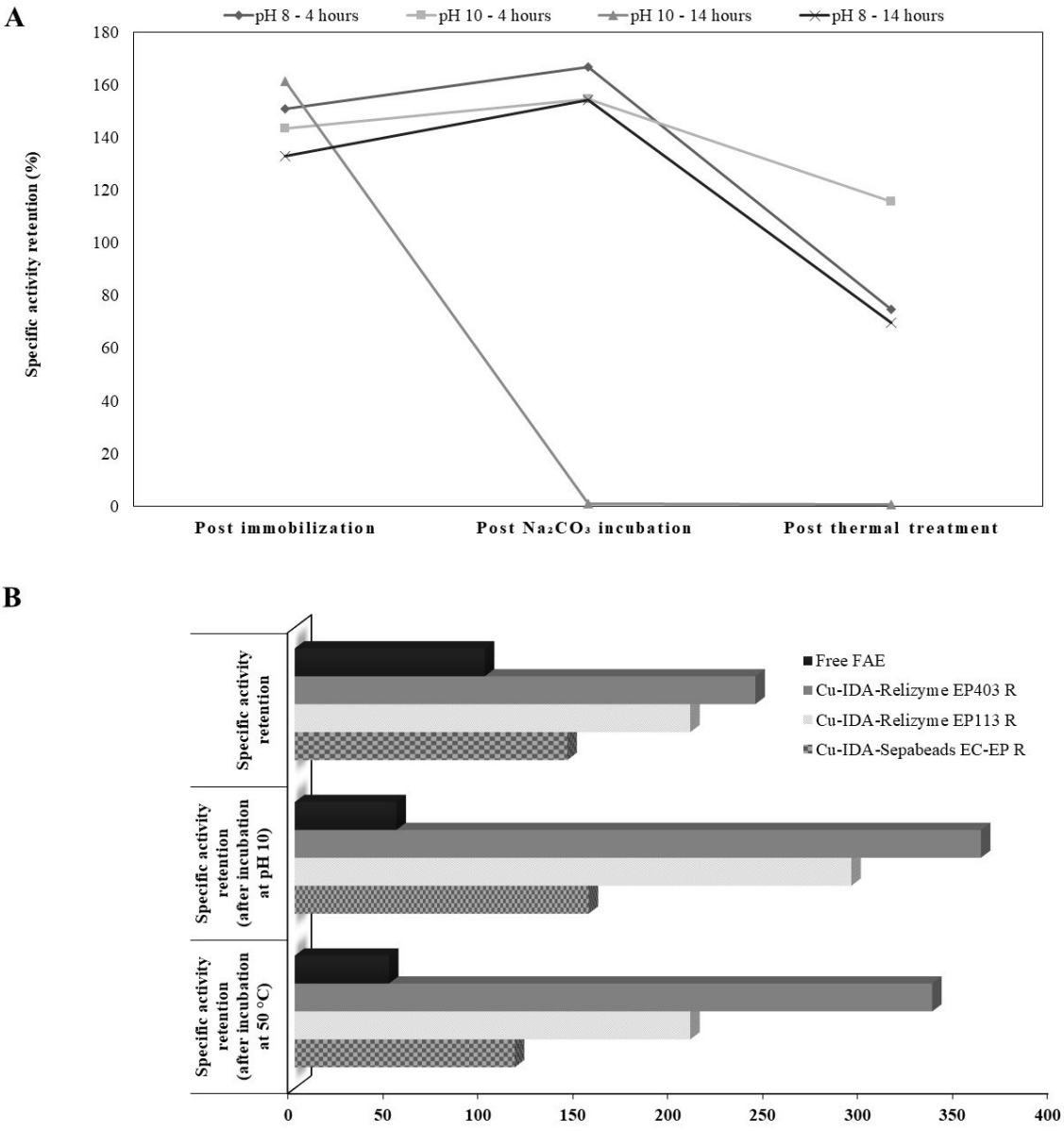

Figure 4.5 (a) Effect of incubation in Na_2CO_3 at selected intervals of time and pH values after thermal incubation for 2 h (50 °C) on the specific activity retention of FAE immobilized into Cu-IDA Sepabeads® EC-EP R (b); Effect of MCA incubation in Na_2CO_3 for 4 h (0.1 mol/L, pH 10) and thermal incubation for 2 h (50 °C) on the specific activity of FAE immobilized on metal chelate epoxy supports with different porosity degree.

Figure 4.5b displays the effect of multi-immobilized FAE into Cu-IDA Sepabeads® EC-EP R, Cu-IDA Relizyme® EP113 R, and Cu-IDA Relizyme® EP403 R. Incubation for 4 h using sodium carbonate buffer at pH 10.0 were defined as multipoint covalent attachment promoting conditions for the remainder of the investigation due to the higher thermostability observed under these settings using Cu-IDA Sepabeads® EC-EP R. Multi-immobilization between the FAE and the functionalized Relizyme® EP113 R and Relizyme® EP403 R supports resulted in a substantial increase on the retention of specific activity of the immobilized enzyme. The highest increment of specific activity retention occurred with the FAE immobilized into the macroporous support Cu-IDA Relizyme® EP403 R (49.0%). However, multi-immobilization between the FAE and Cu-IDA Relizyme® EP113 R also resulted in an increment (40.0%) of enzymatic specific activity retention. Figure 4.5b shows a comparison of the stability of free FAE and multi-immobilized FAE after incubation at 50 °C for 2 h. The free enzyme dilution lost nearly half of its native specific activity after thermal incubation, whereas FAE immobilized into Cu-IDA Sepabeads® EC-EP R lost 25% of its specific activity. Multi-immobilized FAE into supports Relizyme® EP113 R and Relizyme® EP403 R lost only 5% and 7% of its specific activity after thermal incubation, respectively. When incubated at 50 °C for 120 h, FAE from recombinant *Pichia* sp. retained 75% more hydrolytic activity just by immobilization onto magnetic nanoparticles at neutral pH (He et al., 2015). In our study multi-immobilized FAE into Relizyme® EP113 R retained 95% more specific activity than its free FAE counterpart after thermal incubation, which demonstrates one of the benefits of this immobilization/stabilization strategy with this enzyme.

4.4.6. Synthesis of feruloylated oligosaccharides catalyzed by optimally immobilized FAE

Feruloylation in non-conventional media catalyzed by optimized multi-immobilized FAE was evaluated using xylobiose and xylo-oligosaccharides (XOS) as saccharide donors. Cu-IDA Sepabeads® EC-EP R, Cu-IDA Relizyme® EP113 R, and Cu-IDA Relizyme® EP403 R were assessed as immobilization supports. Retention of the bioconversion (%) was defined as the ratio between the bioconversion yield obtained using immobilized FAE to the bioconversion yield obtained in the feruloylation reactions catalyzed by free FAE, multiplied by 100 (Figure 4.6). Using xylobiose as the acyl acceptor, the retention of the bioconversion (21.1 %-26.0 %) was similar with all the investigated immobilization supports. On the contrary, with XOS as the acyl acceptor of the reaction, the retention of bioconversion was only 7.0% with Cu-IDA Sepabeads® EC-EP R, while a substantial increase in the retention of this bioconversion was observed with

FAE immobilized into the other supports with a larger pore size. FAE immobilized onto Relizyme® EP113 R and Relizyme® EP403 R retained 88.4 and 92.9% of the bioconversion of XOS, respectively (Fig. 4.6). Retention of bioconversion results suggest that supports with larger pore size diameter (≥ 50 nm) provide better stabilization against the denaturing effects of organic solvents over the immobilized FAE than mesoporous supports with smaller pore size diameter (≤ 20 nm). The protective effect may be caused by a larger planar surface offered by the supports with larger pores, which might preserve the active conformation of the biocatalyst. The same FAE previously immobilized into an unmodified macroporous epoxy support showed a retention of bioconversion of only 70.5% for the feruloylation of XOS (Tamayo-Cabezas and Karboune, 2018). Moreover, the decrement on the retention of bioconversion yield in the reactions catalyzed by FAE immobilized onto Cu-IDA Sepabeads® EC-EP R with XOS as acyl donor could be attributed to the increased enzyme load onto the support during the optimized experiments, which went up from 10 mg to 22.75 mg of FAE/g of support. It was previously reported that a more spacious surrounding the immobilized FAE increases its specific activity (Thörn et al., 2011).

4.5 Conclusion

The oriented immobilization of a purified preparation of FAE from *H. insolens* into Cu-IDA Sepabeads® EC-EP R was optimized by RSM. Quadratic models described accurately the effect of the selected experimental variables over the FAE activity yield and protein immobilization yield responses while a 2-factor interaction equation characterized the retention of specific activity of the immobilized enzyme. Regression analysis showed that the most significant parameters affecting FAE immobilization into the selected modified epoxy supports were enzyme to support ratio, pH of the immobilization buffer and the interaction between enzyme to support ratio with the molar concentration of the immobilization buffer. Supports with larger pores were the most adequate for FAE from *H. insolens* immobilization and their subsequent stabilization by multipoint covalent attachment. Multi-immobilized FAE into Cu-IDA epoxy macroporous support almost fully retained the feruloylation capabilities of the non-immobilized esterase. These results open the possibility of developing a robust and reliable procedure for the biosynthesis of specific feruloylated bioactive compounds.

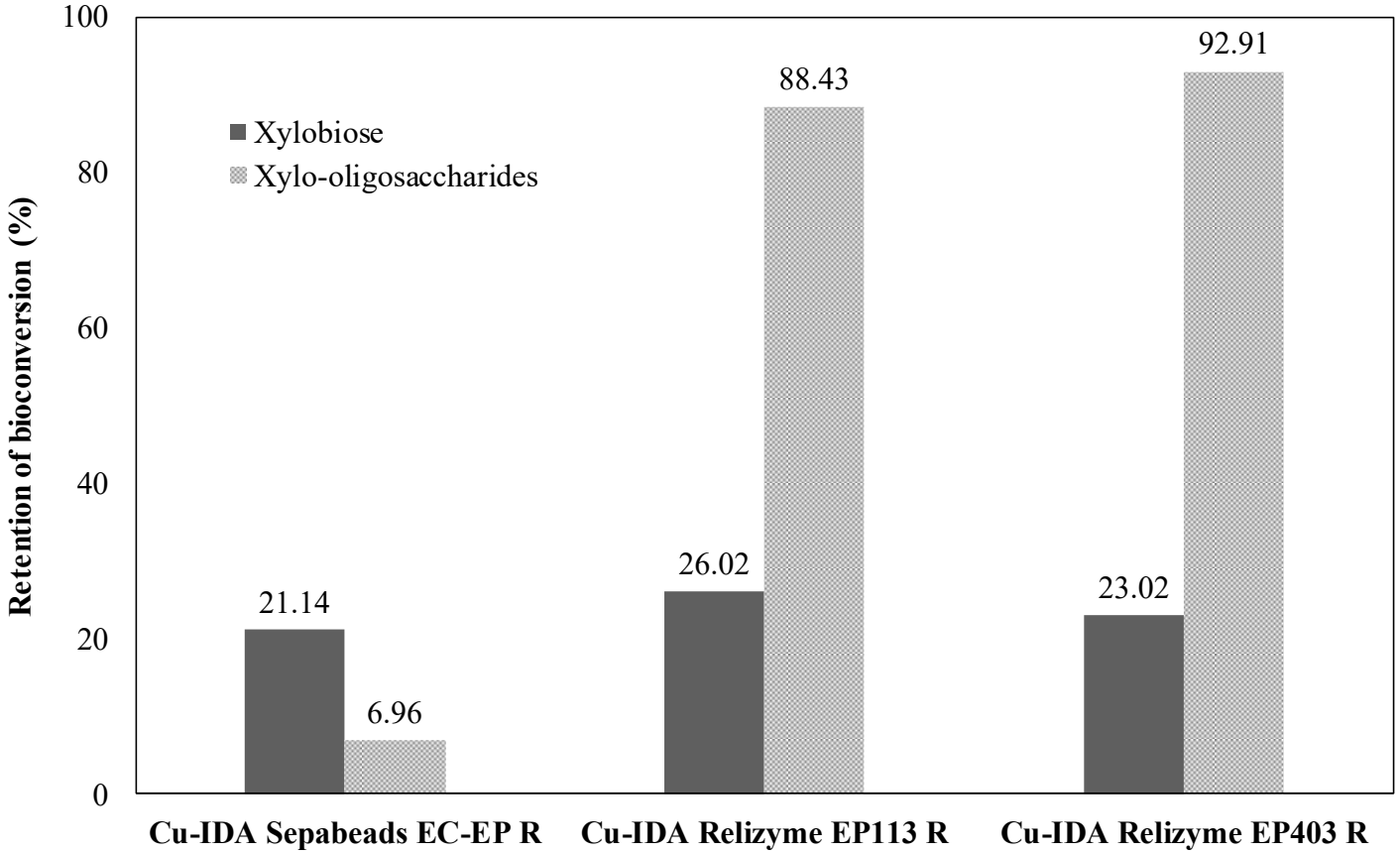

Figure 4.6 Effect of porosity degree and functional group density of the Cu-IDA epoxy immobilization supports on the biosynthesis of feruloylated oligosaccharides catalyzed by immobilized/stabilized FAE.

CONNECTING STATEMENT 3

Chapter III and IV proved the efficiency of free and immobilized FAE-catalyzed esterification reaction for the synthesis of feruloylated oligosaccharides in surfactantless microemulsions. As part of our efforts to develop and modulate the enzymatic-based strategies for the acylation of saccharides, Chapter V focused on the study of an enzymatic approach, in low-solvent reaction media, for the acylation of fructose to lauric and stearic acid catalyzed by commercial immobilized lipases, namely Novozym® 435 or Lipozyme® RM IM. Ball-milling of the substrates was applied to increase the solubility and availability of the acyl acceptor in the reaction.

The results from this study were presented at the 2015 IFT Annual Meeting & Food Expo-The Institute of Food Technologists and submitted to a scientific journal.

Tamayo-Cabezas, J., Carrillo-Montes, J.P., & Karboune, S. (2018). Direct esterification of fructose with medium and long fatty acids catalyzed by immobilized lipases in a low-solvent reaction media. (Submitted).

CHAPTER V. ESTERIFICATION OF FRUCTOSE WITH MEDIUM AND LONG FATTY ACIDS CATALYZED BY IMMOBILIZED LIPASES IN LOW-SOLVENT MEDIA

5.1. Abstract

Synthesis of carbohydrate fatty acid esters catalyzed by immobilized lipases is a pathway to obtain specific isomers from renewable feedstock in comparison to the poor selective chemical esterification. While the use of low-solvent reaction media (≥ 10%) may offer several advantages; the interactive effects of this media with biocatalysts and substrates need to be ascertained to better modulate these interactions towards high catalytic efficiency and substrate availability. Among the investigated co-solvents, the use of *tert*-butanol and DMSO in a mixture of lauric acid substrate/*co-solvent* (90/10; v/v) resulted in high bioconversion yields using either Novozym® 435 or Lipozyme® RM IM as a biocatalyst. Increased hydrophobicity of the Novozym® 435 immobilisation support favoured the bioconversion, while enlarging the surface area of the polar substrate by ball-milling improved the productivity through the enhancement of the fructose availability. A compromise between bioconversion yield (19.7%) and productivity (9.45 µmol/L min) was obtained in the reactions catalyzed by Novozym® 435 using ball-milled fructose at a concentration of 0.2 mol/L. Combining the mechanical ball-milling of the substrates and the low-solvent reaction media approach is expected to enhance and expand the enzymatic synthesis of carbohydrate fatty acid esters.

5.2. Introduction

Surfactants being surface-active ingredients play a critical rule in improving the emulsifying, gelling, film-forming and foaming characteristics of food products (Schramm, 2005). However, chemically-synthesized surfactants have raised attention among consumers and the scientific community because the health concerns associated with some of them and the large carbon footprint of their production. In this regard, bioprocessing approaches, based on the use of the microorganism metabolites or the enzymatic synthesis, arose as 'green alternatives' for surfactant production as they offer high selectivity and enhanced sustainability profile with the use of cheap renewable starting materials and milder conditions. Indeed, biosurfactant molecules can be produced by bacteria (Rendell et al., 1990), yeasts (Mulligan, 2005), fungi (Zavala-Moreno et al., 2014), or enzymatically synthesized (Chang and Shaw, 2009), with considerable structural diversity.

Among the biosurfactants, carbohydrate fatty acid esters (CFAE), a major class, consist of a hydrophilic mono-, di-, or oligosaccharide unit, joined with an ester or glycosidic linkage to a

hydrophobic long-chain hydrocarbon unit. Immobilized lipases, such as Lipase B from *Candida Antarctica* (Novozym® 435) (Casas-Godoy et al., 2016; Chaiyaso et al., 2006; Fukuoka et al., 2011) or lipase from *Rhizomucor miehei* (Lipozyme® RM IM) (Abdulmalek et al., 2012; Dang et al., 2005; Schlotterbeck et al., 1993) have been proven to be suitable biocatalysts for the enzymatic synthesis of CFAE. The key step in the enzymatic synthesis of CFAE is the selection of the appropriate reaction medium that favours the solubility of both substrates, hydrophobic long-chain hydrocarbon unit and hydrophilic glycoside unit, while modulating the properties of lipases towards the synthesis. It has been reported that both the polarity of the solvent and the solvent-enzyme interactions can affect the secondary structure of the enzyme, thus altering its synthetic activity (Li et al., 2015b).

Early studies on the enzymatic synthesis of CFAE identified polar aprotic organic solvents, such as pyridine, dimethyl sulfoxide (DMSO), and dioxane, as suitable co-solvents (Degn and Zimmermann, 2001). In addition, the use of sterically hindered tertiary alcohols in the CFAE production did increase the carbohydrate solubility without affecting the enzymatic activity of the lipase (Watanabe et al., 2001; Zhang et al., 2003). Furthermore, organic solvent mixtures were reported to be appropriate for achieving a compromise between substrate solubility and esterifying activity (Couto et al., 2011). However, there has been an increasing interest in reducing the amount of organic solvent in the reaction media by using low-solvent reaction media (solvent content < 10 %, v/v); or even rescinding its use (solvent-free reaction media and solid-state reactions). These non-conventional reaction systems pose less safety and environmental concerns, require simpler downstream separation, and might provide longer enzyme reusability cycles (Adlercreutz and Hatti-Kaul, 2010).

