DEVELOPMENT OF A NOVEL BIOCATALYTIC APPROACH FOR THE SYNTHESIS OF FERULOYLATED GLYCOSIDES BY FERULOYL ESTERASE EXPRESSED IN SELECTED MULTI-ENZYMATIC EXTRACTS

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SHORT TITLE

Enzymatic Synthesis of Feruloylated Glycosides by Feruloyl Esterase

ABSTRACT

Methyl ferulate and isolated feruloylated non-digestible oligosaccharides (NDOs) from sugar-beet pulp and wheat bran were used as substrates to evaluate the levels of feruloyl esterase (FAE) activity and substrate specificity of twenty multi-enzymatic preparations. The synthesis of feruloylated monosaccharides by FAEs expressed in the six best multi-enzymatic preparations from *Bacillus* spp. (Ceremix), *Humicola* spp. (Depol 740L), *Aspergillus oryzae* (Flavourzyme), *Bacillus amyloliquefaciens* (Multifect P 3000), *Bacillus subtilis* (RP-1), and *Trichoderma reesei* (Depol 670L) was investigated using a surfactant-less organic microemulsion system as reaction medium. The highest bioconversion yield of feruloylated arabinose (37%) and galactose (61%) was obtained using Multifect P 3000 and Depol 670L as biocatalysts, respectively, in n-hexane, 1-butanol and 3-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH buffer reaction mixture (51:46:3, v/v/v). Using n-hexane, 2-butanone and MES–NaOH (51:46:3, v/v/v), Depol 670L resulted in the highest bioconversion yield of feruloylated xylose (37%). Feruloylated arabinose, galactose and xylose demonstrated potential antioxidant properties and their chemical structures were confirmed by APCI-MS. Depol 740L from *Humicola* spp. was enriched on FAE activity (2.5-fold) by ammonium sulfate precipitation. The enriched FAE extract exhibited the highest esterifying activity for the feruloylation of sucrose (13%) in n-hexane, 2-butanone and MES-NaOH (51:46:3, v/v/v). As compared to their corresponding ferulic acid, the feruloylated di- and NDOs demonstrated similar or higher potential antioxidant properties. By varying the media composition, the highest bioconversion yields were obtained in n-hexane, 2-butanone and MES-NaOH (log *P* of 1.8) using arabinobiose (8%), xylobiose (9%) and raffinose (11%) as glycoside substrates. However, using galactobiose as substrate, the highest bioconversion yield (27%) was obtained in n-hexane, 1,4-dioxane and MES-NaOH (log *P* of 1.6). RSM, based on a five level, four-factor central composite rotatable design revealed that enzyme amount and substrate molar ratio were the most important variables for the bioconversion yield of feruloylated raffinose. The optimum synthesis conditions were as follows: 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. The predicted value was 12.0% (119.7 μM) and the actual experimental value was 11.9% (119.4 μM).

RÉSUMÉ

Férulate de méthyle et les oligosaccharides non digestibles (ONDs) féruliques isolés de la pulpe de betterave et du son de blé ont été utilisés comme substrats afin d'évaluer les niveaux d'activité féruloyl estérase (FAE) et sa spécificité dans 20 préparations multi-enzymatiques. La synthèse de monosaccharides féruliques par les FAEs exprimées dans les six meilleures préparations de *Bacillus* spp. (Ceremix), *Humicola* spp. (Depol 740L), *Aspergillus oryzae* (Flavourzyme), *Bacillus amyloliquefaciens* (Multifect P 3000), *Bacillus subtilis* (RP-1), et de *Trichoderma reesei* (Depol 670L) a été étudiée en utilisant un système de microémulsion sans surfactants comme milieu réactionnel. Les rendements les plus élevés d'arabinose (37%) et de galactose (61%) féruliques ont été obtenus en utilisant, respectivement, Multifect P 3000 et Depol 670L comme biocatalyseurs et le mélange n-hexane, 1-butanol et acide 3-(*N*-morpholino) éthane-sulfonique (MES)-NaOH (51:46:3, v/v/v) comme milieu réactionnel. En utilisant n-hexane, 2-butanone et MES-NaOH, Depol 670L démontrait le meilleur rendement de bioconversion de xylose férulique (37%). L'arabinose, galactose et xylose féruliques ont démontré des propriétés antioxydant potentielles et leurs structures chimiques ont été confirmées par APCI-MS. Depol 740L a été enrichie par l'activité FAE (2,5 fois) par précipitation au sulfate d'ammonium. L'extrait FAE enrichi a démontré une activité d'estérification élevée pour la féruloylation du saccharose (13%) dans le mélange n-hexane, 2-butanone et MES-NaOH. Par rapport à l'acide férulique, les di- et ONDs féruliques ont démontré des propriétés antioxydant similaires ou supérieures. En faisant varier la composition du milieu réactionnel, les rendements les plus élevés ont été obtenus dans n-hexane, 2-butanone et MES-NaOH (log *P* 1,8) en utilisant arabinobiose (8%), xylobiose (9%) et raffinose (11%) comme substrat glycosidique. Mais, en utilisant galactobiose, le meilleur rendement (27%) a été obtenu dans n-hexane, 1,4-dioxane et MES-NaOH (log *P* 1,6). RSM, basée sur un composite central rotatif de 5 niveaux et 4 facteurs a démontré que la quantité d'enzyme et le rapport molaire des substrats (acide férulique:raffinose) étaient les variables les plus importantes pour le rendement de raffinose férulique. Les conditions de synthèse optimales ont été déterminées comme suite: T 35°C; rapport molaire des substrats 3:1; teneur en eau 3% (v/v); et quantité d'enzyme 345 unités FAE. La valeur prédite a été 12% (119.7 μM) et la valeur réelle expérimentale a été 11.9% (119.4 μM).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AnFAE: Feruloyl esterase from *Aspergillus niger*

AOS: Arabinooligosaccharide

APCI-MS: Atmospheric pressure chemical ionization–mass spectrometry

[bmim]BF_4: 1-butyl-3-methylimidazolium tetrafluoroborate

[bmim]PF_6: 1-butyl-3-methylimidazolium hexafluorophosphate

CCRD: Central composite rotatable design

DP: Degree of polymerization

$DPPH^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl

ESI-MS: Electrospray-mass spectrometry

E_t: Retention time

FAE: Feruloyl esterase

FoFAE: Feruloyl esterase from *Fusarium oxysporum*

FOS: Fructooligosaccharide

GI: Gastrointestinal

HPLC: High performance liquid chromatography

IL: Ionic liquid

LDL: Low-density lipoprotein

MES: 3-(*N*-morpholino)ethanesulfonic acid

MOPS: 3-(*N*-morpholino)propanesulfonic acid

NaOH: Sodium hydroxide

$(NH4)_2SO_4$: Ammonium sulfate

NDO: Non-digestible oligosaccharide

NSP: Non-starch polysaccharide

MS: Mass spectrometry

RSM: Response surface methodology

SCFA: Short-chain-fatty-acid

StFAE: Feruloyl esterase from *Sporotrichum thermophile*

TLC: Thin layer chromatography

XOS: Xylooligosaccharide

CHAPTER 1
INTRODUCTION

The potential physiological benefits of prebiotic non-digestible oligosaccharide (NDO) compounds to selectively support the intestinal health is now well established (Yuan et al., 2005a). These bioactive compounds have also demonstrated anticarcinogenic, antimicrobial and hypolipidemic properties (Mussatto and Mancilha, 2007). However, most of the NDOs are limited by the low colonic persistence of their prebiotic effect, which reduces their efficiency in the critical distal intestinal region. Phenolated NDOs isolated from wheat and sugar-beet (Ralet et al., 1994a,b) plant cell walls have recently gained interest owing to their potential complementary functional properties, both stimulating the growth of lactic acid bacteria and protecting against oxidative damage (Yuan et al., 2005a,b). Indeed these phenolated glycosides were found to be more effective antioxidants towards low-density lipoprotein (LDL) oxidation than free phenolic acids (Katapodis et al., 2003). Due to their phenolated groups, they may also display antimicrobial, anticarcinogenic and anti-inflammatory properties (Ou and Kwok, 2004). As a result, these bioactive molecules may help reduce the risk of chronic diseases in the distal intestinal region by using the gastrointestinal (GI) route as a potential pathway for the persistent delivery of NDOs and phenolic compounds to the colon