Low-solvent CFAE biosynthesis reduces the amount of organic solvent added to the reaction media down to a point which would not be able to dissolve the carbohydrate substrate but provides a liquid catalytic phase to the reaction system. (Cao et al., 1996; Yan et al., 1999). In addition, selected reaction parameters such as the type of acyl donor, evaluated biocatalysts, substrate molar concentration, and water activity were modulated to control the efficiency of the enzymatic synthesis of CFAE in low-solvent media. It has been reported that the solubility and the availability of the substrate in the enzyme micro-environment can limit the production of CFAE (Nott et al., 2012; Sutili et al., 2013). This was overcome by using the appropriate co-solvent at selected proportion and/or by chemically modifying the fatty acids and the carbohydrates. Ball milling was

identified as a potential approach for enhancing the substrate diffusion and favoring the chemical reactions. However, this approach has not been investigated in enzymatic systems.

The main objective of the present study was mainly to investigate the esterification of unmodified fatty acids to underivatized fructose in low-solvent reaction media (< 10 %, v/v). The effects of the non-polar substrate chain length, the co-solvent type, and the selectivity of the lipase on the bioconversion yield and the productivity of the enzymatic esterification were assessed. In addition, ball milling treatment was evaluated for its efficiency to enhance the bioconversion yield through the improvement of the availability of fructose substrate and the substrate/enzyme interactions. The understanding of the effects of the reaction parameters is a key step for the better modulation of the enzymatic esterification towards the synthesis of the targeted CFAE.

5.3. Materials and methods

5.3.1. Materials

Lipozyme® RM IM immobilized from *R. miehei*, lipase acrylic resin from *C. Antarctica* type B (Novozym® 435), crystalline D-(-)-fructose (≥ 99 %), myristic acid (≥ 98 %), *tert*-butanol (≥ 99.0 %), ethyl acetate (≥ 99.7 %), Triton™ X-100, α-naphtol, and DMSO (≥ 99.9 %) were purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel (20 cm x 20 cm x 0.5 cm) TLC plates 60 F_{254} were purchased from Merck Chemicals (Darmstadt, Germany). Lauric acid (99 %), stearic acid (97 %), high performance liquid chromatography (HPLC) grade acetonitrile, 2-butanone, methanol, n-hexane, toluene, and all other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

5.3.2. Enzymatic esterification in conventional organic solvent reaction media

Esterification reactions of fructose with selected fatty acids (lauric acid, stearic acid) in a conventional organic solvent reaction media were performed using Novozym® 435 or Lipozyme® RM IM as biocatalysts. Reactions were carried out in screw-capped 4 mL amber vials with polytetrafluoroethylene (PTFE) faced rubber caps. Selected amounts of fructose and fatty acid at a ratio of 1:1 were dissolved in *tert*-butanol. Enzymatic reactions were initiated with the addition of 57 mg of biocatalyst for each mL of reaction media. All vials were vacuum sealed and incubated at 55 °C with continuous shaking at 200 rpm for a maximum of 72 h. All reactions were run in triplicate and control trials were carried out to account for unwanted side reactions. The

supernatants of the reaction mixtures were recovered by centrifugation at 6700·x *g* for 5 min. Then, immobilized lipase beads were washed for 3 min with 3 mL of a toluene/ethyl acetate/methanol (10:5:4.5, v/v) solution. Recovered reaction mixtures were combined and stored at -20 °C for further HPLC analysis of end-products.

5.3.3. Enzymatic esterification in low-solvent media

Esterification of fructose with selected fatty acids was carried out in a low-solvent reaction media. The reaction mixture was composed of lauric acid (4.6 mol/L, 90 % v/v) and fructose suspension (0.05 mol/L) in selected co-solvents (10 %, v/v). Four co-solvents (*tert*-butanol, DMSO, 2-butanone and Triton™ X-100) were used to assess the effect of media composition on the bioconversion yield of the esterification reaction. To initiate the reactions, 57 mg or 171 mg of immobilized lipase beads (Lipozyme® RM IM or Novozym® 435) were added to 1 mL of reaction mixtures. The reaction mixtures were incubated at 55 °C with continuous shaking at 200 rpm for a maximum of 72 h. Experiments were run in triplicate. Blanks without enzyme and without glycoside substrate were carried out alongside the esterification reactions to account for possible unwanted side reactions. At selected reaction times, the supernatants were recovered by centrifugation at 6700·x *g* for 5 min, and immobilized lipase beads were washed for 3 min with 3 mL of a toluene/ethyl acetate/methanol (10:5:4.5, v/v) solution. The reaction mixtures were combined and stored at -20 °C for further HPLC analysis of acylation end-products.

5.3.4. Effect of substrate ball-milling

Both reaction substrates were ball-milled in a MM2000 Retsch ball mill operating at a vibrational frequency of 28 Hz for 30 min. The decrease of particle size was assessed using a Hitachi™ TM-3000 tabletop scanning electron microscope (Hitachi High-Technologies Corporation, Japan). The enzymatic esterifications were carried out using crystalline fructose/lauric acid, ball-milled fructose/lauric acid, or ball-milled fructose/ball-milled lauric acid in low-solvent reaction media. Two concentrations of fructose – 0.05 mol/L and 0.5 mol/L – were investigated in the presence of 4.6 mol/L of lauric acid. Reactions were performed as described in section 2.3 using Novozym® 435 as a biocatalyst. After 72 h reactions were stopped and the supernatants were recovered by centrifugation.

5.3.5. Analysis of end-products of esterification reaction

Quantitative analysis of the reaction end-products was performed using a Beckman System Gold® 126 Solvent Module high-performance liquid chromatograph (HPLC) coupled to an Alltech 3300 evaporative light scattering detector (ELSD) operating at 75 ºC with a nitrogen flow of 1.5 L/min (BÜCHI Labortechnik AG, Switzerland). A 20 µL aliquot of the recovered supernatant was injected in the solvent module and separated in a Zorbax SB-C18 reversed-phase column (5 µm, 250 mm x 4.6 mm, Agilent Technologies Canada Inc.; Mississauga, ON). Mobile phase was applied at 0.3 mL/min in a linear gradient with the following profile: 50 % deionized water/50 % acetonitrile/methanol/hexane (90:8:2, v/v) for 5 min, increasing to 100 % acetonitrile/methanol/hexane (mobile phase B) after 10 min and holding for 30 min at the same flow rate before reverting to the initial eluent composition. The mobile phase B composition varied to 70:28:2 (v/v) ratio for the analysis of the reactions with myristic acid as acyl donor. Standard curves were obtained by injecting different concentrations in the range of 1 mmol/L to 5 mmol/L of the purified esters of lauric and myristic acids. Bioconversion yield (%) was calculated from the concentration of the synthesized CFAE divided by the initial concentration of the glycoside substrate, multiplied by 100. Productivity of the reaction (mmol/L min) was calculated from the concentration of the synthesized CFAE divided by the total duration of the reaction. Initial velocity was measured as concentration of esters per minute produced during the lineal fragment of the time-course curve of the esterification reaction (µmol/L min).

5.3.6. Purification of carbohydrate fatty acid esters end-products

Fructose laurate and fructose myristate catalyzed by Novozym® 435 were produced as described in section 2.2, only varying the concentration of substrates and the amount of enzyme added to the reaction (0.2 mol/L and 171 mg, respectively). Reaction products were recovered following the procedure defined in section 2.3. Fructose laurate was purified on TLC F_{254}, using a mobile phase composed of toluene/ethyl acetate/methanol/water (10:5:4.5:0.2, v/v). To identify the bands corresponding to fructose laurate, α-naphtol (R_f mono-ester of 0.54) was used as described previously by Li et al. (2015b). Fructose myristate ester was purified using a Supelclean™ LC-SI normal phase flash chromatography column (Supelco Inc.; Bellefonte, PA) using as eluents pure petroleum ether, followed by a mixture of petroleum ether/ethyl acetate (1:1, v/v), and finally

100% ethyl acetate. Fructose myristate was recovered from organic solvents in a AES2010 SpeedVac® System (Thermo Scientific, Waltham, MA).

Fructose laurate structure was confirmed using electrospray ionization mass spectrometry (ESI MS/MS) in a Waters Synapt™ G2-S (quadrupole Time-of-Flight) high definition mass spectrometer (Waters Ltd.; Brossard, QC) operating at positive ion mode with full scan detection in the m/z range of 100 to 1500. Samples were dissolved in pure acetonitrile prior to injection to the spectrometer. The obtained data was processed using MassLynx® MS software.

5.4. Results and discussion

5.4.1. Structural analysis of carbohydrate fatty acid esters

Fructose laurate was produced by Novozym® 435-catalyzed esterification reaction and purified. The mass spectrometry (LC-ESI-MS) spectra of the recovered reaction end-product showed a major peak at *m/z* value of 385.219, which corresponds to fructose laurate, as well as three minor peaks with *m/z* values of 345.229 $[M+H-H_2O]^+$, 327.077 $[M+H-2H_2O]^+$, and 183.173 (Figure 5.1). The peak found at *m/z* 183.173 might correspond to the fragment of lauric acid without one hydroxyl group (due to the esterification) with a theoretical *m/z* value of 183.174 $[C_{12}H_{23}O]^+$. Additionally, a smaller peak was observed just after that of fructose laurate ester, confirming the formation of fructose laurate isomers in the reaction. Since fructose contains two primary hydroxyl groups (at C_1 and C_6 positions), acylation may occur at both positions at similar rates producing four monoester isomers (Cao et al., 1998). Peaks corresponding to diesters were not observed among the reaction products in the ESI-MS spectra, which coincides with the results obtained with qualitative TLC (data not shown), where only one single spot corresponding to fructose monolaurate was observed. The obtained product profile agrees with the acylation profile reported by Scheckermann et al. (1995) for the Lipozyme® IM20 catalyzed acylation of fructose with lauric acid in 2-methyl-2-butanol.

5.4.2. Enzymatic esterification in organic solvent media

The esterifying efficiency of Novozym® 435 and Lipozyme® RM IM was evaluated in *tert*-butanol organic solvent reaction medium utilizing lauric acid or stearic acid as acyl donors. Two equimolar concentrations – 0.05 mol/L and 0.625 mol/L – of fructose and fatty acid at a molar ratio of 1:1 were investigated. Finding suitable monophasic reaction media for the enzymatic esterification of

glycosides with fatty acid is very problematic due to the inherently weak compatibility of the solubility of these compounds in the same non- aqueous media and the inactivation of most hydrolases in highly polar organic media. *tert*-butanol has been reported of being one of the most adequate solvents not only because of its ability to solubilize fructose more than other non-polar organic solvents but also for being fairly innocuous to the enzymatic activity of the lipase (Cao et al., 1999; Li et al., 2015b). In addition to the lipase specificity, the immobilization support can affect the yield and initial velocity of the reaction. Novozym® 435 is a non-regiospecific recombinant lipase immobilized by adsorption onto a macroporous hydrophobic resin produced from crosslinked methacrylic esters (Brask, 2009; Melgosa et al., 2015). While Lipozyme® RM IM is a *sn 1,3* regiospecific lipase immobilized by electrostatic binding onto a macroporous hydrophilic weak base anion exchange resin, produced from cross-linked phenol-formaldehyde polymers (Jenab et al., 2014).

Table 5.1 shows that the bioconversion yield and the initial velocity of the esterification reaction were dependent on the lipase type and the concentration of substrates. Increasing the substrate concentration resulted in an increase in the initial velocity. These results reveal the limited substrate inhibition in the investigated enzymatic systems. Highest initial velocities (84 µmol/L·min with Lipozyme® RM IM and 85 µmol/L·min with Novozym® 435) were achieved in the esterification reaction system with stearic acid as acyl donor and 0.625 mol/L of substrate concentration. These results reveal the high substrate specificity of both lipases towards longer more lipophilic substrates. Similar enzymatic selectivity was observed for the acylation of fructose in 2-metyhl-2-butanol catalyzed by Novozym® 435 (Olive et al., 2012; Soultani et al., 2001). However, the highest maximum bioconversion yields (18.8 % with Lipozyme® RM IM and 19.2 % with Novozym® 435) were obtained after 72 h in the esterification reaction system with lauric acid and a substrate concentration of 0.625 mol/L. The esterification reaction system with stearic acid and 0.625 mol/L of substrate concentration attained maximum bioconversion yields of 14.4 % (Novozym® 435) and 14.6 % (Lipozyme® RM IM). Indeed, the mass transfer limitations may have been more pronounced with stearic acid, affecting the substrate/product partition in the micro and macro-environment of the biocatalysts, and hence the bioconversion yield (Couto et al., 2010).

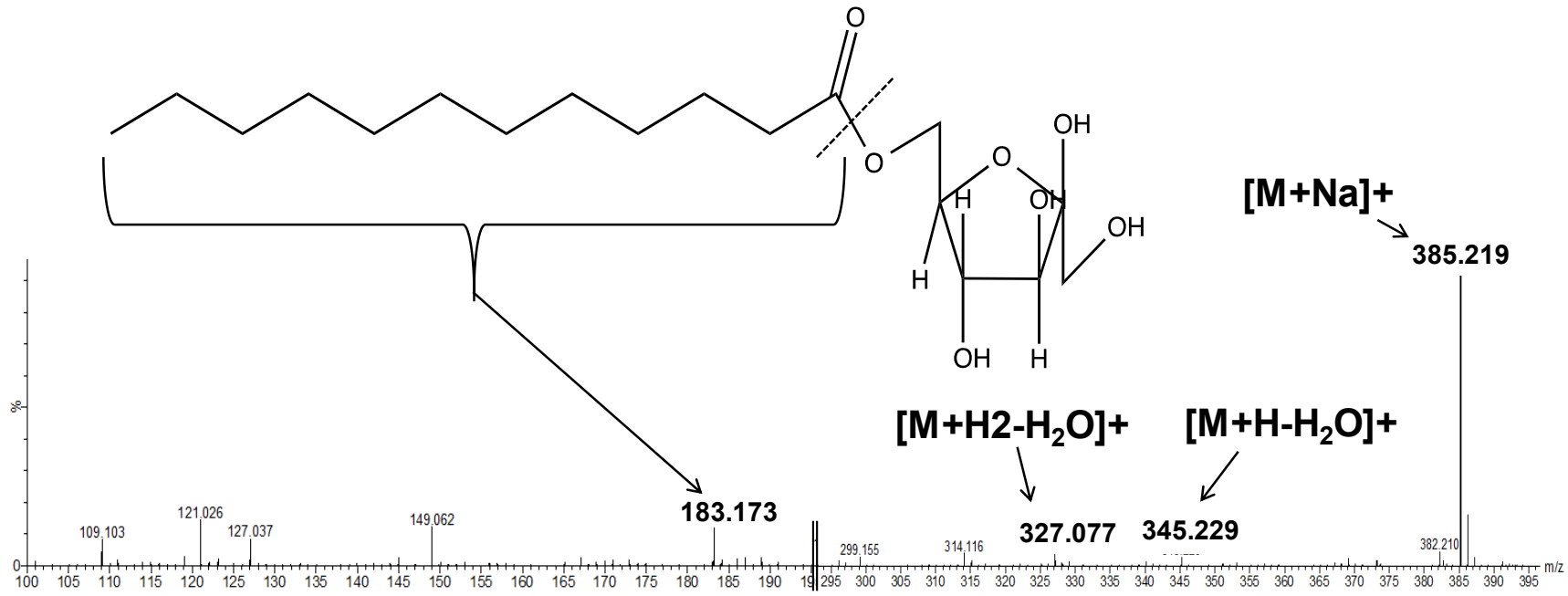

Figure 5.1 LC-ESI-MS/MS spectrum of the fragmentation pattern of fructose laurate obtained by resin immobilized CALB-catalyzed esterification in a reaction media composed of tert-butanol and lauric acid at a ratio of 9:1 (v/v).

Table 5.1 Initial velocity and maximum bioconversion yield of fructose laurate obtained through enzymatic acylation catalyzed by two commercial immobilized lipases in 90% *tert*-butanol.

Acyl donor	Substrates concentration (mol/L)	Enzyme	Bioconversion yield[a] (%)	Initial velocity[b] (μmol/L min)
Lauric acid	0.05	Novozym® 435[c]	16.0[d]	10.3 ± 1.2
Lauric acid	0.05	Lipozyme® RM IM	3.0	8.9 ± 0.1
Lauric acid	0.625	Novozym® 435	19.2	60.3 ± 0.8
Lauric acid	0.625	Lipozyme® RM IM	18.8	62.5 ± 11.8
Stearic acid	0.05	Novozym® 435	14.0	9.8 ± 1.8
Stearic acid	0.05	Lipozyme® RM IM	3.0	1.6 ± 0.1
Stearic acid	0.625	Novozym® 435	14.4	85.1 ± 0.4
Stearic acid	0.625	Lipozyme® RM IM	14.6	84.0 ± 3.0

[a] Maximum bioconversion yield (%). The bioconversion yield was calculated as the concentration of consumed fructose over the initial concentration of fructose, multiplied by 100.

[b] Initial velocity was measured as concentration of esters (μmol/L) per minute produced during the lineal fragment of the time-course curve of the esterification reaction.

[c] Lipase from *Candida Antarctica* type B

[d] Data shown is the average of three determinations

The results also show that at a low substrate concentration (0.05 mol/L), the catalytic performance of the lipases differed markedly. Novozym® 435 esterified 14 % of stearic acid and 16 % of lauric acid with an initial velocity of 9.8 µmol/L·min and 10.3 µmol/L·min, respectively. While Lipozyme® RM IM esterified only 3 % of the substrates with an initial velocity of 1.6 µmol/L·min of fructose stearate and 8.9 µmol/L·min of fructose laurate (Table 5.1). These results may be attributed to the hydrophilic nature of the Lipozyme® RM IM support, which may have favoured not only the achievement of the equilibrium conversion at shorter reaction time, but also the distribution of high concentrations of water and the CFAE in the micro-environment of the support. Which may limit the presence of the acyl donor at the enzyme's active site (Ye et al., 2014). Moreover, the initial moisture content of Novozym® 435 was reported to be 0.7 g/100 g of support (Melgosa et al., 2015), while that of Lipozyme® RM IM was recorded to be in the range of 3.3 g to 3.8 g for every 100 g of support (Jenab et al., 2014; Melgosa et al., 2015). The difference in initial water content between both immobilized biocatalysts may explain the decreased reaction rates at low concentration of substrates, affecting the availability of the non-polar substrate near the enzyme microenvironment. The detrimental effect of an increment of the initial water content on the equilibrium yield for the Novozym® 435 -catalyzed synthesis of fructose laurate in *tert*-butanol at low hexose concentration has been reported by Watanabe et al. (2000).

5.4.3. Enzymatic esterification in low-solvent reaction media

The esterification reaction of fructose with lauric acid catalyzed by Novozym® 435 and Lipozyme® RM IM in the low-solvent reaction media was investigated. It is expected that the partition effects of substrate/product molecules between the micro/macro-environments of lipases to be more pronounced in a low-solvent reaction media with an excess of the non-polar substrate. In addition, the affinity of substrates/products to interact with the immobilization supports of lipase can contribute to this partition effects through adsorption phenomenon (Heinsman et al., 2003; Ye et al., 2016). Indeed, because of the hydrophilicity nature of the resin beads of Lipozyme® RM IM, less swelling of these beads was observed when this lipase was used in the low-solvent media. However, the lipophilic nature of the Novozym® 435 resin polymeric matrix led to more swelling of the beads and expected adsorption effects. Therefore, the water generated during the reaction and the polar substrate are continually transported outside of the Novozym® 435 pores, releasing space for more fatty acid and fructose laurate to be adsorbed into the support beads (Ye et al., 2016).

This ‘swelling’ of the immobilization support beads and the partition effects in low-solvent reaction media may affect the quantification of the unreacted fatty acids or fructose. To overcome this limitation, fructose laurate was produced, purified and used as internal standard for the quantification of our reaction end-products. Due to the unavailability of commercial CFAE standards, several studies estimated the bioconversion yield of the reaction by measuring the amount of unreacted fatty acid in the reaction (Cao et al., 1996; Šabeder et al., 2006; Scheckermann et al., 1995). In addition, a wash step of the beads with a mixture of ethyl acetate and toluene was carried out to recover any product that might have been adsorbed on the immobilization supports of lipases. It is important to point out that, if the bioconversion yields presented in this study were measured based on the amount of unreacted fatty acid in the reaction media, they would have been overestimated by more than 5 times than those obtained through the quantification of the end-products.

Table 5.2 shows the initial velocity and bioconversion yield of esterification reaction systems in low-solvent reaction media catalyzed by Novozym® 435 or Lipozyme® RM IM at two fructose concentrations (0.05 mol/L and 0.625 mol/L). Lipozyme® RM IM was more efficient than Novozym® 435 in the reactions in the presence of a high concentration of fructose. Lipozyme® RM IM catalyzed the bioconversion of 5.3 % of fructose after 72 h at an initial velocity of 52.7 µmol/L min. While Novozym® 435 attained a bioconversion yield of 4.6 % after 72 h with an initial velocity of 40 µmol/L min. (Table 5.2). In contrast, Novozym® 435 outperformed Lipozyme® RM IM in the reactions at low fructose concentrations (0.05 mol/L). Higher bioconversion (13 %) and higher initial velocity of the reaction (8.5 µmol/L min) were obtained with Novozym® 435. Lipozyme® RM IM esterified only 9.1 % of the fructose with an initial velocity rate of 6.8 µmol/L min, which was an improvement compared to the lower bioconversions obtained with this lipase in the reactions with *tert*-butanol and low concentrations of the hexose (Section 3.2). Moisture content of the enzyme support may be tightly bound to the support resin thus unavailable to take part in the reaction. In that case, it has been reported that the solubility of fructose in *tert*-butanol (50 °C) decreases to 0.018 mol/L (Watanabe et al., 2000).

Table 5.2 Bioconversion yield and initial velocity of fructose laurate synthesis obtained through esterification reaction catalyzed by two commercial immobilized lipases in a low-solvent reaction media.

Fructose concentration (mol/L)	Enzyme	Maximum yield[a] (%)	Initial velocity[b] (µmol/L min)
0.05	Novozym® 435	13.0 ± 1.5	8.5 ± 1.7
0.05	Lipozyme® RM IM	9.1 ± 0.4	6.8 ± 1.1
0.625	Novozym® 435	4.6 ± 0.8	40.0 ± 7.2
0.625	Lipozyme® RM IM	6.1 ± 0.9	52.7 ± 7.8

[a] Maximum yield (%). The bioconversion yield was calculated as the concentration of consumed fructose over the initial concentration of fructose, multiplied by 100.
[b] Initial velocity was measured as concentration of esters (µmol/L) per minute produced during the lineal fragment of the time-course curve of the esterification reaction.
[c] Lipase from *C. antarctica* type B

5.4.4. Effect of co-solvent on the enzymatic esterification in low-solvent reaction media

The selection of the co-solvent (10 %, v/v) in a low-solvent- reaction media is a key step as it does affect the behaviour of the catalytic action of the lipase and the availability of substrate in the lipase's micro-environment. In this regard, four types of co-solvents were evaluated including, a low polar protic alcohol (*tert*-butanol), a medium polar ketone (2-butanone), a non-ionic surfactant to promote formation of an interphase (Triton™ X-100), and a dipolar aprotic solvent (DMSO).

Figure 5.2 demonstrates the effect of co-solvents on the fructose laurate ester production catalyzed by Novozym® 435 or Lipozyme® RM IM. The amount of enzyme added to each reaction was adjusted to 12 % (w/w) of the reaction media volume to promote an increment on the initial rate of the esterification reaction. The highest bioconversion yields of the esterification reaction systems catalyzed by Lipozyme® RM IM were achieved in the presence of *tert*-butanol (21 % bioconversion), Triton™ X-100 (21 %) and DMSO (26 %) as co-solvents. While the use of *tert*-butanol and DMSO as co-solvents led to the highest bioconversion yield (16.4 %) among the Novozym® 435-catalyzed esterification reaction systems. However, adding 2-butanone as co-solvent resulted in a decrease of the bioconversion yield to 8.7% and 15% with Novozym® 435 and Lipozyme® RM as a biocatalyst, respectively.

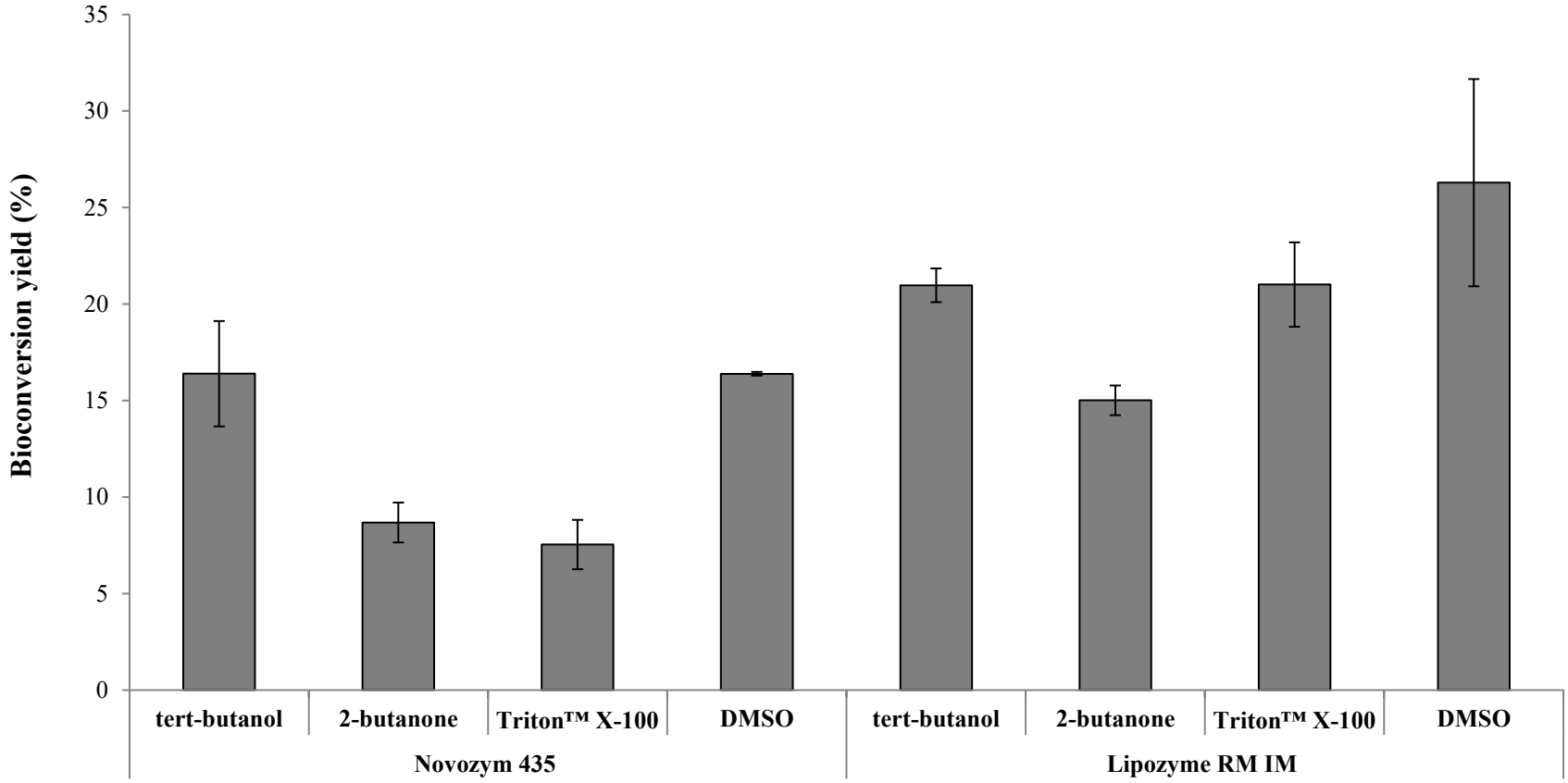

Figure 5.2 Effect of co-solvent (10%, v/v) on the enzymatic production of fructose laurate ester in a low solvent media catalyzed by commercial immobilized lipases

The higher bioconversions obtained with DMSO disregarding the enzyme catalyzing the reaction may be attributed to the higher initial solubilization of the fructose with this co-solvent. DMSO has been previously reported as an appropriate co-solvent at moderate concentrations (10 % to 20 %; v/v) for the enzymatic esterification of fructose and its oligomers with lauric acid (Sagis et al., 2008). Increasing DMSO concentration above 20 % (v/v) in the reaction media has been found to be detrimental for the catalytic activity of the enzyme (Degn and Zimmermann, 2001; Van Kempen et al., 2013). Moreover, a noticeably decrement of fructose solubility in 2-butanone was observed and may account for the lower yields obtained with this co-solvent. Nevertheless, Li et al. (2015b) reported higher bioconversions with 2-butanone over *tert*-butanol for the Novozym® 435 catalyzed lauric acid acylation to fructose in a low-solvent reaction media, even though fructose solubility in 2-butanone was approximately 25 times lower compared to its solubility in *tert*-butanol.

Similar results were reported for the acylation of palmitic acid to fructose catalyzed by Novozym® 435 (Šabeder et al., 2006). However, these studies used an equimolar concentration of substrates (0.8 mmol/mL) which makes us infer that the lower bioconversions obtained in our experiments with 2-butanone as acyl donor are more related to mass transfer limitations and/or conformational changes of the enzyme due to the increased viscosity of the reaction media. The enhanced bioconversion observed with Lipozyme® RM IM in the presence of all the evaluated co-solvents seemed correlated to the hydrophilicity of the support and its higher moisture content. The formation of regions rich in water surrounding the microenvironment of the enzyme benefited its catalytic activity in a reaction system with an excess of the non-polar substrate. Thus, solvation of the fructose and its high partition in the micro-environment of the hydrophilic support may have been favoured, increasing its availability for the acylation reaction. However, the recovery of the products seems to be challenging when the hydrophilicity of the support is high.

In spite of being the second best co-solvent in terms of bioconversion yield, *tert*-butanol was selected rather than DMSO as the co-solvent of choice for the rest of the study due to the better enzyme tolerance towards the alcohol which will extend the number of uses of the biocatalyst (Yan et al., 1999). Additionally, in a recent survey of solvent selection guides DMSO was categorized as a 'problematic' solvent (Prat et al., 2014). Moreover, *tert*-butanol has the potential to be produced from renewable feedstock (Byrne et al., 2016). On the other hand, Novozym® 435 was selected as the biocatalyst of choice for further investigation due to its superior lipophilicity

compared to Lipozyme® RM IM, which may facilitate the downstream separation of substrates, products and enzyme, as well as increase the reusability of the catalyst by repelling polar compounds present in the reaction media, reducing purification and separation times. Additionally, the reduced initial moisture content of Novozym® 435 might help to avoid the need to use molecular sieves for the evaluation, which have been demonstrated to cause potential unwanted side reactions and mass transfer limitations in the reaction system (ter Haar et al., 2010).

5.4.5. Effect of substrates ball-milling on the enzymatic esterification

Figure 5.3 shows that initial fructose crystals size ranged between 200 µm to 400 µm. Micrographs showed that an approximate fifty-fold decrease of the fructose particle size was achieved after 30 min of ball-milling. The ball-milling seems also to promote the shift from the crystalline state to amorphous one with irregular porous surface. To assess the effect of ball-milling of fructose on its enzymatic acylation in a low-solvent reaction system, the esterification reactions were carried out using milled fructose, crystalline fructose, and a milled mixture of fructose/lauric acid at two different concentrations of fructose (0.05 mol/L and 0.5 mol/L). Figure 5.4 shows that milled fructose achieved higher bioconversion yields (7.2 % and 21.9 %) and higher productivity rates (8.3 µmol/L·min and 2.8 µmol/L·min) at fructose concentrations of 0.5 mol/L and 0.05 mol/L, respectively. It can be inferred that the increase of fructose particle surface area because of ball-milling may have improved its solubility in the co-solvent and hence its availability for the enzyme. Ye et al. (2014) reported that reducing sucrose particle size, in parallel with homogenization and ultrasound treatments, increased the concentration of sucrose in the reaction media, causing an increment of the initial reaction rate for Novozym® 435-catalyzed sucrose oleate formation.

Simultaneous milling of reaction substrates showed contradictory results (Figure 5.4). At high fructose concentration, the bioconversion yield (5.9 %) and productivity of the reaction (7.4 µmol/L·min) were higher than those obtained by using crystalline fructose (3.8 % and 4.4 µmol/L·min). On the contrary, simultaneous milling at a fructose concentration of 0.05 mol/L showed lower bioconversion yields (7.7 %) and productivity values (1.1 µmol/L·min) compared to crystalline fructose (16.4 % and 1.9 µmol/L·min). Substantial aggregation of milled substrates, in the presence of lauric acid, inside the ball miller capsule might be the cause of the decrement of bioconversion at low fructose concentration. In addition, the formation of lauric acid clusters might difficult the passage of the sparse fructose particles to the active enzyme of the lipase. Reduction

of the polar substrate particle size alone by ball-milling was established as a preliminary step for further enzymatic esterification trials.

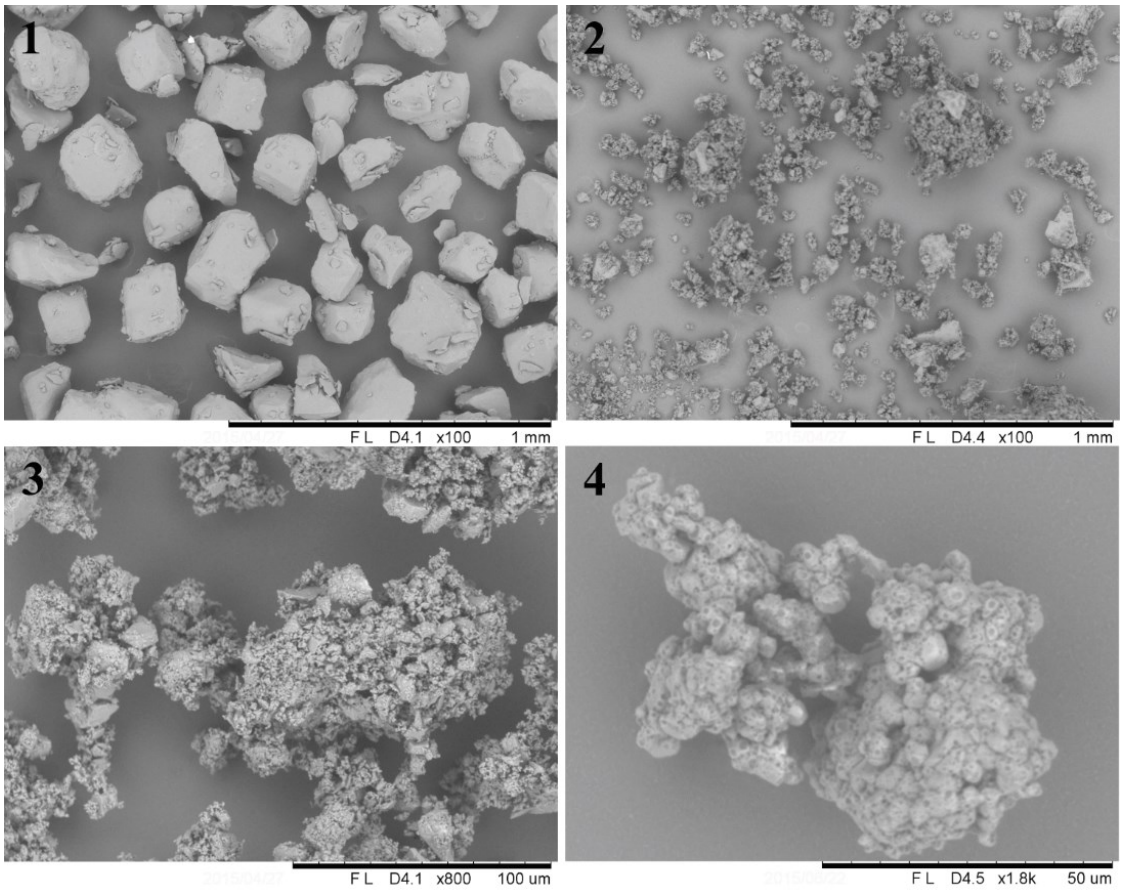

Figure 5.3 Micrographs of crystalline fructose (1) and fine milled fructose particles (2) at 100X magnification. (3) Milled fructose at 800X (4) Milled fructose particle at 1800X.