The isolation of phenolated NDOs from plant cell walls has primarily been based on a mild acid hydrolysis or an enzymatic hydrolysis approach by treatment with glycosyl hydrolases (Ralet et al., 1994a,b; Katapodis et al., 2003). In addition to their very low yield, their structures have not shown good consistency and their phenolated moieties were limited predominantly to ferulic and ρ-coumaric acids. However, enzymatic acylation of phenolic acids with glycosides, via carboxylic ester hydrolase-catalyzed esterification reaction in non-conventional reaction media, may be a valuable approach for the preparation of phenolated NDOs. Earlier work reported in the literature (Compton et al., 2000) has been limited to the esterification of phenolic acids with alkyl fatty chains to produce more hydrophobic phenolated compounds, using lipases as biocatalysts. At the moment, the esterification of phenolic acids with glycosides has been little studied and limited to the use of mono- and disaccharides (Topakas et al., 2005a; Vafiadi et al., 2005,

2006). As far as we are aware, only one investigator has reported the chemoenzymatic esterification of methyl ferulate with four linear arabino-NDOs, containing three to six arabinofuranose units (Vafiadi et al., 2007).

Finding a suitable biocatalyst for the esterification of phenolic acids with NDOs is a key step in the development of such an approach. The lower yields of lipase-catalyzed esterification of phenolic acids with glycosides has been previously reported and ascribed mainly to electronic and/or steric effects (Vafiadi et al., 2005). Recently, feruloyl esterases (FAEs; E.C.3.1.1.73) have gained interest due to their ability to directly catalyze the cleavage of ester bonds between plant cell walls and phenolic acids (Fazary and Ju 2007). However, only a small number of studies (Topakas et al., 2005a; Vafiadi et al., 2005, 2006) have examined the ability of FAEs to catalyze the esterification of phenolic acids with glycosides in non-conventional reaction media. Other limiting factors involved in the synthesis of phenolated NDOs include the non-commercial availability of FAEs and the high cost associated with their single use. Many commercial multi-enzymatic preparations being highly effective for the degradation of plant cell walls may exhibit high levels of FAEs (Mastihubova et al., 2006; Tsuchiyama et al., 2006).

Other key challenges in the enzymatic esterification of phenolic acids with NDOs involve controlling the nature of the reaction medium and parameters (temperature, substrate molar ratio, water content, and enzyme amount) so that enzymatic esterification can be thermodynamically favoured and the FAE properties can be modulated towards the synthesis of phenolated NDOs. Because the reaction media have the ability to alter the reaction equilibrium position, non-conventional systems, with low water activity, are more suited for the synthetic reactions (Topakas et al., 2003a). In addition to the ability of non-conventional media to control thermodynamically the unfavourable hydrolytic reactions, they also increase the solubility of organic substrates and facilitate the enzyme and product recovery (Yadav and Lathi, 2004). Micro- and nanoemulsion systems such as surfactant-less and ionic liquid (IL)-based emulsions have recently gained interest because in contrast to neat organic solvents of similar polarity, they often do not inactivate enzymes, simplifying reactions involving polar substrates (Park and Kazlauskas, 2003; Topakas et al., 2005a).

The main objective of this research was to develop a novel biocatalytic approach for the synthesis of feruloylated NDOs of great health benefits via a FAE-catalyzed esterification reaction.

The specific objectives for the proposed study were:

1. To identify and evaluate, in terms of FAE activity and substrate specificity, the efficiency of commercial multi-enzymatic preparations for the feruloylation of selected monosaccharides.

2. To study the effect of glycoside structures and media composition on the bioconversion yield of selected feruloylated glycosides.

3. To isolate and structurally characterize the feruloylated glycosides as well as evaluate the antioxidant activity of these novel biomolecules.

4. To optimize the reaction parameters for the enzymatic synthesis of a feruloylated raffinose via FAE-catalyzed esterification reaction.

CHAPTER 2
LITERATURE REVIEW

2.1. Plant Cell Wall

In addition to the various food components found within the plant cell such as protein, fat, sugar, and starch, the plant cell wall contains another food constituent known as the non-starch polysaccharides (NSPs) (Harris and Smith, 2006). Depending on the cell type and plant species, these NSPs can be found in the two major groups of wall types known as the primary and secondary walls (Harris and Smith, 2006). In addition, abundant amounts of NSPs can be found in the plant cell walls of fruits, vegetables and whole grain cereals (Harris and Smith, 2006).

Plant NSPs are a very heterogeneous group composed of many monomers including arabinose, xylose and galactose with a degree of polymerization (DP) greater than ten (Sanchez-Castillo et al., 2002). The principal NSPs, making up 90% of the plant cell wall, are referred to as dietary fibres (Selvendran and Robertson, 1990). Moreover, plant NSPs can be separated into cellulose, an unbranched polymer of β-(1-4)-D-glycopyranosyl units, as well as non-cellulosic polysaccharides such as hemicellulose, a branched polymer of β-(1-4)-xylopyranosyl units, and pectin which primarily contains an α-(1-4)-polygalacturonic acid backbone (Sanchez-Castillo et al., 2002).

The complexity of NSPs is further increased by the covalent binding of cinnamic acid derivatives such as ferulic acid or coumaric acids through ester linkages; a reaction formed between the hydroxyl group of a NSP and the carboxyl end of a cinnamic acid derivative (Selvendran and Robertson, 1990).

2.2. Bioactive Molecules Derived from Plant Cell Wall

The various bioactive molecules that can be generated from the plant cell wall as a result of hydrolysis include the dietary fibres, NDOs, phenolic acids, and phenolated NDOs (Sanchez-Castillo et al., 2002).

2.2.1. Dietary Fibres

According to the American Association of Cereal Chemists, dietary fibre is defined as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and

absorption in the human small intestine with complete or partial fermentation in the large intestine" (DeVries, 2003). In addition, dietary fibre provides a wide range of functional properties such as satiation and satiety, normalization of GI transit time and fermentation in the large intestine with the following associated health benefits: fostered weight control, improved GI tract health and colon cancer prevention (The American Dietetic Association, 2008). However, there remains an ambiguity when considering dietary fibres as prebiotics. Even though dietary fibres are not digested and pass intact through the digestive tract until the large intestine, they do not selectively stimulate the growth and/or activity of beneficial bacteria (Gibson and Rastall, 2006). For example, high-polymer inulin (DP between 10 and 65), a naturally occurring polysaccharide belonging to a class of fibres known as fructans, was found to maintain higher total bacterial counts but lower populations of *Bifidobacteria* and *Lactobacilli* when compared to other short-chain carbohydrates (Van Loo, 2004). As a result, dietary fibres are considered as candidate prebiotics since they do not meet all key criteria.

2.2.2. Non-Digestible Oligosaccharides - Prebiotic Compounds

A prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995). More specifically, prebiotics are non-digestible carbohydrates that are fermentable by selective beneficial bacteria in the hindgut, namely *Bifidobacterium* and *Lactobacillus* spp. As a result, the stimulated bacteria in the colon become the prevailing species over all other intestinal bacteria (Fooks et al., 1999). For centuries, prebiotic compounds have been a fundamental component of the human diet. However, it is only recently that their nutritional properties were documented. In fact, it was only in the mid-1990s that the term "prebiotic" came into existence (Gibson and Rastall, 2006). Presently, prebiotic compounds are finding increasing use in functional foods due to their heat resistance and few stability issues (Gibson and Rastall, 2006).

NDOs can be generated from the plant cell wall as a result of the hydrolysis from the NSPs (Mussatto and Mancilha, 2007). These short-chain sugar molecules have numerous functional and physiological benefits. NDOs are particularly recognized for their

prebiotic effects and for the reason that they can be easily incorporated into many processed food products (Mussatto and Mancilha, 2007).

2.2.2.1. Types and Structures of Prebiotics

According to IUB-IUPAC nomenclature, oligosaccharides are defined as "saccharides containing between 3 and 10 sugar moieties" (Mussatto and Mancilha, 2007). However, no physiological and/or chemical rational has been determined for setting such limits (Voragen, 1998). Therefore, oligosaccharides are low molecular weight carbohydrates that can be classified as digestible or non-digestible (Mussatto and Mancilha, 2007). NDOs include glycosides in which fructose, galactose, glucose, and/or xylose form the monosaccharide units. From a structural point of view, NDOs are non-digestible due to the β-glycosidic linkage type formed between the monosaccharide units, making them non-digestible by human digestive enzymes (Mussatto and Mancilha, 2007). Furthermore, prebiotic activity greatly depends on the chemical structure of NDOs, in particular the type of hexose moieties, their DP, their glycosidic linkages, and degree of branching (Voragen, 1998). Prebiotic NDOs can be produced by one of three processes: (1) extraction from natural sources (e.g., beet, starch, cow's milk, and soybean); (2) controlled hydrolysis of polysaccharides (e.g., starch, inulin and xylan); and (3) enzymatic synthesis using hydrolases, transglycosylases and isomerases (Sako et al., 1999). Sako and colleagues (1999) described thirteen classes of commercially available NDOs that possess bifidogenic functions (Table 1). The chemical differences between these NDOs may be explained by the length of the repeating units, monosaccharide composition, branching structure, and purity (Mussatto and Mancilha, 2007).

2.2.2.2. Functional Properties and Health Benefits

The mechanisms of action of prebiotics involve the following proposed scientific explanations: modification to the microbial population dynamics and/or metabolic activity (Gibson and Rastall, 2006). Upon ingestion, NDOs are capable of withstanding digestion in the small intestine and persist through the large intestine until reaching the ileocaecal region in a rather unchanged form (Oku et al., 1984; Nilsson et al., 1988). Once in the colon, NDOs can act as dietary bulking agents or become available substrates for the indigenous intestinal microflora (*Bifidobacteria* spp.). The resulting fermentation of monomers contributes towards the production of lactic acid and short-chain-fatty-acids

Table 1. Commercially available non-digestible oligosaccharides with bifidogenic functions.

Compound[a]	**Molecular structure**[b]
Cyclodextrins	$(Gu)_n$
Fructooligosaccharides	$(Fr)_n$ -Gu
Galactooligosaccharides	$(Ga)_n$ -Gu
Gentiooligosaccharides	$(Gu)_n$
Glycosylsucrose	$(Gu)_n$ -Fr
Isomaltooligosaccharides	$(Gu)_n$
Isomaltulose (or palatinose)	$(Gu\text{-}Fr)_n$
Lactosucrose	Ga-Gu-Fr
Lactulose	Ga-Fr
Maltooligosaccharides	$(Gu)_n$
Raffinose	Ga-Gu-Fr
Soybean oligosaccharides	$(Ga)_n$ -Gu-Fr
Xylooligosaccharides	$(Xy)_n$

[a] Adapted from Sako et al., 1999.

[b] Ga, galactose; Gu, glucose; Fr, fructose; Xy, xylose.

(SCFAs), which decrease the pH and, in turn, may result in the reduced numbers of pathogenic microorganisms (Morisse et al., 1993). In addition, NDOs have also shown to have an anti-adhesive activity effect against pathogens. Other possible health benefits of prebiotics in the colon include increased mineral uptake, regulation of immune reactions, trophic and anti-neoplastic effects of SCFAs, and reduced toxic microbial metabolites (Gibson and Rastall, 2006). As a result, such anticipated benefits may reduce the risk of acute gastroenteritis, cancer risk, osteoporosis, and may also have an effect on lipid regulation.

It has been suggested that *Bifidobacteria* and *Lactobacilli* play a significant role in inhibiting and providing improved protection against pathogenic microorganisms such as *Escherichia coli*, *Campylobacter* and *Salmonella* spp., and/or their toxins (Hui et al., 1994). The possible mechanisms include their ability to lower the gut pH, compete for nutrients, their natural antimicrobial excretion, and competitive effects for occupation of colonisation sites (Gibson et al., 1997). Several experimental studies *in vivo* have demonstrated that prebiotics may modulate the functioning of the gut-associated immune system (Kelly-Quagliana et al., 2003; Roller et al., 2004a,b). In a double-blind, placebo-controlled study, the effect of oligofructose supplementation in infants 6 to 24 months of age on gut microflora and well-being was determined. After four weeks of intervention, the test group had an improved bacterial colonisation. Other observed effects included less fever, flatulence, diarrhoea, and regurgitation (Waligora-Dupriet et al., 2005).

Research in experimental animal models has demonstrated that prebiotics also possess anticarcinogenic properties (Pool-Zobel et al., 2002). They have the ability to reduce genotoxic enzyme activity as well as the incidence of azoxymethane, a chemical that brings about crypt foci and tumours in the colon (Pool-Zobel et al., 2002). The mechanism by which prebiotics act chemopreventively may be due to their ability to produce lactic acid producing bacteria and SCFA butyrate and the induction of apoptosis in transformed cells (Pool-Zobel et al., 2002). Pierre and colleagues (1997) observed that life-long feeding of inulin and oligofructose in rats suppressed the formation of colonic crypt foci and tumours and developed an improved gut associated lymphoid tissue. In addition, prebiotic fed rats had an increased apoptotic index meaning that colonic cells with defective DNA were more efficiently eliminated (Hughes and Rowland, 2001). The

experimental evidence from animal models suggests the possibility that prebiotics may reduce the risk of cancer in humans. However, human dietary intervention studies warrant further study.

Several *in vivo* studies have shown, without understanding the mechanism, that adding galactooligosaccharides and/or fructooligosaccharides (FOSs) to the diet can increase the bioavailability of calcium and magnesium, which are essential for good bone structure and preventing progressive conditions such as osteoporosis (Chonan et al., 2001; Bornet et al., 2002; Roberfroid, 2002). Prebiotics may also have beneficial effects on lipid metabolism. Studies have found that both FOSs and xylooligosaccharides (XOSs) reduce blood lipid levels (Bornet et al., 2002; Roberfroid, 2002). Even though positive findings have been obtained, the exact mechanism of action is still unknown.

2.2.3. Phenolated Non-Digestible Oligosaccharides

Phenolated glycosides such as feruloylated NDOs are a group of bioactive molecules that can be released from the plant cell wall as a result of the hydrolysis from the NSPs (Selvendran and Robertson, 1990). These ester linked bioactive molecules have been the subject of recent interest because, compared to phenolic acids and NDOs, they possess both antioxidant and prebiotic functional properties.

2.2.3.1. Types and Structures of Phenolated Non-Digestible Oligosaccharides

Numerous feruloylated tri- to octa-NDOs from plant cell walls have been isolated and the nature of their ester linkages has been extensively studied (Ralet et al., 1994a,b). The glycosyl residue composition of isolated feruloylated NDOs includes arabinose, galactose and/or xylose (Ralet et al., 1994a,b). From a structural point of view, phenolated NDOs are non-digestible due to the β-glycosidic linkage type formed between the glycosyl units, making them non-digestible by human digestive enzymes (Mussatto and Mancilha, 2007). Moreover, phenolated NDOs display antioxidant activities due to the phenolic acid moiety esterified to the NDO. In maize bran, the ferulic acid in the isolated feruloylated tri- and tetrasaccharide was reported to be ester-linked to *O*-5 of the arabinose residue (Allerdings et al., 2006). In addition, the structural characteristics of the feruloylated NDOs prepared from sugar-beet pulp and wheat bran revealed that the ferulic acid was ester-linked to *O*-5 of the arabinose residue, while in sugar-beet pulp it was found mostly

linked to *O*-2 of arabinose residues (Ralet et al., 1994a). The chemical differences among phenolated NDOs include chain length, monosaccharide and phenolic acid composition, degree of branching, and proportion of phenolic compound (Mussatto and Mancilha, 2007).