5.4.6. Effect of substrate concentration on the bioconversion yield

The bioconversion yield and the productivity of the enzymatic synthesis of milled fructose laurate ester in a low-solvent reaction system were determined at selected fructose concentrations (Figure 5.5). Increasing the fructose concentration from 0.05 mol/L to 0.2 mol/L resulted in similar bioconversion yield of 20 % to 23 %; whereas the productivity increased from 2.8 µmol/L·min to 9.5 µmol/L·min. Further increase of the fructose concentration to 0.3 mol/L led to a significant decrease in the bioconversion yield and the productivity to 10.5 % and 7.6 µmol/L·min, respectively. Beyond fructose concentration of 0.3 mol/L, the bioconversion yield remained more or less constant; and as expected the productivity increased to reach 13.1 µmol/L·min at 0.5 mol/L.

Reduced bioconversion yields at higher concentrations of acyl acceptor can be attributed to the fact that a layer of small particles of fructose adsorbed to the surface of the enzyme support, causing enzyme inhibition and/or mass diffusional limitations (Ye and Hayes, 2012). At 0.2 mol/L of fructose concentration, it was possible to achieve a compromise between the productivity (9.45 μmol/L·min) and the bioconversion yield (19.7 %). Cao et al. (1997) noted that an increase in the glucose concentration led to a higher bioconversion of glucose palmitate in a reduced solvent reaction media, but with lower amounts of palmitate esters produced. In contrast, a study investigating the biosynthesis of ethylglucoside monooleate determined that an excess of glucoside resulted in a highly viscous reaction medium and a lower yield of monooleate in a solvent-free media (Wei et al., 2003).

The effect of the fatty acid concentration on the bioconversion yield of the esterification reaction was analyzed at three different lauric acid concentrations: 4.6 mol/L, 6.9 mol/L, and 9.2 mol/L. Fructose molar concentration was set at 0.2 mol/L. According to the results (Figure 5.6), increasing the ratio negatively affected the acylation of fructose. The highest bioconversion yield (19.7 %) was obtained with 4.6 mol/L of lauric acid. Seconded by the bioconversion obtained with a concentration of 6.9 mol/L (17.56 %); and finally, by adding 9.2 mol/L (12.66 %) (Figure 5.6). Scheckermann et al. (1995) found that increasing the lauric acid/fructose molar ratio up to 15:1 increased the production of fructose palmitate on the lipase-catalyzed acylation of palmitic acid to fructose in a low-solvent reaction media. In another publication, fructose laurate biosynthesis peaked at a molar ratio of 2:1 (lauric acid:fructose). The poor bioconversion yield obtained at higher lauric acid:fructose molar ratio may have been caused by the increased viscosity of the media due to the excess of fatty acid, limiting the polar substrate mass diffusion (Li et al., 2015a).

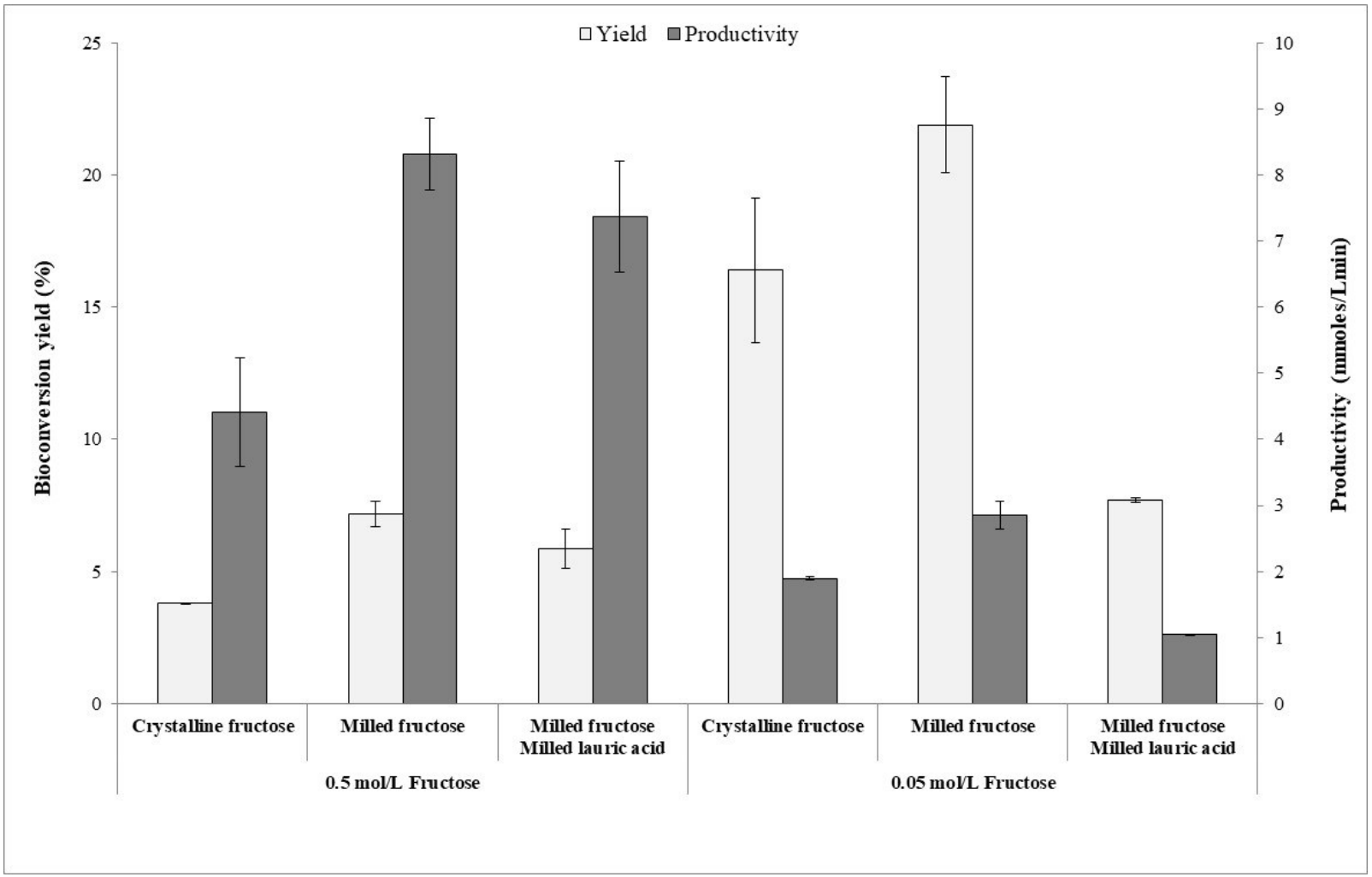

Figure 5.4 Effect of substrate particle size reduction on the bioconversion yield and reaction productivity of fructose laurate esterification catalyzed by Novozym® 435 in a low-solvent media (10% *tert*-butanol).

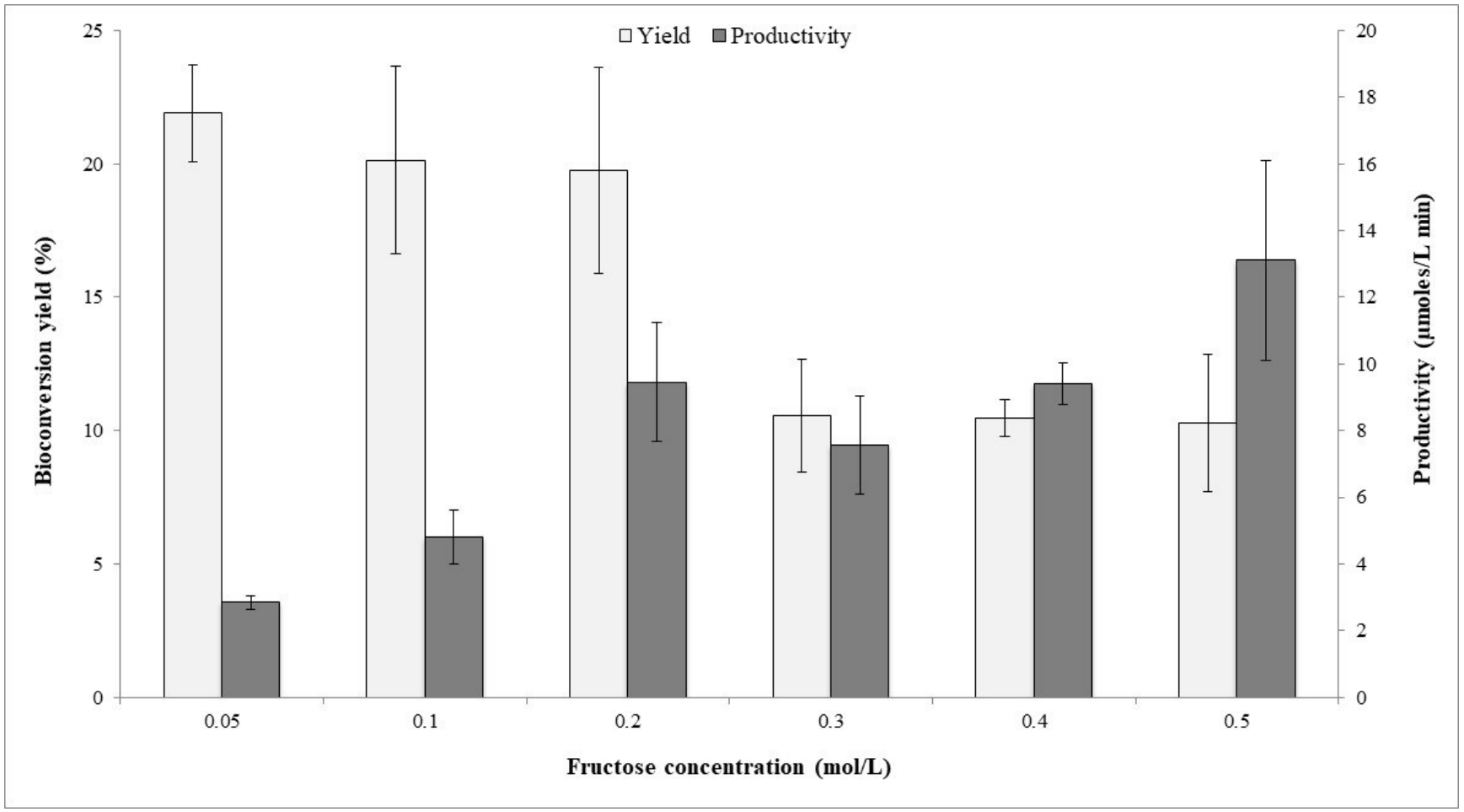

Figure 5.5 Effect of glycoside concentration on the bioconversion yield and reaction productivity of fructose laurate esterification catalyzed by Novozym® 435 in low-solvent media composed of lauric acid and *tert*-butanol at a ratio of 9:1 (v/v).

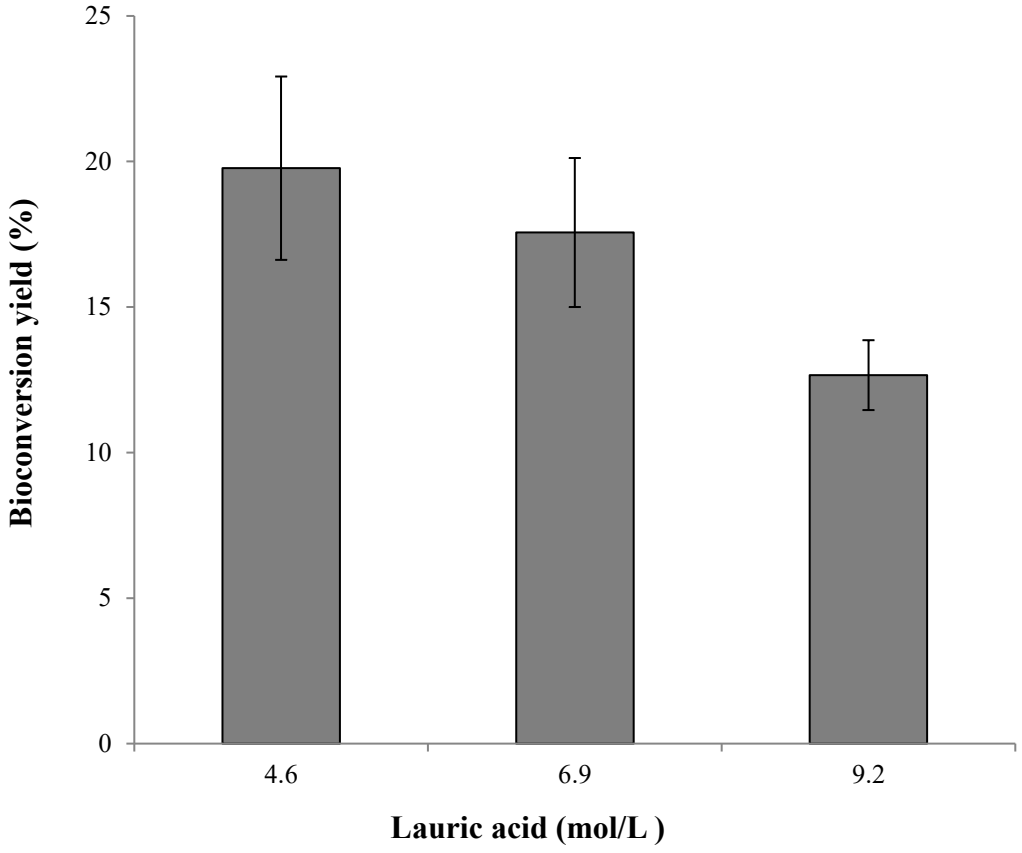

Figure 5.6 Effect of lauric acid molar concentration on the bioconversion yield of fructose laurate enzymatic synthesis catalyzed by Novozym® 435 in a low-solvent reaction media (10% *tert*-butanol).

5.4.7. Time course for the synthesis of fructose esters in a low-solvent reaction media

Figure 5.7 shows the time course of fructose laurate and fructose myristate synthesis catalyzed by Novozym® 435. Fatty acid concentration was established at 4.6 mol/L while milled fructose concentration was set at 0.2 mol/L. Higher conversions were obtained after 72 h, with 18.02 % of the fructose esterified to lauric acid, while esterification to myristic acid achieved a 17.14 % yield (Figure 5.7). A lower yield (~ 10 %) for the Novozym® 435-catalyzed biosynthesis of fructose myristate in the presence of an excess of myristic acid in the reaction media has been reported (Scheckermann et al., 1995). Interestingly, in the present study, two thirds of the ester products were synthetized during the first 8 h of reaction time in both acylation experiments. One of the causes for the slight variation of bioconversion yield may be a difference in the viscosity of the acyl donors at 55 ºC, as the melting point of lauric acid is 43.8 ºC while that of myristic acid is 54.4 ºC. Moreover, Soultani et al. (2001) reported a decrease in the bioconversion yield of the reaction upon increase the fatty acid aliphatic chain size. The decrement might be due to the saturation of the enzyme active site by a large amount of the acyl donor, which can restrict the access of fructose to the active site of the biocatalyst.

5.5. Conclusion

Per a one-factor-at-a-time evaluation, the best substrate concentrations for the biosynthesis of fructose laurate in a low-solvent reaction media using 10% of *tert*-butanol (v/v) were defined at 0.2 mol/L of milled fructose and 4.6 mol/L of lauric acid. Increased hydrophobicity of the Novozym® 435 support favoured bioconversion while enlarging the surface area of the polar substrate by ball-milling improved fructose availability for the esterification reaction. By applying these conditions, it is possible to obtain a similar bioconversion to that obtained using DMSO as a co-solvent in the same reaction setting. Low-solvent media (< 10 %, v/v) allows for a reduction of solvent waste and avoids extra concentration/purification steps of the reaction products, improving the efficiency of the process. This study is believed to lay out the foundation for the development of free-solvent biosynthetic processes applied to food and cosmetic industries.