2.2.3.2. Functional Properties and Health Benefits

The recent interest in phenolated NDOs is due to their complementary functional properties, which may provide better protection against diseases than each of these bioactive molecules separately (Yuan et al., 2005a,b). Studies reveal that water-soluble feruloylated NDOs encourage *in vitro* growth of *Bifidobacterium bifidum* and protect rat erythrocytes against *in vitro* oxidative damage (Yuan et al., 2005a,b). In addition, feruloylated NDOs isolated from bran compared to free ferulic acid, were more effective antioxidants towards the oxidation of LDLs and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) free radical scavengers (Katapodis et al., 2003). Consequently, phenolated NDOs may increase the safe delivery of phenolic compounds to the colon, reducing the risk of chronic diseases. The health-promoting properties of phenolated NDOs to reduce the risk of certain diseases such as cancer have stimulated the interest in the preparation of such novel molecules with improved prebiotic activity and other functional properties.

2.3. Production of Phenolated Non-Digestible Oligosaccharides

The preparation of phenolated NDOs can be achieved through one of two routes: (1) through mild acid hydrolysis or enzymatic hydrolysis using glycosyl hydrolases of plant cell wall polysaccharides; or (2) by enzymatic synthesis through esterification. However, the isolation of these bioactive molecules has been based primarily on a mild acid hydrolysis or enzymatic hydrolysis approach.

2.3.1. Isolation from Plant Cell Wall Polysaccharides

The mild acid hydrolysis of plant cell wall NSPs from maize bran, sugar-beet pulp, wild rice, and wheat bran has the advantage of being affordable and simple (Ralet et al., 1994a,b; Allerdings et al., 2006). Allerdings et al. (2006) isolated and structurally identified three novel complex feruloylated XOSs from maize bran insoluble dietary fibre after treatment with trifluoroacetic acid. The most complex isolated heteroxylan side-chains from maize bran included the feruloylated tetrasaccharides α-D-xylopyranosyl-

(1→3)-α-L-galactopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose and α-D-galactopyranosyl-(1→3)-α-L-galactopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose, as well as the feruloylated trisaccharide α-L-galactopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose.

The drawbacks, however, emerging from the mild acid hydrolysis approach include the random nature of the hydrolysis and poor yield, which is due to the low percentage of ferulic acid present in plant cell wall polysaccharides. For example, wheat bran contains 0.4–0.7% w/w of ferulic acid (Clifford, 1999). Other disadvantages include the heterogeneous and limited structures obtained (Allerdings et al., 2006). In fact, the limited structures of phenolated NDOs are due to the fact that ρ-coumaric and ferulic acid are the predominant phenolic acids found in plant cell wall NSPs (Lequart et al., 1999). In addition, the mild acid hydrolysis approach mainly yields feruloylated arabinose and galactose as monosaccharides (Ralet et al., 1994b).

Enzymatic hydrolysis of plant cell wall NSPs has the advantage, when compared to mild acid hydrolysis, of providing specific hydrolysis under mild operating conditions (Ralet et al., 1994a). For instance, the main isolated phenolated NDOs include feruloylated di-, tri-, tetra-, hexa-, hepta-, and octaarabinose as well as tri- and tetraxylose (Ralet et al., 1994a,b). However, the low yield and limited structures of phenolated NDOs have not been overcome. Indeed, the structures of phenolated side-chains in plant cell wall NSPs seem to be more complex than previously thought. Yuan et al. (2005a,b) prepared feruloylated NDOs from wheat bran insoluble dietary fibre by treatment with xylanases from *Bacillus subtilis*. Moreover, the hydrolysis of wheat bran using FAEs and glycosyl hydrolases from *Humicola insolens* also resulted in the production of feruloylated NDOs (Faulds et al., 2004). However, the hydrolysis of wheat bran by a thermostable endoxylanase only released 6% of the cinnamoyl-oligosaccharides (Lequart et al., 1999).

2.3.2. Enzymatic Esterification

In order to overcome the low yield and limited structures of phenolated NDOs obtained through the two preceding routes, enzymatic synthesis through esterification is a promising route for the preparation of phenolated NDOs. Enzymatic esterification of

glycosides provides a solution to the poor selectivity of chemical synthesis and is becoming of increased importance in the field of biotechnology (Vafiadi et al., 2007). Until now, most of the glycoside acylations were mainly limited to the incorporation of hydrophobic alkyl chains into mono-, di- and trisaccharides using lipases and proteases (Compton et al., 2000; Pérez-Victoria and Morales, 2006). At the moment, the esterification of glycosides with phenolic acids has been little-studied and literature has only achieved low (18%) to moderate (40%) conversions of monosaccharides to phenolated derivatives (Topakas et al., 2005a; Vafiadi et al., 2005, 2006). But most importantly, no research has been carried out on the optimization of the esterification reaction of phenolic acids with NDOs. As far as we are aware, only one example of chemoenzymatic transesterification has been reported (Vafiadi et al., 2007). Vafiadi and colleagues (2007) carried out the feruloylation of four linear arabino-NDOs, containing three to six arabinofuranose units, using a type C FAE from *Sporotrichum thermophile* (StFaeC) in a ternary water-organic mixture consisting of n-hexane, t-butanol and water. The enzymatic transesterification of NDOs with hydroxycinnamic acids by FAEs provided a novel enzymatic approach for the synthesis of functional and biodegradable materials. As a result, further research regarding the optimization of the esterification reaction of phenolic acids with NDOs is warranted.

2.4. Esterification of Glycosides

The key challenges involved in the enzymatic esterification of glycosides with phenolic acids involve choosing the best biocatalyst, the nature of the reaction media as well as the various reaction conditions involved.

2.4.1. Nature of Biocatalysts

Due to the complexity, the structural diversity and the multiple attachment sites of saccharides, the regioselective chemical esterification of glycosides can be a challenge (Pérez-Victoria and Morales, 2006). As a result, the use of enzymes as an alternative to the chemical catalyst has helped tremendously. The main types of biocatalysts used to catalyze the esterification of glycosides include the hydrolases (e.g., lipases, proteases and FAEs) and the transferases (e.g., glycosyl-transferases) from plant or microbial origin (Monsan and Paul, 1995).

Lipases and proteases are a versatile group of hydrolases that are widely used in esterification reactions because of their high specificity and selectivity (Martín et al., 2008). Pérez-Victoria and Morales (2006) carried out the enzymatic acylation of the non-reducing NDOs raffinose, melezitose, 1-ketose, and stachyose with vinyl laurate as the acyl donor. The esterification reactions were catalyzed in tert-butanol/pyridine mixtures with lipases from *Candida antarctica* B (Novozym 435) and *Thermomyces lanuginosus* (Lipozyme TL IM) as well as in pyridine with the alkaline protease from *Bacillus licheniformis* (subtilisin Carlsberg). The three enzymes produced high isolated yields of the sugar monoesters and only small amounts of diesters. In addition, Riva et al. (1988) synthesized a series of esterified tri- and tetrasaccharides by using subtilisin in dimethylformamide and trichloroethyl butyrate as the esterifying agents. Ferrer et al. (2002) synthesized long-chain fatty acid esters of maltotriose using a lipase from *T. lanuginosus* in a medium comprising of two miscible solvents (2-methyl-2-butanol/dimethylsulfoxide).

On the other hand, FAEs (EC 3.1.1.73) possess an active site that has high affinity for cinnamic acid derivatives such as ferulic acid and are therefore the most promising biocatalysts for the synthesis of phenolated NDOs (Fazary and Ju, 2007). Topakas et al. (2005a) carried out the first example of enzymatic feruloylation of a monosaccharide. StFaeC was able to catalyze the transfer of the phenolic acid from methyl ferulate to L-arabinose in numerous surfactant-less microemulsions. The StFaeC catalytic behaviour for the transesterification of methyl phenolic (e.g., ferulic, ρ-coumaric, caffeic, and sinapinic) esters with 1-butanol in various surfactant-less microemulsions was affected by the system composition and to a lesser degree by the phenolic substrate employed. However, in all media compositions used, the transesterification of methoxylated and hydroxylated derivatives of cinnamic acid was proficiently catalyzed by StFaeC. Nonetheless, the ferulic acid ester demonstrated to have the best bioconversion yield. Approximately, a 40% conversion of L-arabinose to its feruloylated derivative was achieved in the n-hexane, t-butanol and water ternary system ratio of 47.2:50.8:2.0 (v/v/v). As opposed to the enzyme's natural hydrolytic activity in aqueous medium, the synthetic activity can be favoured in a low water-content system. Similarly, Vafiadi et al. (2005) were able to map the hydrolytic and synthetic selectivity of StFaeC using alkyl

ferulates in order to catalyze the feruloyl group transfer to L-arabinose in a n-hexane, t-butanol and water ternary system (53.4:43.4:3.2, v/v/v). Lengthening of the alkyl chain of the ferulate substrates increased the hydrophobicity, resulting in a significant lower synthetic activity of the type C FAE. A comparable conversion yield for the transesterifcation of ferulic acid methyl ester with L-arabinobiose under the catalysis of StFaeC in a detergent-less microemulsion system of n-hexane, t-butanol and water (47.2:50.8:2.0, v/v/v) was also obtained by Vafiadi and colleagues (2006).