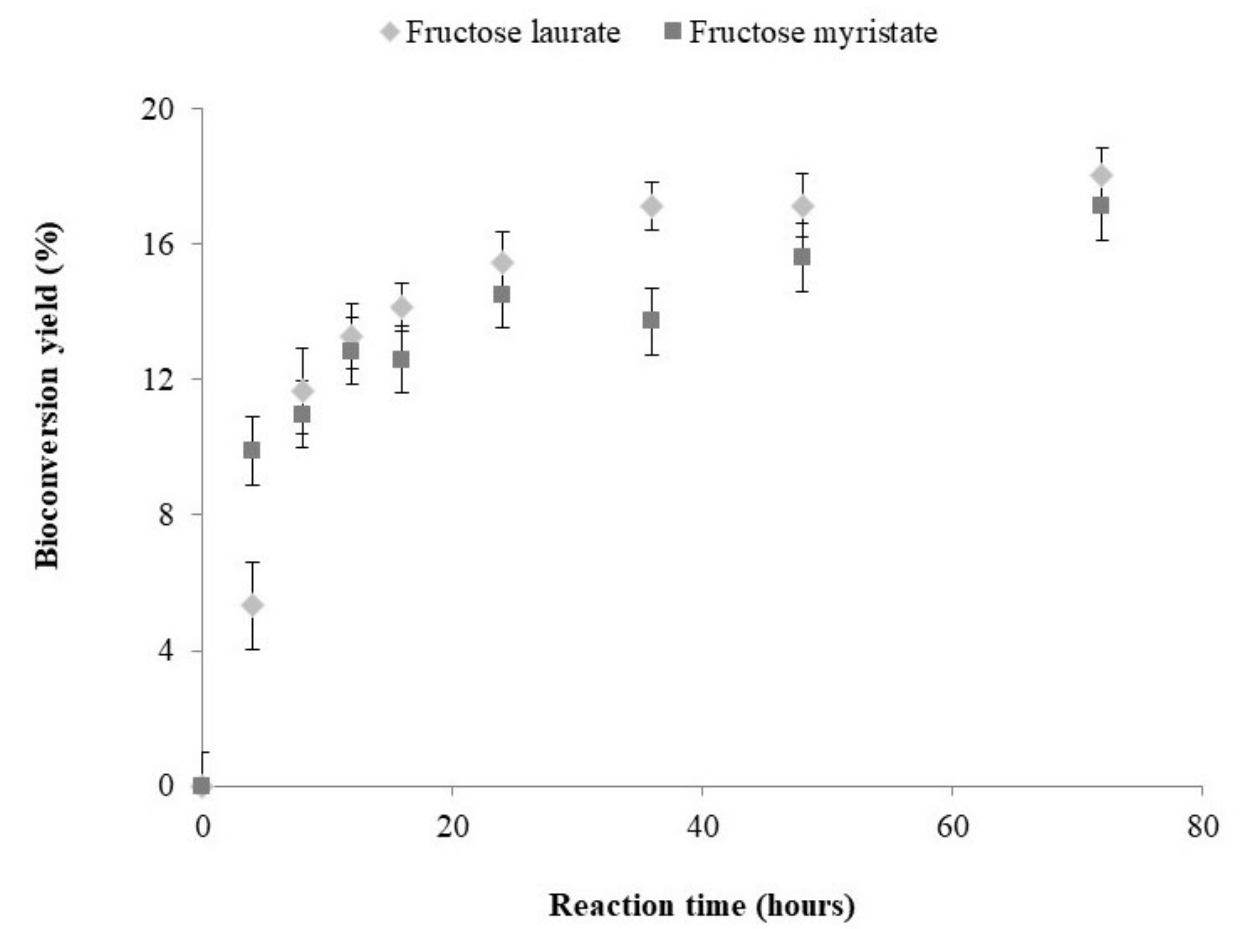

Figure 5.7 Time-course bioconversion yield of fructose laurate and fructose myristate biosynthesis catalyzed by Novozym® 435 in a low-solvent media (10% *tert*-butanol).

CONNECTING STATEMENT 4

In Chapter V, the low solvent immobilized lipase-catalyzed esterification reaction was investigated for the acylation of fructose to medium and long saturated fatty acids. Chapter VI continued the one-parameter-at-a-time optimization of the previous chapter by evaluating the effect of the acyl acceptor chain length and the use of molecular sieves as a water controlling agent on the bioconversion yield. Addition of oleic acid to the reaction media was applied as an alternative to reduce the mass diffusional limitations in the reaction system. At this point, optimization of the low solvent approach by RSM using a five-level and three-variable CCRD was applied not only to maximize the productivity and bioconversion of the reaction but also to gain assessment of the significance and interaction effects of the variables, including fructose concentration, immobilized lipase concentration, and oleic acid content. A reusability study of the immobilized biocatalyst was performed setting the variables at levels to maximize each of the responses of the design. Lastly, acylation end-products were characterized by MS.

Tamayo-Cabezas, J., Carrillo-Montes, J.P., & Karboune, S. (2018). Lipase-catalyzed synthesis of myristic acid esters in a low solvent reaction media: effect of acyl acceptor, optimization of the bioconversion and reusability of the biocatalyst (*To be submitted*).

CHAPTER VI. LIPASE-CATALYZED SYNTHESIS OF MYRISTIC ACID ESTERS IN A LOW SOLVENT REACTION MEDIA: EFFECT OF ACYL ACCEPTOR, OPTIMIZATION OF THE BIOCONVERSION AND REUSABILITY OF THE BIOCATALYST

6.1. Abstract

Enzymatic synthesis in organic solvents attracted a high interest as a tailored alternative for sugar esters production because of its process simplification, waste reduction, and the possibility to label the product as “natural”. However, aprotic solvents required to dissolve the polar substrates are not only detrimental for the enzyme, but their substitution have been re-examined due to environmental and human health considerations. Herein, a thorough study of the immobilized lipase-catalyzed esterification of myristic acid in low solvent reaction media (10 %, v/v) is presented. The chain length of the acyl acceptor was reported to be inversely proportional to the reaction yield. Application of molecular sieves proved unsuccessful in low solvent media due to their saturation and the mass transfer limitations that arose from their use. A 1:1 ratio of oleic to lauric/myristic acid in the reaction decreased the viscosity and the melting point of the media but also decreased the esterification yield by half. RSM based on a 5-level and 3-factor central composite design was used as statistical optimization tool which helped us to determine the reduced quadratic models that accurately described the yield and productivity of the reaction. Maximum reaction yield (22%) was obtained using 0.2 mol/L of fructose and 5% (w/v) of enzyme in a reaction media composed of 10% *tert*-butanol, 10% oleic acid, and 80% myristic acid. Reaction parameters that lead to maximum yield (25.5%) without addition of oleic acid were 0.2 mol/L of fructose and 6% (w/v) of enzyme. With the latter conditions, Lipozyme® RM IM catalyzed 6 consecutive reactions before showing reduction of its synthetic activity. ESI-MS analysis confirmed the selectivity of this enzyme for saturated fatty acids over unsaturated fatty acids.

6.2. Introduction

Surfactants are key ingredients in the food industry, influencing not only the texture but also the appearance of food products. Sugar-based surfactants made from renewable materials have experienced a growing demand due to their improved biodegradability and lower ecotoxicity compared to petroleum-based surfactants (Jurado et al., 2013; Steber, 2007). Chemical synthesis has provided a wide range of commercially-available sugar-based surfactants such as alkyl polyglycosides (APG). However, APG synthesis is an energy-demanding process requiring the use of base catalysts to produce complex mixtures of several amphiphilic species (Pantelic and Cuckovic, 2014). These limitations have raised interest among both food scientists and food

processors to develop alternative synthetic pathways for sugar-based surfactants. Lipase-catalyzed synthesis of carbohydrate fatty acid esters (CFAE) has been extensively studied and is a promising route which can lead to process simplification, production of specific structures, waste reduction, and the possibility to label the product as "natural".

One of the main challenges of lipase-catalyzed fatty acid acylation of sugars is the difficulty to solubilize the two substrates in a monophasic reaction media. Some publications have investigated using mixtures of moderately polar solvents with water-miscible aprotic solvents such as dimethyl sulfoxide (DMSO) to increase the solubility of the polar substrate (Pérez-Victoria and Morales, 2006; Sagis et al., 2008). However, categorization of DMSO as a 'problematic' solvent due to its environmental and health concerns (Prat et al., 2014), along with observed deactivation of lipases by this solvent (van Kempen et al., 2014) reduces the sustainability profile of this strategy. On the other hand, a solvent-free or a low-solvent enzymatic synthesis approach eliminates or reduces the use of organic solvent to a minimum, which would be unable to dissolve the carbohydrate substrate, but maintains a role as a reaction coadjuvant. The use of solvent-free and low solvent media for the enzymatic acylation of simple sugars has been shown to increase the productivity of the process and the number of cycles of reutilization of the enzyme (Yan et al., 2001; Ye and Hayes, 2012).

Myristic acid and its derivatives have been evaluated for their potential in both the health and food sectors. In terms of health applications, they may act as carriers to deliver potentially targeted therapeutics across the blood-brain barrier (Li et al., 2011; Shen et al., 2013). Moreover, myristic acid sugar esters have been reported to inhibit the growth of common food-related pathogens. Karlová et al. (2010) found that adding myristic acid fructose esters at a concentration of 1.25 mmol/L strongly reduced the growth of *Bacillus cereus* and *Fusarium culmorum*. Additionally, sucrose myristate displayed inhibitory activity against Gram-positive bacteria (Zhao et al., 2015). However, solvent-free or low-solvent acylations should take place at temperatures higher than 60°C to avoid mass transfer limitations that arise due to the solidification of myristic acid, which is not a suitable condition for the biocatalysts as the optimum temperature range for resin-immobilized lipases is 30-60°C. It can be hypothesized that the addition of oleic acid may help to overcome this issue by reducing the melting point of the reaction media while offering multi-products.

In our previous work, the acylation of fructose with myristic acid catalyzed by either Novozym® 435 or immobilized lipase from *R. miehei* (Lipozyme® RM IM) in a low-solvent reaction media was investigated using 10% (v/v) of *tert*-butanol as reaction co-adjuvant (Tamayo-Cabezas et al., 2018). The effects of various reaction parameters such as concentration and particle size of the polar substrate were assessed on a one-parameter-at-a-time basis. However, the understanding of the interactive effects of selected reaction parameters may contribute to a better modulation of the acylation and the end-product profile. The main objective of the current study was to investigate the lipase-catalyzed acylation of fructose and fructooligosaccharides (FOS) with myristic acid in low solvent reaction media. The first part of the study focused on evaluating the effect of the acyl acceptor in the reaction yield and productivity. Then, the addition of a water-controlling agent followed by use of a mixture of fatty acids were assessed. The optimization of the bioconversion yield and productivity of the synthesis of myristic acid esters in low solvent media was then carried out. Using the identified optimal reaction conditions that maximize the reaction yield, the reusability of the enzyme was evaluated. Finally, an analysis of the acylation profile of the reaction products by ESI-MS was performed.

6.3. Experimental

6.3.1. Materials

Lipozyme® RM IM, Novozym® 435, crystalline D-(-)-fructose (≥ 99%), myristic acid (≥ 98%), oleic acid (≥ 99% GC), *tert*-butanol (≥ 99.0%), ethyl acetate (≥ 99.7%), and molecular sieves 4Å 8-12 mesh were purchased from Sigma Chemical Co. (St-Louis, MO). FOS commercial products Fibrulose® F97 and Fibrulose® L85 were kindly donated by Cosucra Groupe Warcoing SA (Warcoing, Belgium). 1-kestose was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Sep-Pak® Vac Aminopropyl (NH_2) 6 cc (500 mg sorbent) cartridges were purchased from Waters Ltd (Brossard, QC). High performance liquid chromatography (HPLC) grade acetonitrile, methanol, n-hexane, toluene, and all other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

6.3.2. Direct enzymatic esterification in low solvent media

Esterification of fructose with selected fatty acids was carried out in low solvent reaction media composed of *tert*-butanol and myristic acid substrate (1:9, v/v), according to the identified optimal

reaction medium for the direct esterification of lauric acid with D-fructose (Tamayo-Cabezas et al., 2018). Two concentrations (0.05 mol/L and 0.2 mol/L) of powdered fructose were used. First, fructose was added to *tert*-butanol in 4 mL amber vials with PTFE-faced rubber caps; then the mixture was incubated at room temperature under continuous shaking in a Forma Scientific orbital incubator shaker for 30 min at 40 rpm. To initiate the reaction, 171 mg/mL of immobilized lipase beads（Lipozyme® RM IM or Novozym® 435） and 4.6 mol/L of myristic acid were added. All vials were vacuum sealed and incubated at 55 °C with continuous shaking at 200 rpm for 72 h. Experiments were run in triplicate. Blanks without enzyme and without glycoside substrate were carried out alongside the esterification reactions to account for possible unwanted side reactions.

6.3.3. Ball-milling of glycoside substrate

Reduction of fructose and 1-kestose particle size was performed in a MM2000 Retsch ball mill operating at a vibrational frequency of 28 Hz for 30 min. Confirmation of the decrease in fructose particle size was observed on a Hitachi™ TM-3000 tabletop scanning electron microscope (Hitachi High-Technologies Corporation, Japan).

6.3.4. Analysis of esterification end-products

Quantitative analysis of the reaction end-products was performed using a Beckman System Gold® 126 Solvent Module high-performance liquid chromatograph (HPLC) coupled to an Alltech 3300 evaporative light scattering detector (ELSD) (BÜCHI Labortechnik AG, Switzerland). A 20 µL aliquot of the recovered reaction product solution was injected into the solvent module with the ELSD detector operating at 75 ºC with a nitrogen flow of 1.5 L/min. Separation was performed on a Zorbax SB-C18 reversed-phase (RP) column (5 µm, 250 mm × 4.6 mm, Agilent Technologies Canada Inc.; Mississauga, ON). Mobile phase was applied at 0.3 mL/min in a linear gradient with the following profile: 50% deionized water/50% acetonitrile/methanol/hexane (70:28:2, v/v) for 5 min, increasing to 100% acetonitrile/methanol/hexane (mobile phase B) after 10 min and holding for 30 min at the same flow rate before reverting to the initial eluent composition. Standard curves were constructed by injecting different concentrations of purified fructose myristate in the range of 1 mmol/L to 5 mmol/L. Recovery and the purification of the reaction end-products were performed following the procedure described by Tamayo Cabezas et al. (2017). Bioconversion yield (%) was calculated from the concentration of the synthesized fructose/FOS ester of myristic acid divided by the initial concentration of the glycoside substrate, multiplied by 100. Productivity

of the reaction (mmol/L min) was obtained from the ratio of synthesized CFAE by the duration of the esterification reaction.

6.3.5. Effect of acyl acceptor on the enzymatic synthesis of myristic acid esters

To study the effect of the chain length of the acyl acceptor in the bioconversion yield and productivity of the reaction, FOS varying in their degree of polymerization (1-kestose, Fibrulose® F97 and Fibrulose® L85) were used as glycoside substrates at 0.05 mol/L and/or 0.2 mol/L. Fibrulose® FOSs were evaluated two different concentrations (0.05 mol/L and 0.2 mol/L) while milled 1-kestose was only added at the latter concentration. Each acyl acceptor was evaluated using either Lipozyme® RM IM or Novozym® 435 as biocatalyst of the reaction. Esterification reactions proceeded according to the method described in section 2.2.

6.3.6. Effect of molecular sieves on the enzymatic synthesis of myristic acid esters

The effect of the addition of a water control agent, such as molecular sieves, on the bioconversion yield and productivity of fructose/FOSs enzymatic esterification with myristic acid was investigated by adding molecular sieves 4Å 8-12 mesh at a ratio of 10% (w/v) to the reaction vials prior to incubation at 55 °C. Sieves were previously activated at 200 °C in a vacuum oven. Reactions were performed following the procedure described in Section 2.2, using the most efficient enzyme and acyl acceptor from the previous esterification trials at a concentration of 0.2 mol/L.

6.3.7. Effect of oleic acid addition on the enzymatic synthesis of myristic acid esters

Oleic acid was added to the reaction media to overcome potential mass diffusion limitations of the principal acyl donor – myristic acid – into the lipase support and to reduce the viscosity of the reaction media. The effect of the addition of oleic acid on the yield and productivity of the enzymatic synthesis of fructose myristate esters was studied using three selected concentrations of oleic acid in the reaction media: 5%, 20%, and 50% (v/v). Reactions were performed following the procedure described in section 2.2, using the most efficient enzyme and glycoside substrate at a concentration of 0.2 mol/L.

6.3.8. Experimental design for the optimization of the enzymatic synthesis of myristic acid esters

RSM was used to optimize the levels of variables known to influence the enzymatic esterification of myristic acid in low solvent reaction media, and to assess the interactions of these variables and their effect on the bioconversion yield and productivity of the acylation. A five-level, three-variable central composite rotatable design (CCRD) with six center points, eight factorial points, and six- star points was used as the experimental design. The independent variables included in the design were percentage of oleic acid (v/v) in the reaction media, fructose concentration (mol/L), and percentage of immobilized lipase in the reaction media (w/v). Experimental ranges of the variables were selected based on results obtained with preliminary experiments. The experimental and coded values for each level of these variables are detailed in Table 6.1. Reactions were performed using the most efficient enzyme and glycoside substrate from previous esterification trials and reaction products were quantified following the procedure described in Section 2.4.

Table 6.1 Levels and experimental range and levels of the independent variables used in RSM in terms of actual and coded values.

		Range of levels				
Variables		-α	-1	0	+1	+α
A.	Oleic acid (%, v/v)	0	8.1	20	31.9	40
B.	Fructose (mol/L)	0.02	0.2	0.5	0.8	1
C.	Enzyme (%, w/v)	5	9.1	15	20.9	25

6.3.9. Statistical analysis

Bioconversion yield (%) and productivity of the reaction (μmol/L min) were selected as the responses of the RSM design. The obtained experimental data was fitted into a second order polynomial equation by multiple regression analysis using the software Design-Expert 8.0.2 (Stat-Ease, Inc., Minneapolis, MN, USA). The proposed quadratic models for the optimization of the dependent responses have the following general structure:

$$Y_n = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where Y_n is the predicted response, β_0 is the intercept; β_1, β_2, β_3 are the linear terms; β_{12}, β_{13}, β_{23}, are the interaction terms; β_{11}, β_{22}, β_{33} are the quadratic terms; and X_{1-3} are the independent variables of the experimental design. Statistical testing of the proposed optimization models was evaluated using analysis of variance (ANOVA). Model fitting accuracy was expressed by the adjusted and predicted R^2, and its statistical significance was determined by Fisher's test. The proposed models were validated by performing three replicates at selected experimental conditions, aiming to maximize each of the responses with and without addition of oleic acid.

6.3.10. Reusability of the biocatalyst

The potential of biocatalysis in a low solvent reaction media to increase the reusability of the enzyme was assessed by measuring the variation of the bioconversion yield after successive esterification reactions. Experimental esterification conditions were defined by specific optimization criteria using the models obtained in Section 2.9. After each esterification cycle (72 h), the immobilized lipase beads were rinsed three times with 10 mL of acetone per rinsing cycle. Then, the beads were recovered by filtration and transferred to a convection oven for 5 min (60 °C) to let excess of acetone evaporate.