2.4.2. Nature of Reaction Media

Finding a suitable reaction medium for the enzymatic esterification of glycosides is problematic due to the inherently low solubility of these compounds in most-non-aqueous media and the inactivation of most hydrolases in highly polar organic media, in which the two polar substrates exhibit appreciable solubility. In conventional aqueous media, hydrolysis reactions are thermodynamically favoured. However, in non-conventional media with low water activity, esterification reactions can be achieved by a mass action effect (Raab et al., 2007). Moreover, because enzymatic reaction systems have the ability to alter the equilibrium position, non-conventional media are particularly suited for synthetic reactions. A number of non-conventional media employed for esterification reactions include: (1) monophasic composed of water miscible solvents; (2) biphasic composed of two water immiscible solvent phases; and (3) micro- and nanoemulsions, which are a dispersion of two immiscible liquids stabilized by surfactants or other alcohols or solvents (Decastro et al., 1992; Ikushima et al., 1996; Knez and Habulin, 2002; Yadav and Lathi, 2004). In addition to the ability of non-aqueous environments to control thermodynamically the unfavourable reactions in water, they also increase the solubility of organic substrates and facilitate enzyme and product recovery (Yadav and Lathi, 2004). Recently, much focus has been placed on the use of micro- and nanoemulsion systems such as surfactant-less and IL-based emulsions due to the nature of the substrates and biocatalyst but also because there is less diffusional limitation of substrates as compared to the biphasic non-conventional media (Yadav and Lathi, 2004).

Several researchers have employed surfactant-less microemulsions, such as ternary water-organic mixtures consisting of n-hexane, 1-butanol and water, due to the fact that these

reaction systems represent "thermodynamically stable and optically transparent dispersions of aqueous microdroplets in hydrocarbon solvent" (Khmelnitsky et al., 1988; Vulfson et al., 1991; Topakas et al., 2005a). In fact, these systems have already been efficiently employed for the esterification or transesterification of various phenolic acids catalyzed by extracellular FAEs from *Fusarium oxysporum* (FoFAE) and *S. thermophile* (Topakas et al., 2003a,b, 2004, 2005a). Therefore, the dilemma of reaction product separation and enzyme reuse can be solved effortlessly by the use of surfactant-less microemulsions as reaction media (Khmelnitsky et al., 1988). Moreover, the presence of large amounts of polar alcohol allows high solubility of the moderately polar phenolic acids (Topakas et al., 2005a).

ILs are considered as clean alternatives to classical organic solvents (Lozano et al., 2004). In terms of their physico-chemical properties, ILs, such as 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) and 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF_4), are salts that become liquid below 100ºC or close to room temperature (Martín et al., 2008). They comprise of large heterocyclic organic cations and anions that can be varied in order to obtain different physico-chemical properties (Martín et al., 2008). ILs, unlike conventional organic solvents, are non-volatile, non-flammable, have excellent chemical and thermal stability (Yang and Pan, 2005), and have the potential to be recycled and reused (Mohile et al., 2004). ILs can also directly solubilize a number of enzymes and substrates (e.g., glycosides) that normally cannot be dissolved in organic solvents (Kim et al., 2003). Furthermore, ILs are capable of carrying out nucleophilic substitution reactions (Kim et al., 2003) and enzymes, such as hydrolases, maintain their activity while suspended (Martín et al., 2008). Studies have revealed that, when compared with conventional organic solvents, the regioselectivity of enzyme catalysis in the esterification of sugars is significantly enhanced in ILs (Kim et al., 2003). Kim et al. (2003) reported that the selective enzymatic esterification of glycosides carried out in two ILs including [bmim]PF_6 and 1-methoxyethyl-3-methylimidazolium hexafluorophosphate occurred more quickly and gave higher isolated yields (92% and 89%, respectively) than those in conventional organic solvents.

2.4.3. Reaction Conditions

The last challenge involved in the enzymatic esterification of glycosides with phenolic acids involves controlling the numerous reaction conditions so as to modulate enzyme activity and favour the synthesis of the targeted molecule. The most important reaction conditions that may affect enzymatic esterification include temperature, substrate concentration and water content. However, optimum reaction conditions depend greatly on the source of the biocatalyst.

Depending on the source of the biocatalyst, enzymatic esterification reactions have been performed at temperatures between 25 and 75°C with optimum temperatures ranging between 35 and 60°C (Topakas et al., 2003a,b, 2005a; Hatzakis and Smonou, 2005; Vafiadi et al., 2005; Tsuchiyama et al., 2006; Vafiadi et al., 2009). Substrate concentration is also an important reaction condition that can affect the progression of an enzymatic esterification reaction (Vafiadi et al., 2005, 2006; Tsuchiyama et al., 2006; Vafiadi et al., 2009). Interestingly, Vafiadi et al. (2005) demonstrated that the enzyme activity of StFaeC increased as the concentration of methyl ferulate equally increased. Similarly, the L-arabinose feruloylation rate also increased to a conversion of 35%. However, a notable opposite effect was seen with an increase in L-arabinose concentration, suggesting that an excess amount of phenolic acid is required to favour the enzymatic esterification reaction. Lastly, Vafiadi and colleagues (2009) studied the effect of different water contents (%) added on the bioconversion yield of the esterification reaction catalyzed by a FAE from *Aspergillus niger* (AnFaeA) in two ILs. The results revealed that the enzymatic esterification reaction increased with an increase in water content up to 15%, where the optimum conversion value (6-9%) was obtained.

2.5. Feruloyl Esterases

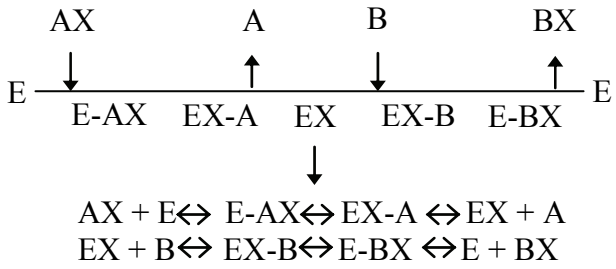
FAEs (EC 3.1.1.73), also known as cinnamoyl ester hydrolases, ferulic acid esterases and hydroxycinnamoyl esterases are a subclass of the carboxylic acid esterases (EC 3.1.1) that have the ability to catalyze the cleavage of ester bonds between plant cell wall NSPs and phenolic acids such as ferulic, caffeic, sinapic, and ρ-coumaric acid (Faulds and Williamson, 1993; Tarbouriech et al., 2005). In fact, since 1990, the amount of research on microbial FAEs has increased considerably due to their significant roles as

biotechnological tools for several industrial and medicinal applications (Fazary and Ju, 2007). FAEs from mesophilic and thermophilic sources can be sub-classified into four types A, B, C, and D, based on their amino acid sequence identity, ability to hydrolyze hydroxycinnamic acid methyl esters and capacity to release 5,5'-diferulic acid from substrates and plant cell wall materials (Fazary and Ju, 2007). Moreover, the nomenclature of FAEs refers to the enzyme microbial source as well as the FAE type (Fazary and Ju, 2007).

2.5.1. Catalytic Mechanism of Action

AnFaeA is one of the most carefully studied α/β-hydrolases (Fazary and Ju, 2007). Its catalytic triad comprises of three specific amino acids including serine, histidine and aspartic acid (McAuley et al., 2004). The protein with the closest sequence to the type A FAE is a lipase from *Rhizomucor miehei*, which has an overall sequence identity of 32% (Aliwan et al., 1999). Cygler and Schrag (1997) showed that lipases and esterases catalyze similar chemical reactions. Moreover, AnFaeA possesses a serine-lipase-like active-site motif and both share significant structural homology (McAuley et al., 2004). However, lipases compared to esterases act at an interface (Cygler and Schrag, 1997).