6.3.11. Purification and structural characterization of the myristic acid esters

The reaction end-products were purified by solid phase extraction (SPE) chromatography with a moderately polar Sep-Pak® Vac aminopropyl reverse phase columns using as eluents acetonitrile, followed by ethyl acetate, and finally hexane. CFAE were recovered from organic solvents in a Thermo Scientific AES2010 SpeedVac® System (Waltham, MA). Myristic acid ester production and acylation profile in low solvent media was confirmed using ESI LC/MS system comprised of an Agilent 1200 HPLC module coupled to a G6224A time-of-flight Agilent spectrometer（Agilent Technologies Canada Inc.，Mississauga，ON) operating in negative ion mode with scan detection in the m/z range of 100–3000. An aliquot of 0.5 µL of sample was injected and separation was performed on a Zorbax RP column (4.6 mm x 150 mm). Mobile phase was applied at a constant flow of 0.5 mL/min in a linear gradient with the following profile: initial composition of 90 % 50 mM ammonium formate/10 % pure methanol changing to 5 % 50 mM ammonium formate /95 % pure methanol after 10 min. Capillary potential was 3 kV and the fragmentor operated at 120 V. The obtained data was processed using Agilent MassHunter® MS software.

6.4. Results and discussion

6.4.1. Effect of the chain length of the acyl acceptor

Selected fructose-based glycosides varying in their degree of polymerization (DP) were evaluated as suitable acyl acceptors for the low solvent acylation reaction of myristic acid catalyzed by immobilized lipases. The acyl acceptors include Fibrulose® F97 (DP range from 2 to 20), Fibrulose® L85 (DP range from 2 to 10), the trisaccharide 1-kestose, and fructose. Fibrulose FOS are derived from chicory-extracted inulin, which undergoes an enzymatic hydrolytic procedure to reduce their DP; while 1-kestose (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl α-D-glucopyranoside) is synthesized by glycosyltransferases from filamentous fungi acting on sucrose. The investigated acylation reactions were catalyzed either by Lipozyme® RM IM or Novozym® 435 at two different glycoside substrate concentrations (0.05 mol/L and 0.2 mol/L). Reaction media composition and parameters were selected based on previous results proven effective for the direct esterification of lauric acid in low solvent reaction media (Tamayo-Cabezas et al., 2018).

Table 6.2 shows the bioconversion yield and the productivity attained with these experimental conditions. After 72 h of reaction, the highest bioconversion yields were obtained with ball-milled fructose and Fibrulose® L85. At low concentration of glycoside substrate (0.05 mol/L), reactions catalyzed by Novozym® 435 esterified 31.2 % of the initial Fibrulose® L85, while reactions catalyzed by Lipozyme® RM IM esterified 8.7 % of the initial glycoside concentration. At the same concentration (0.05 mol/L); 19.2 % and 41.7 % of fructose was converted to fructose myristate in the reactions catalyzed by Lipozyme® RM IM and Novozym® 435, respectively. Increasing the concentration of fructose to 0.2 mol/L resulted in a higher bioconversion in the reactions catalyzed by Lipozyme® RM IM (27.5 %) compared to the yields obtained using Novozym® 435 (18.2 %). Contrary to what was observed in the experiments with d-fructose, bioconversion in the reactions catalyzed by Novozym® 435 at high concentration of Fibrulose® L85 (0.2 mol/L) decreased to 7.4 %, but nevertheless was higher that the bioconversion attained with Lipozyme® (4.8%). However, productivity of the esterification doubled in the reactions catalyzed by Lipozyme® RM IM at high concentration of Fibrulose® L85. On the other hand, productivity remained constant (3.6 %) in the reactions catalyzed by Novozym® 435 disregarding the concentration of Fibrulose® L85 added to the reaction.

Table 6.2 Bioconversion yield and productivity of fructose myristate synthesis obtained through esterification reaction catalyzed by two commercial immobilized lipases in a low solvent reaction media.

Acyl acceptor	Concentration (mol/L)	Bioconversion yield [a] (%)		Productivity [b] (µmol/L min)	
		Lipozyme® RM IM[c]	Novozym® 435[d]	Lipozyme® RM IM	Novozym® 435
Fructose	0.05	19.2 ± 1.5	41.7 ± 2.3	2.2 ± 0.2	4.8 ± 0.3
	0.2	27.5 ± 2.2	18.2 ± 1.8	12.7 ± 0.1	8.4 ± 0.8
Fibrulose L85	0.05	8.7 ± 0.7	31.2 ± 2.3	1.0 ± 0.1	3.6 ± 0.3
	0.2	4.8 ± 0.2	7.4 ± 0.2	2.2 ± 0.1	3.4 ± 0.1
Fibrulose F97	0.05	0.2 ± 0.02	0.2 ± 0.01	0.02 ± 0.001	0.02 ± 0.001
	0.2	n.d.	n.d.	n.d.	n.d.
1-kestose	0.2	1.9 ± 0.1	n.d.	0.8 ± 0.02	n.d.

[a] Maximum bioconversion yield (%). The bioconversion yield was calculated as the concentration of consumed fructose over the initial concentration of fructose, multiplied by 100.
[b] Productivity was measured as concentration of synthesized esters (µmol/L) per minute divided by the duration of the reaction.
[c] Lipase from *Rhizomucor Miehei*
[c] Lipase acrylic resin from *Candida Antarctica* type B

Very low bioconversion yields were observed in the reactions with Fibrulose® F97 or 1-kestose as acyl acceptors. The limited availability of these glycoside substrates in the immobilized lipases' micro-environments may have led to the observed low yields. Indeed, Fibrulose® F97, in a powder form with a minimum oligofructose content of 95%, and 1-kestose have limited solubility in alcohols and ketones. Blecker et al. (2002) determined the average DP of Fibrulose® F97 to be 5.5. The steric hindrance of the kestose and the fructose-based oligomers at the lipase's active site may have also limited their esterification with myristic acid. Novozym® 435 catalyzed acylation of inulin-derived FOS (average DP of 4.4) with fatty acids using a mixture of *tert*-butanol and DMSO in amounts enough to solubilize the reactants have been reported in the literature (Van Kempen et al., 2013). The reported bioconversions after 69 h of reaction time were 36 % for the acylation experiments with lauric acid, while 52 % of the FOSs esterified to palmitic acid.

To improve the availability of the 1-kestose, it was ball-milled to reduce the size of its crystals. No improvement in the bioconversion yield was observed after ball-milling the kestose. Myristic acid acylation was only attained in the reactions catalyzed by Lipozyme® RM IM with a bioconversion of 1.9 % and a productivity of 0.8 µmol/L min after 72 h of reaction time. In contrast, the particle size reduction of the acyl acceptor proved to be successful for the low solvent lauric acid acylation of fructose (Tamayo-Cabezas et al., 2018). This may reveal the significant contribution of the steric hindrance of the kestose substrate at the lipase's active site to the low bioconversion yield.

The effect of the immobilization support nature of the enzyme on the synthesis of CFAE was also elucidated. The reduced bioconversion yields of the Fibrulose® L85-based acylation reactions catalyzed by Lipozyme® RM IM can be explained by the hydrophilicity of the anion exchange resin used as a support for this enzyme. Fibrulose® L85 is a syrup (75.5 °Brix) containing 85 % FOS on dry basis. The water content of the oligofructose syrup (~15 %) along with the hydrophilic nature of Lipozyme® RM IM support may have favoured the adsorption of polar compounds present in the reaction media into the resin and limited the presence of the nonpolar substrate in the microenvironment of the immobilized enzyme. However, the reaction equilibrium yield decreased due to the increased water content near the enzymes. Traditionally, acylation of FOS by transesterification is preferred over direct esterification due to the increased reactivity of the ester group over the carboxyl group, and the facilitated removal of the aldehyde group generated as a by-product of the reaction compared to water generated by direct esterification (Plou et al., 2002).

However, esterification has certain benefits over transesterification, such as the direct application of natural-occurring fatty acids instead of chemically-produced vinyl esters, or avoidance of potential inactivation of the biocatalyst, possibly caused by aldehydes released as a sub-product of transesterification reactions (Weber et al., 1995).

The effect of water removal on the Lipozyme® RM IM-catalyzed acylation reactions was assessed. Due to the hydrophilicity nature of Lipozyme® RM IM immobilization support, its initial water content was higher as compared to Novozym® 435 (Jenab et al., 2014). Flores et al. (2002) reported that a decline in the glucose laurate esterification synthesis rate may be caused by the water accumulation and not by the limited sugar dissolution rate. Activated microporous molecular sieves have been used as water removal agents in the esterification or the transesterification of fructose and FOS with fatty acids in solvent-based reaction media (Casas-Godoy et al., 2016; Šabeder et al., 2006). Table 6.3 shows the results obtained using microporous molecular sieves (8-12 mesh) as a water controlling agent at a concentration of 10 % (w/w). Overall, addition of molecular sieves negatively affected the yield and productivity of the reaction.

Compared to the reactions without sieves, fructose myristate bioconversion decreased by 84 % while myristic acid acylation of Fibrulose® L85 decreased by 56 %. Similarly, productivity of the reactions with molecular sieves decreased compared to the ester production rate obtained without the water controlling agent. No esterification could be observed in the reactions where Fibrulose® F97 was used as acyl acceptor. The addition of molecular sieves to the reaction media at the aforementioned concentration and granulometry may have a detrimental effect on the mass transfer of the substrates and reaction products. Šabeder et al. (2006) used microporous molecular sieves for the biosynthesis of fructose palmitate in low solvent reaction media and found that at molecular sieve concentrations ranging from 12.1 % to 35.6 % (w/w), it was challenging to maintain uniform stirring of the reaction mixture. The observed operational limitation coincided with a decrease in the bioconversion yield of the esterification reactions (Šabeder et al., 2006).

Table 6.3 Comparison of bioconversion yield and productivity of fructose myristate synthesis obtained through esterification reaction catalyzed by Lipozyme® RM IM in a low solvent reaction media.

Acyl acceptor	Concentration (mol/L)	Bioconversion yield [a] (%)		Productivity [b] (µmol/L min)	
		Without MS[c]	With MS	Without MS	With MS
Fructose	0.2	27.5 ± 2.2	4.5 ± 1.0	12.7 ± 0.1	2.1 ± 0.1
Fibrulose® L85	0.2	4.8 ± 0.2	2.1 ± 0.1	2.2 ± 0.1	1.0 ± 0.02
Fibrulose® F97	0.2	n.d.	n.d.	n.d.	n.d.

[a] Maximum bioconversion yield (%). The bioconversion yield was calculated as the concentration of consumed fructose over the initial concentration of fructose, multiplied by 100.
[b] Productivity was measured as concentration of synthesized esters (µmol/L) per minute divided by the duration of the reaction.
[c] Molecular sieves (MS)

6.4.2. Effect of oleic acid addition on the enzymatic acylation of myristic acid

Our previous study showed that Lipozyme®RM IM-catalyzed synthesis of fructose laurate in a low solvent reaction media was more effective at 55 °C (Tamayo-Cabezas et al., 2018). Nevertheless, low solvent enzymatic acylation of fructose with myristic acid at this temperature may be problematic due to its proximity to the melting point of the fatty acid (54.4 °C). Moreover, the optimum temperature range for the ester hydrolysis catalyzed by Lipozyme® RM IM is 30 °C to 50 °C (Novozymes A/S, 2016). It can be hypothesized that substituting partially the myristic acid in the reaction with oleic acid may overcome the substrate diffusional limitations caused by the myristic acid solidification. Indeed, oleic acid melting point (14 °C) is drastically lower than that of myristic acid, and the potential side esterification reactions are minimized due to the reported chemoselectivity of Lipozyme® RM IM towards saturated fatty acids. Mukherjee et al.（1993) studied the esterification of unsaturated fatty acids with *n*-butanol in the presence of myristic acid catalyzed by an immobilized lipase from *M. miehei*. It was found that fatty acids having specific positions of the first *cis* double bond from the carboxyl were strongly discriminated against myristic acid. Selmi et al. (1998) selected Lipozyme® RM IM as the catalyst to assess the effect of the fatty acid unsaturation on the synthesis of triglycerides in a solvent-free media They reported that the initial velocity and rate of triacylglycerol synthesis with oleic acid were lower compared to the values obtained with myristic acid as acyl donor, concluding that the higher the number of unsaturation of the fatty acid, the lower the rate of synthesis and the final yield (Selmi et al., 1998).

However, a balance between both fatty acids must be established as the solubility of the sugar esters will be affected by the addition of a more nonpolar substrate to the reaction media (Gumel et al., 2011). Figure 1 shows that the increment of oleic acid in the reaction media was inversely proportional to the bioconversion yield and the productivity of the reactions. A decrement of 60 % on the bioconversion yield was obtained when half of the myristic acid was replaced with oleic acid. When 25 % of the myristic acid in the reaction was replaced with oleic acid, the average bioconversion yield decreased by 28 % compared to the control without oleic acid (Figure 6.1). The use of 5% of oleic acid led to similar bioconversion yield than that achieved without oleic acid; while no solidification of the reaction media was observed in the reaction systems containing 5 % of oleic acid, revealing the potential of these reaction systems containing oleic acid to be

scaled up. It is important to note that no additional ester peak was detected upon the addition of oleic acid (data not shown).

6.4.3. Optimization of the enzymatic acylation of myristic acid

6.4.3.1. Analysis of variance and validation of the predictive models

To optimize the concentration of the independent variables, namely oleic acid concentration, fructose concentration, and enzyme concentration, and to simultaneously assess their interactions on the bioconversion yield and productivity of the enzymatic acylation reaction of myristic acid, a central composite circumscribed rotatable design was selected. This design was chosen due to its excellent prediction accuracy across a defined design space, and a reduced number of trials compared to a full factorial design (Tobias and Trutna, 2012). Trials were performed in random order and each response point of the design corresponds to the average of three replicates. Fructose was selected as the acyl acceptor of the reaction because of its higher solubility in the low solvent reaction media allowing the assessment of the influence of the selected variables on the esterification reaction. The real and coded levels of the design with the experimental and the model-predicted responses are detailed in Table 6.4.

Along the design points, maximum bioconversion (20.8 %) was obtained with design trial No. 7 (8.1 % oleic acid, 0.2 mol/L of fructose, and 20.9 % of Lipozyme® RM IM). Minimum reaction yield (3.1 %) was obtained with the experimental conditions of design point No. 5 (20 % oleic acid, 1 mol/L of fructose, and 15 % of Lipozyme® RM IM). Independent variables parameter values applied in trial No. 20 (8.1 % oleic acid, 0.8 mol/L of fructose, and 20.9 % of Lipozyme® RM IM) resulted in the highest reaction productivity, with a rate of 11.8 μmoles/L min. Lowest ester productivity (5 μmol/L min) was attained upon the application of the conditions of trial No. 15 (20 % oleic acid, 0.025 mol/L of fructose, and 15 % of Lipozyme® RM IM). The analysis of variance (ANOVA) for the design is shown in Table 6.5. The best-fitting models were evaluated for significance (F-test values, P values, lack of fit). The high F-values obtained for each of the models along with low P-values implies that reduced quadratic models are significant for the description of both responses. The regression equations obtained gave the level of bioconversion yield and productivity achieved as a function of oleic acid, powdered fructose and lipase starting concentrations in the reactions.

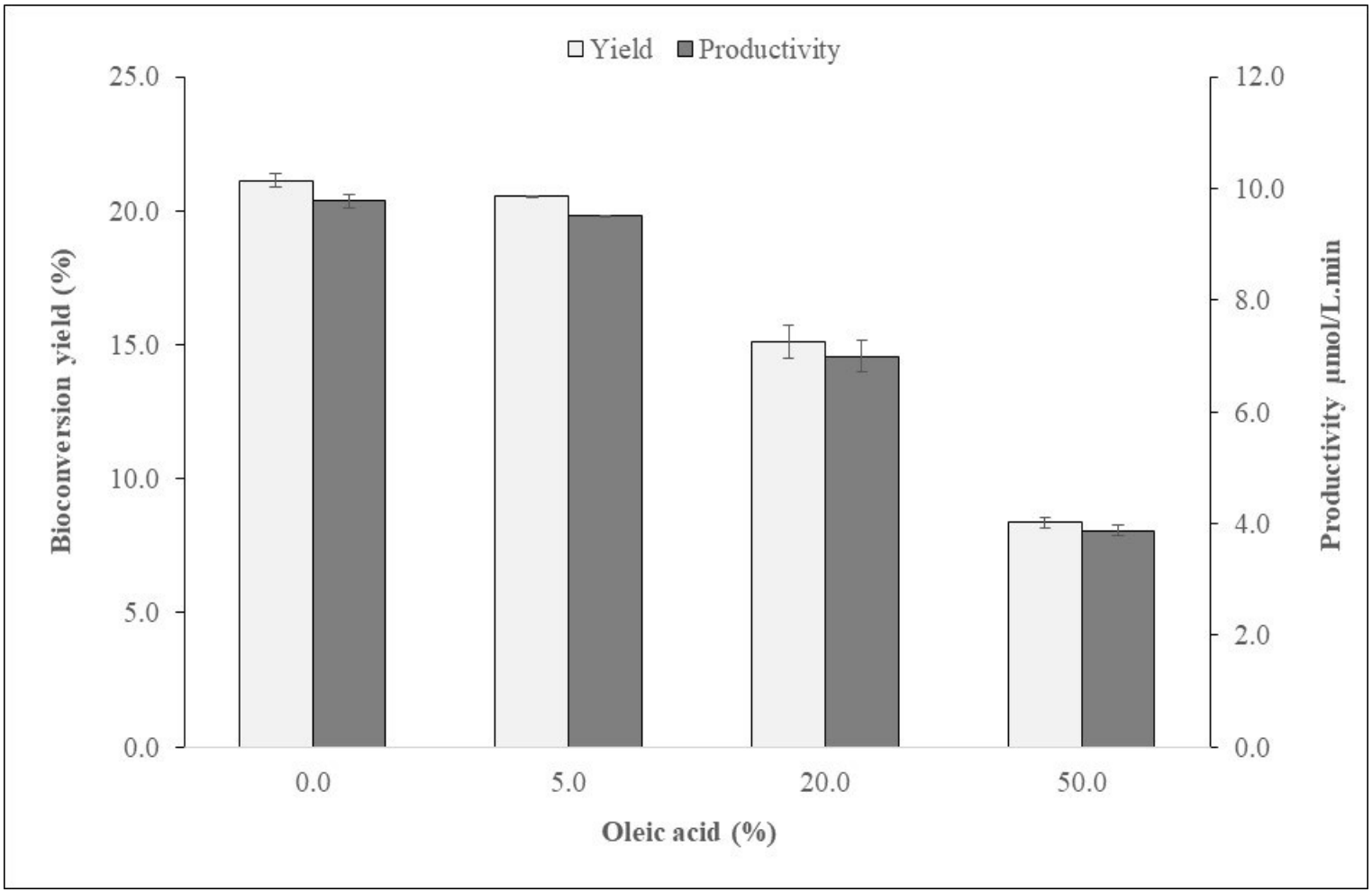

Figure 6.1 Effect of oleic acid content in the reaction media on the bioconversion yield and reaction productivity of fructose myristate esterification catalyzed by Lipozyme® RM IM in a low solvent reaction media composed of lauric acid and tert-butanol at a ratio of 9:1 (v/v)

Table 6.4 Results of 5-level 3-variable central composite rotatable design for the optimization of the Lipozyme® RM IM-catalyzed synthesis of myristic acid esters of fructose in a low solvent reaction media showing observed and predicted responses.