The ping-pong bi-bi mechanism (Figure 1), defined as a non-sequential mechanism, is the most generally accepted mechanism for esterase-mediated esterification reactions (Chulalaksananukul et al., 1990; Martinelle and Hult, 1995; Yong and AlDuri, 1996; Mukesh et al., 1997). Catalysis commences with the first substrate, being the phenolic acid, binding to the enzyme in order to form a binary complex. The first product, being water, is then released to form an acyl-enzyme (Bousquet-Dubouch et al., 2001). Subsequently, the second substrate, being the glycoside, binds to the acyl-enzyme in order to yield the second product, being 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.



*Adapted from Bousquet-Dubouch et al., 2001.

Figure 1. Ping-pong bi-bi mechanism.

2.5.1.1. Kinetic Parameters

In order to calculate the catalytic efficiency and affinity of the enzyme towards the substrates, the Michaelis-Menten equation, derived from the ping-pong bi-bi mechanism can be employed (Equation 1).

$$v_o = \frac{V_{max}[AX_o][B_o]}{K_{m(B)}[AX_o] + K_{m(AX)}[B_o] + [AX_o][B_o]} \qquad (1)$$

*Adapted from Bousquet-Dubouch et al., 2001.

The kinetic parameters involved in the esterification rate equation include initial velocity (v_o), substrate concentrations (AX_o, B_o), maximum reaction rate (V_{max}), and the dissociation constants of an enzyme–substrate complex; in which the concentration at which the rate of the enzyme reaction is half V_{max} ($K_{m(AX)}$, $K_{m\,(B)}$).

2.5.2. Microbial Sources

The non-commercial availability of FAEs and the high cost associated with their single use have been limiting factors in their application for the synthesis of phenolated NDOs. As a result, FAEs are generally produced from a variety of microbial sources including bacteria and fungi species with different FAE activity (mU/mg) such as *Streptomyces*, *Schizophyllum, Neocallimastix, Penicillium, Trichoderma, Talaromyces, Piromyces, Aspergillus, Aureobasidium, Bacillus, Clostridium, Neurospora, Humicola,* and *Fusarium* spp. (Fazary and Ju, 2007).

More recently, the use of multi-enzymatic preparations exhibiting FAE activity from different microbial sources for the production of alkyl ferulates has been investigated (Vafiadi et al., 2008). For example, enzyme preparations with FAE activity from *Humicola* spp., *T. lanuginosus* and *A. niger* have been employed (Mastihubova et al., 2006; Vafiadi et al., 2008). Enrichment of the commercial multi-enzymatic preparations including Ultraflo L, Depol 740L and Depol 670L involved precipitation with different aggregating agents such as ammonium sulfate ($(NH_4)_2SO_4$) in order to concentrate and enrich the FAE (Vafiadi et al., 2008).

2.5.3. Substrate Specificity

The specificity of FAEs from mesophilic and thermophilic fungi have been examined by using a wide range of substrates, including methyl phenylalkanoates and short-chain aliphatic acid esters of ρ-nitrophenol, in order to probe the active site of FAEs (Topakas et al., 2005b).

Topakas et al. (2005b) revealed that the mesophilic FoFaeA showed specificity for methoxylated substrates while the thermophilic StFaeB and StFaeC preferred hydroxylated compounds. FoFaeB and StFaeB demonstrated similar specificities in terms of the benzene ring methoxy and hydroxyl substitutions. However, differences with respect to the position of the substitution were noticeable. StFaeC displayed specificity for both a methoxy and hydroxyl substitution on the substrate. In addition, the type C enzyme required the 4-position of the benzoic ring to contain a hydroxyl group (Fazary and Ju, 2007). Moreover, StFaeC demonstrated highest catalytic efficiency on 4-hydroxy-3-methoxy cinnamate, indicating that this type of FAE may perhaps be the most promising biocatalyst for the feruloylation of aliphatic alcohols and oligo- or polysaccharides (Topakas et al., 2005b).

The FAE substrate specificity of two forms of FAEs from *A. niger* has also been determined by assessing its hydrolytic activity on feruloylated NDOs isolated from sugar-beet pulp and wheat bran (Ralet et al., 1994a). Ralet and colleagues (1994a) demonstrated that both FAE-I and FAE-III exhibited different specificities towards plant cell wall materials. Both enzymes exhibited affinity for the arabinose residue attached to the ferulic acid in the furanose form. In addition, maximum activity was seen on feruloylated

trisaccharides. FAE-I showed no preference for the type of linkage involved. However, FAE-III exhibited higher affinity for the ferulic acid attached to the *O*-5 rather than the *O*-2 of the arabinofuranose ring. Interestingly, the DP had a significant effect on catalysis. For example, a reduced rate of hydrolysis was seen in feruloylated NDOs with a DP > 3.

2.5.4. Effect of pH and Temperature

Two factors affecting enzyme activity include temperature and pH. Too high of a temperature can destroy an enzyme. However, below the critical range, an increase in temperature increases its activity (Fazary and Ju, 2007). It has been shown that FAEs from thermophilic microorganisms present an increase in their thermal stability when compared to FAEs produced from mesophilic microorganisms (Fazary and Ju, 2007). In order for catalysis to take place, enzyme flexibility is required. However, resistance to thermal degradation, as shown in the case of FAEs from thermophilic microorganisms, is due to their capability of resisting the movement of their more compact chains. Therefore, since the movement of their chains becomes energetically more favourable with an increase in thermal energy, enzyme catalysis can take place at elevated temperatures (Topakas et al., 2005b). As a result, thermophilic FAEs have the potential of being effectively used in high-temperature applications, which may be too high for mesophilic FAEs (Topakas et al., 2005b).

Various studies have shown that microbial FAEs have a broad pH and temperature range (Fazary and Ju, 2007). However, FAEs often show optimal activity between pH 5-8 and thermostability between 30-65ºC (Fazary and Ju, 2007). Topakas et al. (2005a) were able to determine the effect of pH and temperature on enzyme activity and stability. The results revealed that the StFaeC was optimal at pH 6 and 55ºC. Increasing the pH from 6 to 8 displayed a 20% drop of the peak activity. Furthermore, the enzyme remained stable at a pH range of 6-10 and maintained 80% of its activity at pH 10. In terms of temperature effect, StFaeC retained 82% of its activity at 60ºC and displayed half lives of 133 and 55 min at 55 and 60ºC, respectively.

Topakas et al. (2003b) similarly determined the effect of pH and temperature on enzyme activity and stability. In this case, the optimum pH was determined by measuring the enzyme activity over the pH range 3-10 and optimum temperature was determined by

assaying the enzyme activity at various temperatures between 30 and 70ºC. The FoFAE-I revealed to be optimally active at pH 7 and 55ºC. When pH and temperature were altered, the enzyme displayed more than 70% of the peak activity in pH 8 and remained stable in the pH range of 6-8. Moreover, roughly 60% of the maximum activity was retained at pH 10. Results were comparable to those reported for esterases from other mesophilic microorganisms (Christov and Prior, 1993; Donaghy and McKay, 1997).

2.6. Structural Characterization of Phenolated Non-Digestible Oligosaccharides

The structural characterization of phenolated NDOs can be confirmed by quantitative analysis using high performance liquid chromatography (HPLC) and by qualitative analysis using thin layer chromatography (TLC) and mass spectrometry (MS).

2.6.1. Thin Layer Chromatography

Topakas et al. (2005a) qualitatively characterized the enzymatic synthesis of feruloylated L-arabinose by spotting the reaction mixture on aluminum sheets coated with Silica gel 60. The solvent system used for the resolution of the feruloylated product formed was chloroform:methanol:water (65:15:2, v/v/v). A UV lamp was used to detect the phenolic component of the product while the aniline-hydrogen phthalate reagent was employed to detect the sugar component.