Run[a]	Oleic acid (X_1)		Fructose (X_2)		Enzyme (X_3)		Bioconversion yield (%)[b]		Productivity (µmol/L min)	
	Actual	Coded	Actual	Coded	Actual	Coded	Experimental	Predicted	Experimental	Predicted
1	31.9	+1	0.2	-1	20.9	+1	12.6	12.9	6.5	6.2
2	20.0	0	0.5	0	15.0	0	7.9	8.2	9.4	9.6
3	20.0	0	0.5	0	15.0	0	8.3	8.2	9.8	9.6
4	20.0	0	0.5	0	15.0	0	8.0	8.2	9.4	9.6
5	20.0	0	1.0	+α	15.0	0	3.1	4.3	7.1	7.6
6	20.0	0	0.5	0	15.0	0	9.2	8.2	10.9	9.6
7	8.1	-1	0.2	-1	20.9	+1	20.8	20.5	10.7	11.0
8	20.0	0	0.5	0	15.0	0	7.9	8.2	9.3	9.6
9	0.0	-α	0.5	0	15.0	0	10.5	11.8	12.4	13.0
10	31.9	+1	0.8	+1	20.9	+1	3.5	3.1	6.6	6.4
11	8.1	-1	0.2	-1	9.1	-1	20.7	20.3	10.6	10.5
12	20.0	0	0.5	0	15.0	0	8.6	8.2	10.2	9.6
13	20.0	0	0.5	0	25.0	+α	7.4	7.7	8.7	8.8
14	40.0	+α	0.5	0	15.0	0	4.3	4.6	5.0	6.2
15	20.0	0	0.0	-α	15.0	0	18.7	26.2	1.1	8.1
16	31.9	+1	0.8	+1	9.1	-1	3.7	3.1	6.8	6.2
17	8.1	-1	0.8	+1	9.1	-1	5.2	4.1	9.6	9.6
18	31.9	+1	0.2	-1	9.1	-1	14.5	14.3	7.5	7.4
19	20.0	0	0.5	0	5.0	-α	6.7	7.6	7.9	8.2
20	8.1	-1	0.8	+1	20.9	1	6.3	5.7	11.8	11.5

[a] Runs were performed in random order.

[b] The experimental results are an average percentages of triplicate trials within ± 10% error.

Table 6.5 The analysis of variance (ANOVA) for central composite rotatable design.

Parameters and interactions	Bioconversion Yield		Productivity	
	F-Value	P[a]	*F*-Value	P
Model	70.95	< 0.0001	17.35	< 0.0001
Oleic acid concentration (X_1)	75.87	< 0.0001	118.28	< 0.0001
Fructose molar concentration (X_2)	470.82	< 0.0001	0.54	0.4777
Enzyme concentration (X_3)	0.020	0.8917	0.89	0.3678
X_1X_2	15.31	0.0029	0.10	0.7547
X_1X_3	1.68	0.2243	3.04	0.1120
X_2X_3	1.17	0.3042	2.11	0.1770
$(X_2)^2$	64.31	< 0.0001	6.82	0.0260
$(X_3)^2$	0.67	0.4318	4.18	0.0681
Lack of Fit	5.03	0.0504	1.51	0.3313
Mean	8.89		8.97	
R^2	0.98		0.93	
Adjusted R^2	0.97		0.88	
Predicted R^2	0.89		0.78	
Coefficient of variance	10.16		7.71	
Adequate Precision	24.93		17.18	

[a] $P < 0.05$ indicates statistical significance.

The equations that represented an adequate model for the yield of the lipase-catalyzed fructose ester of myristic acid synthesis (Y_1) and productivity of the esterification reaction (Y_2) are:

(1) $Y_1 = 8.19 - 2.13A – 6.5B + 0.034C + 1.25AB – 0.41AC + 0.35BC + 2.49B^2 – 0.2C^2$

(2) $Y_2 = 9.61 - 2.03A – 0.17B + 0.18C – 0.079AB – 0.43AC + 0.36BC - 0.62B^2 – 0.38C^2$

Where A is oleic acid percentage in the reaction media; B is fructose molar concentration and C is lipase concentration.

The coefficient of determination (R^2) was calculated to be 0.98 for the esterification yield model (Y_1) and 0.93 for the productivity model (Y_2). These values imply that the models could explain 98 % and 93 % variability of the responses, respectively. The regression analysis also revealed that the predicted and the adjusted R^2 values of both models are in reasonable agreement, with a difference of 0.1 or less between them. If the model contains multiple non-significant terms or inadequate samples size, then the adjusted R^2 may be significantly different (± 0.2) than the predicted R^2. Adequate precision measures the signal to noise ratio, with an adequate precision value greater than 4 indicating that the proposed model can be used to navigate the design space. The values of adequate precision obtained were 24.93 for the bioconversion yield model and 17.18 for the productivity model.

The linear term of oleic acid content and the fructose molar concentration, their interaction, and the quadratic term of the fructose concentration were the most significant terms of the proposed bioconversion yield model. Productivity was statistically influenced predominantly by the linear term of the oleic acid content of the reaction media and the quadratic term of the fructose molar concentration in the reaction. Figure 6.2 shows the plots between the predicted vs the actual experimental response values of the experimental design. The purpose of this diagnostic graph was to visually detect which design point or design range had not been accurately predicted by the proposed response model equations. Each point in the graph corresponded to a design point. All design points gathered closely to the diagonal line of the graph correlation between the experimental and the predicted response results, indicating that the proposed equations accurately described the relationship between the independent variables and the selected responses.

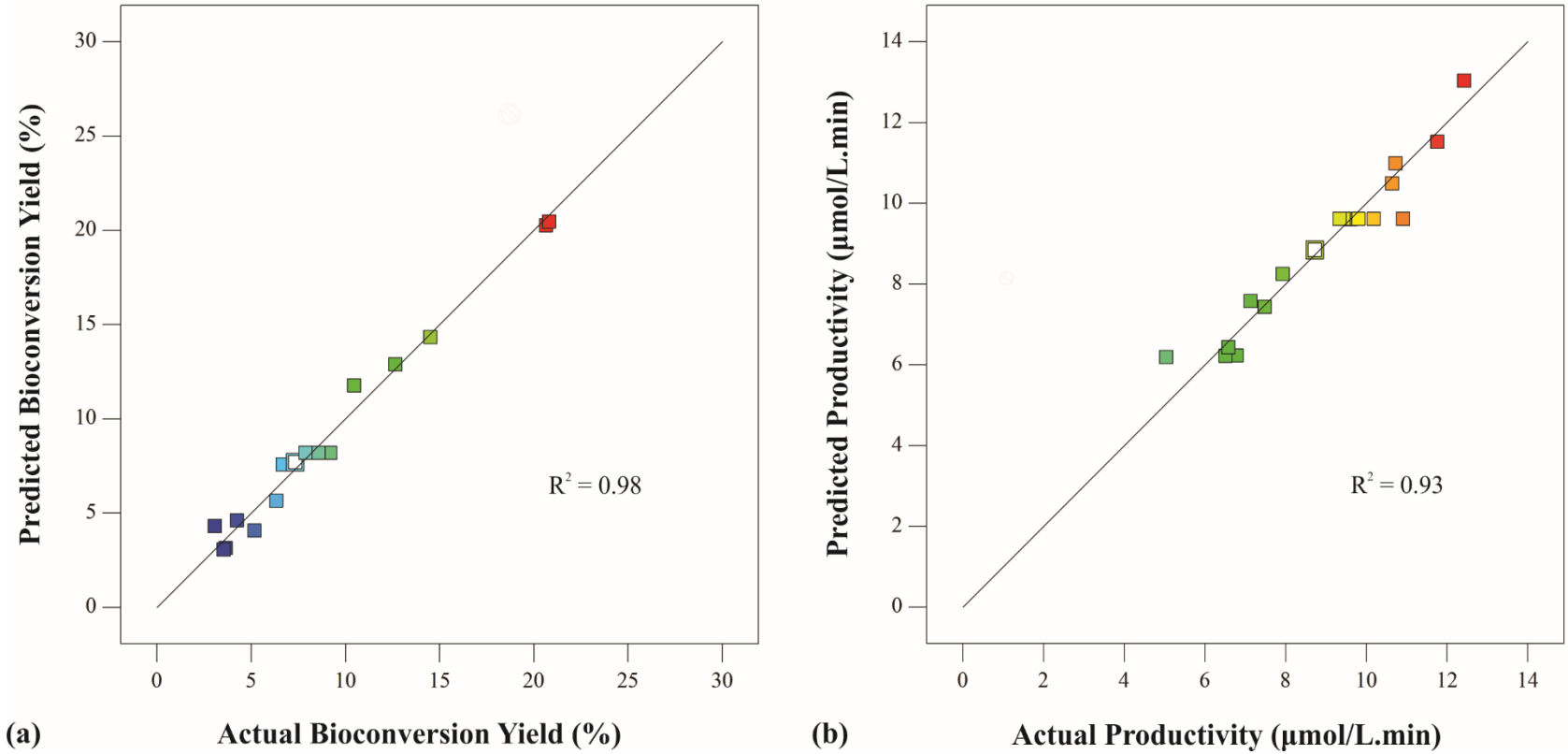

Figure 6.2 Predicted vs. actual bioconversion yield (%) and productivity (µmol/L min) model diagnostic plot

Table 6.6 Confirmation report of the proposed optimization model for the biosynthesis of fructose myristate in a low solvent reaction media.

	Variable levels							
	Oleic acid (%, v/v)	Fructose (mol/L)	Enzyme (%, w/v)	Response	95% PI[a] low	Predicted mean	Data mean[b]	95% PI high
Condition 1	10.00	0.20	5.06	Yield	17.68	20.45	22.03	23.22
Condition 2	0.00	0.20	6.10	Yield	19.58	22.96	25.48	26.35
Condition 3	0.00	1.00	25.00	Productivity	9.43	12.66	13.91	15.87
Condition 4	10.00	0.90	25.00	Productivity	8.40	10.70	11.54	13.01

[a] PI: prediction interval

[b] Data mean is the average response from three replicates of each experimental condition

Confirmation experiments were run under four different sets of variable conditions inside and outside the defined design space to validate the proposed response models. Optimization criteria applied to designate the values of the independent variables were aimed to maximize one response at a time in the presence or absence of oleic acid in the reaction media. The experimental conditions selected for the confirmation experiments are detailed in Table 6.6, along with the lower and upper prediction intervals, the predicted mean and the experimental mean. Experimental means (either yield or productivity results) are the average of three replicates obtained for each set of experimental conditions. It can be observed that all the experimental means obtained fell inside the established prediction intervals (α risk = 0.05) thus validating the proposed optimization models.

6.4.3.2. Effects of reaction parameters

The significance of fructose concentration on the proposed model for the bioconversion yield of the reaction can be better understood with the series of two-dimensional contour plots shown in Figure 6.3. The upper row of plots shows the effect of enzyme at three different concentrations. With the center and axial values of this variable seen across the design space of the two other variables. As can be observed, the distribution of colors remains constant in all the upper row plots, meaning the bioconversion yield is inappreciably affected even by increasing the enzyme concentration in a 130 %. The lower row of contours shows the effect of fructose concentration at the center and axial values for this variable across the design space of the other two independent variables. In the proposed model, bioconversion yield is inversely proportional to the amount of fructose added to the reaction vials. When initial fructose concentration is 0.8 mol/L (positive axial value), enzyme concentration is low and oleic acid concentration is high, the bioconversion yield decreases to 3.5 %. Keeping the same concentrations of enzyme and oleic acid but decreasing the fructose concentration to its lower axial value (0.2 mol/L), the bioconversion yield increases to 16 %. The bioconversion yield increases to 21.3 % at this fructose concentration if the oleic acid concentration is decreased to its lower axial value (8.1 %, v/v).

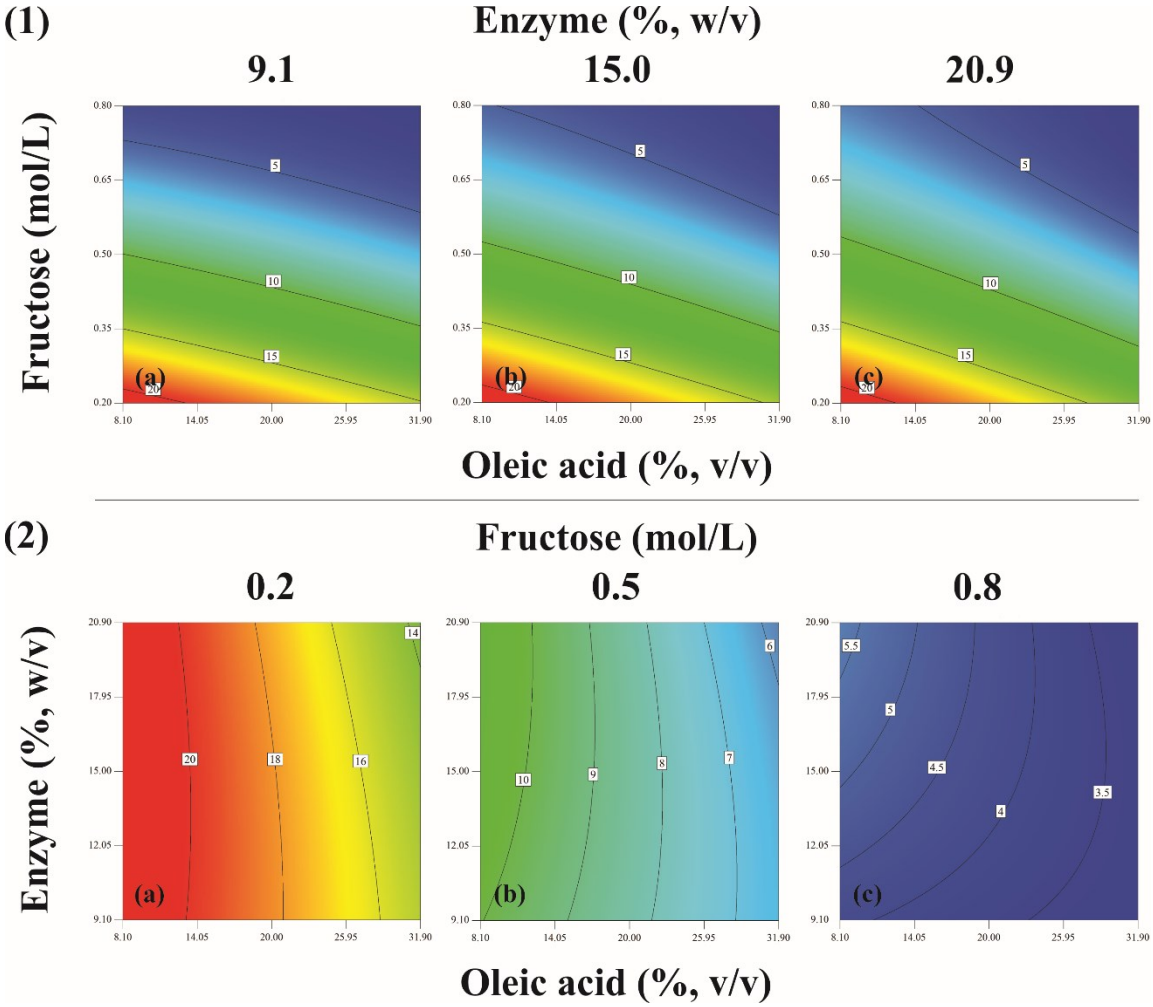

Figure 6.3 Contour plots showing the interaction of the independent variables on the bioconversion yield response quadratic model, numbers in squares indicate the bioconversion obtained by myristic acid acylation of fructose catalyzed by Lipozyme® RM IM in a low solvent reaction media composed of myristic acid (90%) and tert-butanol (10%)

6.4.4. Reusability of the immobilized lipase

Reusability of the immobilized lipase in the myristic acid acylation reaction system was evaluated. Figure 6.4 shows that the bioconversion yield and the productivity increased during five esterification cycles (equal to 360 h), peaking at the fifth cycle. It can be hypothesized that the fructose accumulated inside the pores of the immobilized enzyme may have led to an apparent increased in the bioconversion yield obtained through the five cycles even though the immobilized enzyme was thoroughly washed three times with acetone after each esterification cycle. Results confirm the benefit accomplished by low solvent reaction media on the stability of Lipozyme® RM IM. It was previously reported that this enzyme was able to catalyze four consecutive cycles (528 h) of fructose oleate synthesis in solvent-free media without loss of activity (Ye et al., 2014). Zhong et al. (2013) evaluated the stability of Lipozyme® RM IM in the solvent-free synthesis of diacylglycerols of lauric acid and found that only 15% of the original catalytic activity was lost after 6 consecutive batches (18 h).

6.4.1. Structural characterization of myristic acid esters of fructose

Esterification end-products produced using the identified optimal conditions by RSM were purified by RP SPE chromatography, and analyzed by ESI LC-MS. Purification was confirmed with HPLC ELSD prior to the spectrometric analysis. LC-ESI-MS negative ion full scan showed two peaks with a retention time of 12.2 min and 14.9 min aside the myristic acid and the oleic acid standards (data not shown). Figure 6.5a shows the ESI-MS negative ion scan spectrum of the acetonitrile-extracted fraction analyzed at 12.2 min of retention time. ESI-MS negative ion spectra of the same fraction at 14.9 min of retention time is shown on Figure 6.5b.