2.6.2. High Performance Liquid Chromatography

HPLC has become one of the most commonly used separative techniques due to its wide range of rapid analysis for analytes and simple automation (Nollet, 2000). Allerdings et al. (2006) isolated and structurally identified four complex heteroxylan side-chains acylated with ferulic acid and one arabinosyl ester of ρ-coumaric acid from maize bran after acidic hydrolysis. The hydrolysate was applied to an Amberlite XAD-2 column and the isolated fraction was concentrated and applied to a Bio-Gel P-2 column for further purification. Finally, semi-preparative reverse phase HPLC using a Nucleosil 100-5 C18 HD column equipped with a UV-detector was used to further separate the isolated fraction into four compounds. Elution was carried out using an acetonitrile and water gradient at a flow rate of 4 mL/min followed by detection of phenolic compounds at 325 nm. Feruloylated and ρ-coumaroylated oligo- and monosaccharides were identified using

HPLC coupled to electrospray-mass spectrometry (ESI-MS) working in the positive-ion mode.

2.6.3. Mass Spectrometry

MS has proven to be a very powerful analytical technique for various applications. Vafiadi et al. (2007) structurally characterized the chemoenzymatic synthesis of feruloylated arabinooligosaccharides (AOSs) by ESI-MS^n. ESI-MS^n using positive ion mode was employed to verify the degree of feruloylation and the linkage position of the feruloyl group(s) on the four linear AOSs. In other words, the specificity of the StFaeC in this kind of synthetic reaction was determined. Full MSs of all products revealed that all AOS substrates were mono-ferulated on the primary hydroxyl group situated on the non-reducing arabinofuranose ring (Vafiadi et al., 2005, 2006). Mono-feruloylation was shown by the singly charged $[M+Na]^+$ molecular ions of the feruloylated AOSs with ratios of *m/z* 613 (feruloylated arabinotriose), 745 (feruloylated arabinotetraose), 877 (feruloylated arabinopentaose), and 1009 (feruloylated arabinohexaose) (Vafiadi et al., 2007).

2.7. Assessment of Phenolated Non-Digestible Oligosaccharides Functional Properties

While studies exploring the benefits of dietary phenolic compounds dominate the scientific literature in the field of antioxidants, a variety of phenolated NDOs also show promise as free radical scavengers.

2.7.1. Antioxidant Activity

As part of normal metabolism, free radicals such as superoxide and nitric oxide are produced in the body and can be potentially damaging (Webb, 2006). Due to the antioxidant nature of phenolic compounds, they have gained much interest over the years (Balasundram et al., 2006). Their antioxidant mode of action depends on their capability of acting as electron donors, electron acceptors, decomposers of peroxide and hydroperoxides, metal activators and deactivators, and UV absorbers (Balasundram et al., 2006).

Literature on the assessment of the antioxidant potential of phenolated NDOs has shown to be limited. However, Katapodis and colleagues (2003) evaluated the antioxidant activity of a feruloylated oligosaccharide from wheat flour arabinoxylan against $DPPH^{\bullet}$ reduction assay and copper-catalyzed peroxidation of LDL. The results revealed that the feruloyl arabinoxylotrisaccharide exhibited profound $DPPH^{\bullet}$ reduction activity with an antiradical efficiency of 0.035 (x 10^{-3}). Moreover, the feruloylated oligosaccharide dose-dependent inhibition of the copper-induced oxidative modification of human LDL was almost complete at 32 μM. In this case, the glycoside esterification of ferulic acid exerted a protective role against copper-catalyzed oxidation of LDL. The results reveal that the feruloyl arabinoxylotrisaccharide antioxidant activity may be of great importance in the prevention or reduction of atherosclerosis progression.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

Sugar-beet pulp was kindly provided by Lantic Inc. (Taber, AB, Canada). Wheat bran was purchased from a local grocery store. Depol 670L and Depol 740L multi-enzymatic preparations were from Biocatalysts Limited (Cardiff, UK). Lipase A, Lipase PS30, Lipase M, CGT Amano, RP-1, Biozyme F10 SD, Newlase 11 and YL-15 multi-enzymatic preparations were a gift from Amano Enzyme Inc. (Nagoya, Japan). Multifect P 3000 and Laminex BG multi-enzymatic preparations were from Genencor International (Rochester, NY, USA), while AMG, Flavourzyme (Type A), Gamanase and Ceremix multi-enzymatic preparations were from Novo-Nordisk Biochem (Franklinton, NC, USA). Cellulase and Diazyme L-200 multi-enzymatic preparations were from Iogen Corporation (Ottawa, ON, Canada) and Solvay Enzymes Inc. (Elkhart, IN, USA), respectively. Arabinobiose, galactobiose and xylobiose were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). FOS and XOS were a gift from Quadra Chemicals Ltd. (Burlington, ON, Canada) and Shandong Longlive Bio-Technology Co., Ltd. (Shandong, China), respectively. Sucrose was purchased from MP Biomedicals, LLC (Solon, OH) while ethanol was obtained from Commercial alcohols (Brampton, ON). Silica gel 60 plates, bovine serum albumin, sodium hydroxide (NaOH), dialysis tubing, $(NH_4)_2SO_4$, and HPLC grade formic acid, acetic acid, acetonitrile, chloroform, methanol, and n-hexane were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 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).

3.2. Determination of Feruloyl Esterase Activity in Commercial Multi-Enzymatic Preparations

3.2.1. Preparation of Feruloylated Non-Digestible Oligosaccharide Substrates

The isolation of feruloylated NDOs from sugar-beet pulp and wheat bran was carried out according to a modification of the procedure described by Ralet et al. (1994a) and Bunzel et al. (2001), respectively. After autoclaving at 121°C for 45 min, wheat bran (10 g) was destarched using two subsequent treatments with α-amylase (1 mL, pH 6, 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 in order 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, whereas 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 an oven incubator. The isolation of feruloylated NDOs from these selected plant cell walls (0.8 g) was carried out using the multi-enzymatic driselase (0.01 g/mL), containing a set of fungal glycosyl hydrolases but not FAE, as biocatalyst. The hydrolytic reactions were carried out at 37°C with continuous shaking at 150 rpm in an orbital incubator shaker (Forma Scientific, Inc., Marjetta, OH, USA). After 24 h hydrolysis, absolute ethanol (4×10 mL) was added to the hydrolysates to precipitate the polymeric fragments. The ethanol soluble fractions containing the feruloylated NDOs were recovered by centrifugation at 8000*g* for 20 min using a centrifuge (model J2-21; Beckman Coulter, Fullerton, CA, USA) and concentrated under vacuum to 8 mL using an Automatic Environmental Speed Vac system (Savant Instruments, Inc., Holbrook, NY, USA).

3.2.2. Feruloyl Esterase Activity Assay

The hydrolytic activity of FAEs expressed in the selected multi-enzymatic preparations was assayed using methyl ferulate and feruloylated NDOs isolated from sugar-beet pulp and wheat bran as substrates, according to a modification of the method of Bunzel et al. (2001). Prior to the enzymatic reaction, stock solutions of methyl ferulate (33 μM) and feruloylated NDOs (2 to 4 mM) from sugar-beet pulp and wheat bran were prepared in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer at 100 mM and pH 6. The FAE activity assay was carried out at 37°C in a 1 mL cuvette. The assays were initiated by the addition of 0.1 mL of multi-enzymatic preparations at appropriate dilutions (0.41–40.9 mg protein) to 0.7 mL of substrate solution. The concentration of the consumed feruloylated substrates was monitored spectrophotometrically at 335 nm over a reaction period of 20 min using a DU-650 spectrophotometer (Beckman Instruments, Inc., San Raman, CA, USA) equipped with a thermocontrolled sample compartment. Reaction rate

was calculated from the slope of the curve of absorbance versus time using a molar extinction coefficient of 3940/M.cm for the esterified ferulic acid. This value was determined from the absorbance of standard solutions of methyl ferulate in the reaction mixture. Control trials without the enzyme were carried out in tandem with the enzymatic reactions to monitor for potential chemical side reactions. All assays were run in triplicate. One enzymatic unit of FAE activity (1 U) was defined as the amount of enzyme consuming 1 μmol of the esterified ferulic acid per minute under the above conditions. The protein concentration was determined by Hartree–Lowry assay using bovine serum albumin as a standard. The specific activity was defined as enzymatic units of FAE activity per milligram of protein.