Expected mass to charge ratio (m/z) values of the most common negative ion adducts for fructose myristate monoester are 389.2545 $[M-H]^-$, 425.2312 $[M+Cl]^-$, 435.2599 $[M+HCOO]^-$, and 452.2501 $[M+NO_3]^-$; while the typical negative ion adducts for fructose myristate diester have an expected m/z value of 599.4528 $[M-H]^-$, 635.4295 $[M+Cl]^-$, 645.4583 $[M+HCOO]^-$, and 662.4485 $[M+NO_3]^-$ Spectrums depicted in Figures 5a and 5b shows the chlorine $[M+Cl]^-$, formate $[M+HCOO]^-$, and nitrate adducts $[M+NO_3]^-$ corresponding to fructose monoester of myristic acid and fructose diester of myristic acid, respectively Peaks on the diester spectrum display nearly 5 times less intensity than the peaks on the monoester spectrum. Even though monoacylation was favoured, it is important to point out that diacylated products were obtained at a substantial

proportion in the low solvent reaction media. Similar myristic acid acylation profile was obtained in a solid-phase system by Cao et al., (1998). Whereas lipase-catalyzed acylation of fructose and FOS in conventional solvent-based reaction media containing tert-butanol produced mostly mono-esters (Degn and Zimmermann, 2001; Tükel et al., 2013; Van Kempen et al., 2013).

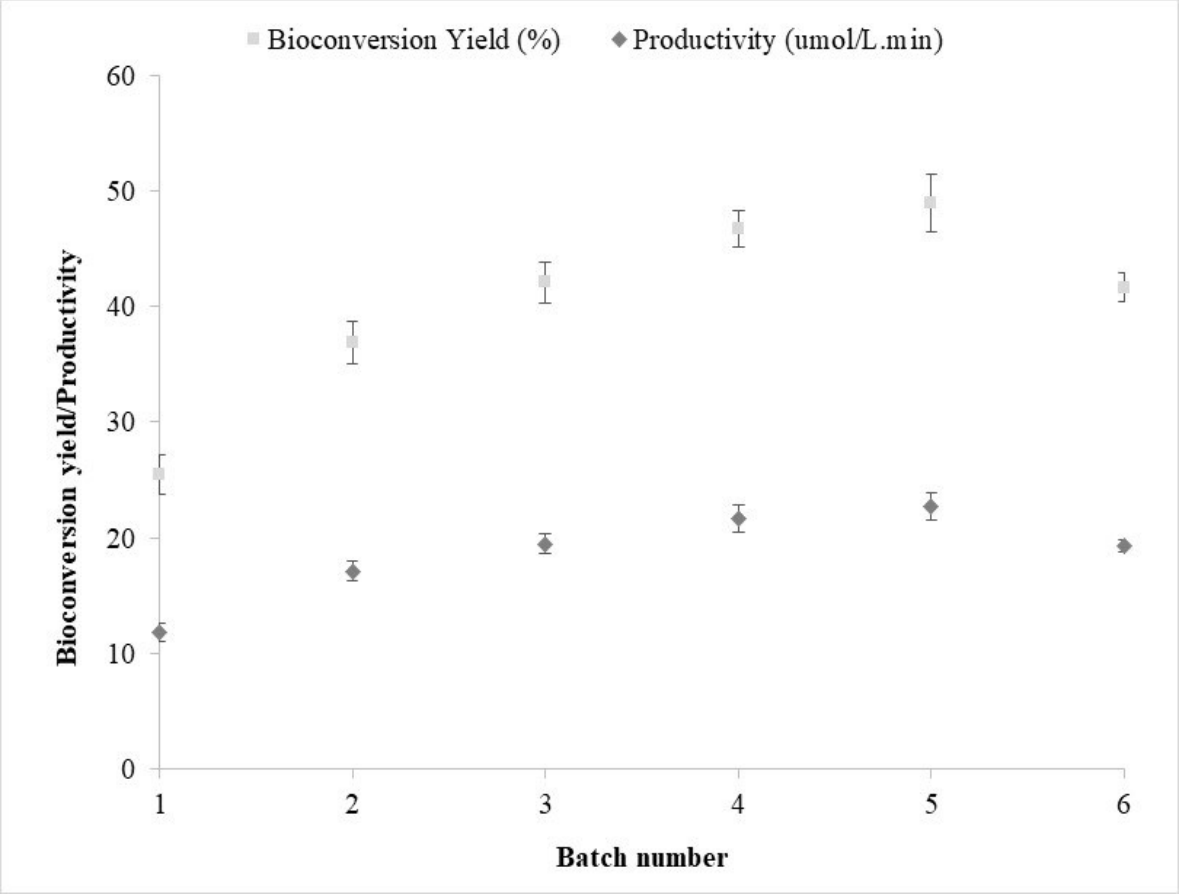

Figure 6.4 Reusability of Lipozyme® RM IM for the synthesis of fructose myristate in a low solvent reaction media composed of myristic acid (90%) and tert-butanol (10%) v/v

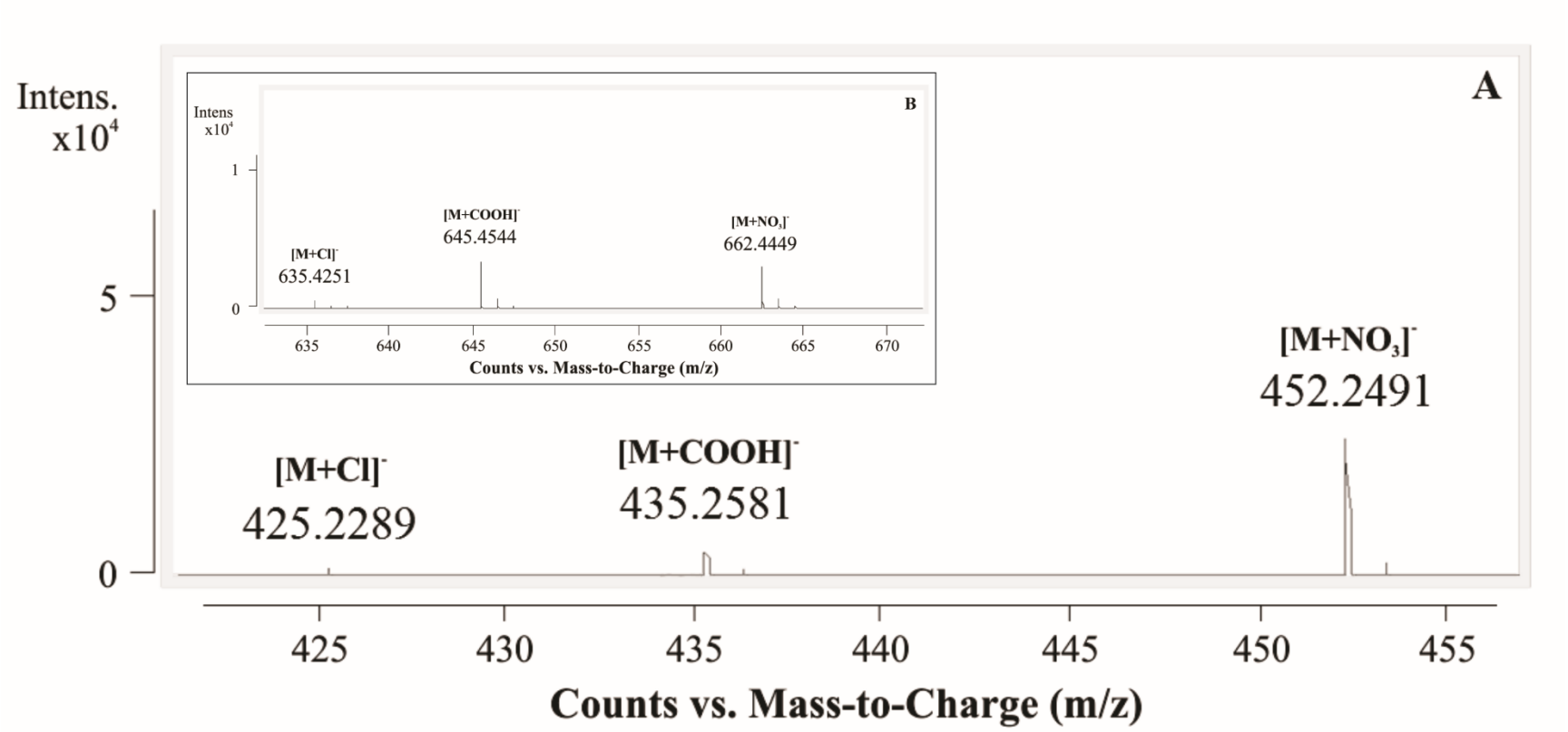

Figure 6.5 LC-ESI-MS spectra of the fragmentation pattern of the Lipozyme® RM IM-catalyzed myristic acid acylation of fructose in a reaction media composed of *tert*-butanol, oleic acid, and myristic acid at a ratio of 1:1:9 (v/v): (a) monoester formation, (b) diester formation.

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6.5. Conclusion

Low solvent reaction media with a co-solvent content of 10% of tert-butanol and 90% of acyl donor content was studied as potential reaction media for the enzymatic synthesis of myristic acid esters. Increasing the chain length of the acyl acceptor was found to reduce the availability of the glycoside substrate for the esterification reaction due to the lower rate of dissolution of the oligosaccharides in the reaction media. Molecular sieves were not suitable controlling-water agents in the presence of excess fatty acid in the reaction media due to their saturation and the mass transfer limitation caused by their addition to the reaction vial. The inclusion of oleic acid to the reaction media reduces the dissolution of the polar substrate. However, it allows for the use of low solvent reaction media containing fatty acids with melting points higher than 50 °C at conditions closer to the optimum working temperature of the enzyme, favouring reusability of the immobilized lipase. RSM optimization determined the optimum experimental conditions for the synthesis of myristic acid esters of fructose in low solvent media with and without addition of oleic acid. Bioconversions to fructose myristate under these conditions were similar than those attained using aprotic solvents as co-solvents. Six consecutive reaction cycles without addition of enzyme between reactions. MS spectra of the reaction products confirmed the selectivity of Lipozyme® RM IM for saturated fatty acids. This study proposes a novel more-sustainable alternate methodology for the acylation of C_{14} or longer saturated fatty acids.

CHAPTER VII. GENERAL SUMMARY AND CONCLUSIONS

The focus of this research was on the development of novel biosynthetic approaches to produce acylated mono- and oligosaccharides catalyzed by immobilized carboxyl ester hydrolases. The acylation of saccharides with bioactive moieties has attracted attention as a viable means of obtaining value-added products from low cost renewable sources. Widespread application of diverse acyl groups coupled with additional functionalities to mono- and oligosaccharides has been implemented in the pharmaceutical, food and cosmetic industries. Despite the abundance, low cost and natural origin of the substrates, the different attachment possibilities of the acyl donor to the saccharide unit, with each potential coupling leading to the synthesis of a specific compound with unique physicochemical properties, poses a challenge. The development of selective and reproducible enzymatic processes in non-conventional reaction media is of great importance.

Therefore, the esterification of selected oligosaccharides with ferulic acid catalyzed by a purified FAE from *H. insolens* was studied in six different surfactant-less microemulsions. The reaction medium composed of n-hexane/2-butanone/water favoured the acylation of all the evaluated oligosaccharides, while xylobiose was determined to be more effective as an acyl acceptor compared to raffinose and XOS, confirming the direct effect of carbohydrate length on the FAE-catalyzed feruloylation of oligosaccharides. Covalent attachment of the FAE to epoxy supports proved to be the best immobilization strategy for this enzyme. The inclusion of metal-chelate functional groups through a two-step partial chemical modification of the epoxide in the supports improved protein immobilization and the retention of FAE hydrolytic activity. Compared to its free counterpart, immobilized FAE onto metal-chelate epoxy supports retained up to 35 % of the feruloylated oligosaccharide synthetic activity. A model biocatalytic approach based on the immobilized FAE-catalyzed esterification reaction is paramount to produce well-defined non-digestible phenolated oligosaccharides with nutraceutical functionalities at a preparative level.

Reusability of the enzyme is a key characteristic of economically viable enzymatic processes. Immobilization of FAE from *H. insolens* on mesoporous metal-chelate epoxy supports was optimized using a 5-level 4-variable design RSM, namely mg of FAE/g of support, immobilization buffer pH, immobilization buffer molarity and immobilization time. Regression analysis showed that enzyme to support ratio, pH of the immobilization buffer and the interaction between enzyme to support ratio with the molar concentration of the immobilization buffer were the most significant parameters affecting FAE immobilization. In addition, it was shown that supports with larger pores enhanced FAE from *H. insolens* immobilization and their subsequent stabilization by multipoint

covalent attachment. Further, multi-immobilized FAE into Cu-IDA epoxy macroporous support almost fully retained the feruloylation capabilities of the non-immobilized esterase. These results show potential in further developing a robust and reliable procedure for the biosynthesis of specific feruloylated bioactive compounds.

One of the main challenges to overcome in the lipase-catalyzed acylation of saccharides with lipophilic moieties is the selection of a solvent capable to solvate both substrates without affecting the catalytic properties of the enzyme. Using a one-factor-at-a-time evaluation, the optimal parameters for the biosynthesis of fructose laurate in a low-solvent reaction media such as substrate concentration, type of co-solvent, and enzyme were determined. The increased hydrophobicity along with the low water content of the Novozym® 435 support favoured bioconversion, while ball-milling the polar substrate enlarged its surface area, improving fructose availability for the esterification reaction. By applying these conditions, a similar bioconversion was obtained compared to that using the polar aprotic DMSO as a co-solvent in the same reaction setting. The use of low-solvent media ultimately improves process efficiency by reducing solvent waste and avoiding extra downstream concentration/purification steps of the reaction products.

Low solvent reaction media was studied as potential media for the enzymatic synthesis of myristic acid esters of fructose and FOS. Molecular sieves were not suitable controlling-water activity in the presence of such an excess of fatty acid. A method to optimize experimental conditions for the synthesis of myristic acid esters of fructose in low solvent media with and without addition of oleic acid was determined using a 5-level and 3-factor central composite design RSM. Similar bioconversions to fructose myristate were obtained under the optimal conditions compared to those using aprotic solvents as co-solvents. The application of the proposed low-solvent methodology along with the optimum acylation parameters results in incrementing bioconversions for five consecutive reaction cycles without addition of enzyme between reactions.

Overall, this research contributes to the scientific knowledge for the effective synthesis of acylated mono- and oligosaccharides with potential complementary functionalities. Optimized methods resulted in an improved immobilization of FAE and led to an increase in the synthesis of fructose myristate in low-solvent media. Taken together, the overall experimental findings will contribute to lay the ground for the development of potential sustainable specific enzymatic processes applicable to food, pharmaceutical and cosmetic industries.

CHAPTER VIII. CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

8.1. Contributions to Knowledge

The major contributions to knowledge of this study are:

1. This is the first study to evaluate the hydrolytic activity of two pure FAE preparations on naturally occurring feruloylated oligosaccharides isolated from wheat bran and sugar beet, as well as methyl ferulate. The outcome of this assessment was used to select the most appropriate FAE preparation for ferulic acid acylation of oligosaccharides in non-conventional media.
2. For the first time, the enzymatic ferulic acid acylation of xylobiose, raffinose, XOS in selected surfactantless microemulsions catalyzed by a pure FAE was investigated. Results helped us compared the synthetic specificity of the enzyme when compared to previous experiments performed using FAE expressed in multi-enzyme preparations as biocatalysts.
3. For the first time pure FAE was successfully immobilized by covalent attachment onto epoxy meso- and macroporous supports. Five commercial immobilization supports were also used to evaluate their performance on the immobilization of the non-selective purified FAE. Design of a reliable immobilization protocol for FAE is of key importance to establishing a feasible commercial application for the immobilized biocatalyst.
4. This is the first study to demonstrate the activation-stabilization effect on the FAE attained by immobilization onto metal chelate-epoxy supports. Hydrolytic activity of the FAE immobilized on the modified epoxy support over methyl ferulate was higher than the activity attained by its non-immobilized counterpart.
5. For the first time immobilized FAE catalyzed the ferulic acid acylation of xylobiose and XOS in surfactantless microemulsion, and its performance was compared to the feruloylation reaction results obtained using free FAE. To our knowledge this is one of various FAE-immobilization strategies already published. However, literature regarding enzymatic reactions catalyzed by immobilized FAE evaluated the catalytic efficiency of the biocatalyst on hydrolysis reactions or synthesis of alkyl ferulates.
6. This is the first study to evaluate the reusability of the immobilized lipase for the synthesis of myristic acid fructose ester using low-solvent reaction media. Reduction of the polar aprotic solvent required to solubilize the polar acyl acceptor exerts a positive effect on the number of cycles catalyzed by the lipase.

7. The issue of quantifying reaction end-products in esterification reactions catalyzed by lipases immobilized onto hydrophobic supports using an excess of fatty acid as reaction media was addressed for the first time. Application of our proposed recovery process will help reduce the overestimation of the bioconversion.

8.2. Recommendations for Future Research

- Application and reusability studies of the metal chelate-epoxy-FAE on hydrolytic, esterification, and trans-esterification reactions.
- Thorough evaluation of the effects of the immobilization on the selectivity and specificity of the FAE.
- Assessment of the potential bioactive and functional properties of the synthesized acylated products, with a special focus on the properties of the products showing multiple acylation.
- Evaluation of the application of physical treatments prior to the enzymatic reaction (homogenization) or during the reaction (ultrasound) to either reduce mass diffusional limitations or improve the availability of the polar substrate in the hydrophobic environment of the immobilized enzyme.
- Screening of other desiccants rather than molecular sieves (i.e.: clay, silica gel, calcium sulphate) for the control of water in the low solvent reaction media proposed for the acylation of fructose and FOS, which would help assess the effect of water on the reaction equilibrium.

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