3.3. Enrichment of Selected Multi-Enzymatic Preparation with Feruloyl Esterase Activity

Enrichment of the selected multi-enzymatic preparation (Depol 740L from *Humicola* spp.) with FAE activity was carried out by ultrafiltration and using saturated $(NH_4)_2SO_4$, according to a modification of the method reported by Donaghy and McKay (1997) and Vafiadi et al. (2008). Prior to both ultrafiltration and ammonium sulfate precipitation (516 g/L), Depol 740L (5-15 mL) was diluted in a 15-45 mL MOPS buffer at 100 mM and pH 6. Depol 740L was concentrated at 8°C using a stirred ultrafiltration unit (Millipore, Amicon system) fitted with a 10 kDa-molecular-mass-cut off membrane, followed by freeze-drying (Labconco Corp., Kansas City, Mo) the multi-enzymatic retentate for 24 hrs. The ammonium sulfate precipitation reaction was carried out at 8°C and stirred gently for 2 hrs. The precipitated fractions containing FAE activity were recovered by centrifugation at 8000*g* for 25 min using a Beckman centrifuge (Model J2-21, Inc., Fullerton, CA) and re-suspended in MOPS buffer (1.5 mL, 100 mM, pH 6). The multi-enzymatic precipitate was dialysed against MOPS buffer (5 mM, pH 6) using a membrane with a cut off of 6-8 kDa for 48 hr-period, followed by freeze-drying for 24 hrs.

3.4. Esterification Reaction

Enzymatic synthesis of feruloylated glycosides was carried out using a surfactant-less organic microemulsion as reaction medium, according to a modification of the method reported by Vafiadi et al. (2006). Prior to the enzymatic reaction, a stock solution of ferulic acid (30 mM) was prepared in 2-butanone, while those of mono- (33.33 mM), di-

and NDOs (100 mM) were prepared in 3-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH buffer (20 mM, pH 6). Defined amounts of substrate stock solutions were diluted with a sufficient amount of n-hexane to obtain a final substrate molar ratio of phenolic acid to glycoside of 3:1 in the surfactant-less organic microemulsion mixture composed of n-hexane, 2-butanone and MES-NaOH buffer at a ratio of 51:46:3 (v/v/v). The enzymatic esterification reaction was carried out in 25-mL Erlenmeyer flasks and initiated by the addition of 2–8 mg proteins of pre-purified multi-enzymatic preparations for the feruloylation of monosaccharides and of 8.9-14.1 mg protein of the enriched Depol 740L for the feruloylation of di- and NDOs. All flasks were vacuum sealed and incubated at 35°C with continuous shaking at 70 rpm in an orbital incubator shaker. Control trials, without enzyme, were carried out in tandem with the enzymatic reactions, which were run in duplicate. At defined time intervals over a 3- and 6-day period, the concentration of the consumed monosaccharides was measured using the dinitrosalicylate assay (Leung and Thorpe, 1984), whereas that of the formed feruloylated mono-, di- and NDOs was quantified by HPLC.

3.5. Analysis of the Reaction Components

3.5.1. Analysis of the Reaction Components of the Monosaccharide Feruloylation

Quantitative analysis of reaction mixtures was carried out, according to the modification of the method of Topakas et al. (2005a). A Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an ultraviolet diode-array detector (Model 168) was used. The separation was performed on a Zorbax SB-C18 reversed-phase column (5 μm, 250×4.6 mm, Agilent Technologies Canada Inc.; Mississauga, ON), using a linear gradient from 100% water/formic acid mixture (8.5:0.5, v/v) to 80% acetonitrile for a period of 40 min at a flow rate of 0.7 mL/min. The scanning of the reaction components was performed in the region of 190-400 nm at 1 s intervals, whereas the detection was carried out simultaneously at 280 and 300 nm. The bioconversion yield (%) was calculated as the concentration of consumed ferulic acid or formed feruloylated monosaccharides divided by the initial concentration of monosaccharides, multiplied by 100.

3.5.2. Analysis of the Reaction Components of Di- and Non-Digestible Oligosaccharide Feruloylation

Quantitative analysis of reaction mixtures of di- and NDO feruloylation was carried out, according to a modification of the modified method of Couto et al. (2010). A Waters HPLC system (Model 25P, Waters Corp., Milford, MA) equipped with a photodiode array detector (Model 2998) and refractive index detector (Model 2414) was used. The separation was performed on a Zorbax SB-C18 reversed-phase column (5 μm, 250×4.6 mm, Agilent Technologies Canada Inc.; Mississauga, ON), using an isocratic elution of 100% water/formic acid (8.5:0.5, v/v)/acetonitrile mixture (80:20, v/v) for a period of 16.1 min at a flow rate of 0.7 mL/min. Injected sample volume was 20 μL and detection of reaction components was performed by UV detection at 260 and 320 nm and by refractive index detection (temperature 30°C, sensitivity 64). The scanning of the reaction components was performed in the region of 210-400 nm at 1 s intervals. The calibration curves were constructed with selected di- and NDO standards. The bioconversion yield (%) was calculated from the concentration of the synthesized feruloylated di- and NDOs divided by the initial concentration of di- and NDOs, multiplied by 100%.

Qualitative analysis of the feruloylated NDOs, isolated from plant cell wall, and the reaction components of the esterification reaction was carried out by TLC, according to a modification of the method of Topakas et al. (2005a). Aliquots (5 μL) of reaction mixtures were spotted on silica gel 60 plates. The developing solvent consisted of a mixture of chloroform–methanol–acetic acid–water (5:2:2:1, v/v/v/v). The bands corresponding to glycosides and their feruloylated derivatives were detected under visible light after spraying with methanol–sulfuric acid mixture (95:5, v/v) and heating at 100°C.

3.6. Isolation and Structural Characterization of Feruloylated Glycosides

The molecular structures of feruloylated glycosides (mono-, di- and NDOs) were characterized using HPLC interfaced to atmospheric pressure chemical ionization–mass spectrometry (APCI-MS). The APCI-MS system (ThermoFinnigan, San Jose, CA, USA) was 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 to 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.

3.7. Assessment of the Efficiency of Selected Feruloyl Esterases for the Synthesis of Feruloylated Monosaccharides

The efficiency of FAEs expressed in the six best multi-enzymatic preparations from *Bacillus* spp. (Ceremix), *Humicola* spp. (Depol 740L), *Aspergillus oryzae* (Flavourzyme), *Bacillus amyloliquefaciens* (Multifect P 3000), *B. subtilis* (RP-1) and *Trichoderma reesei* (Depol 670L) for the synthesis of feruloylated monosaccharides was investigated as described previously (Section 3.4). Two surfactant-less organic microemulsion systems composed of n-hexane, 2-butanone or 1-butanol and MES-NaOH buffer at a ratio of 51:46:3 (v/v/v) were used as reaction medium.

3.8. Effect of Glycoside Structures on Bioconversion Yield

To study the effect of glycoside structure on the bioconversion yield of the feruloylated glycosides, selected mono-, di- and NDOs were investigated as glycoside substrates. The monosaccharides, including D-arabinose, D-galactose, D-xylose, were used as glycoside substrates for the synthesis of feruloylated monosaccharides. While L-arabinobiose, L-galactobiose, L-xylobiose, α-lactose, and sucrose were investigated as substrates for the synthesis of feruloylated disaccharides. The synthesis of feruloylated NDOs was studied using D-raffinose, FOS and XOS as glycoside substrates. The enzymatic synthesis was carried out using a surfactant-less organic microemulsion as reaction medium as described previously (Section 3.4).

3.9. Effect of Reaction Media on Bioconversion Yield of Feruloylated Di- and Non-Digestible Oligosaccharides

Selected surfactant-less organic microemulsions and IL-based emulsions were investigated as reaction media for the enzymatic feruloylation of di- and NDOs. The surfactant-less organic microemulsion systems comprised of n-hexane/2-butanone, 1-butanol or 1,4-dioxane/MES-NaOH (51:46:3, v/v/v). Selected IL-based emulsions were also used as reaction media for the feruloylation of di- and NDOs, including: n-

hexane/[bmim]BF_4/MES-NaOH (51:46:3, v/v/v), [bmim]PF_6/2-butanone/MES-NaOH (51:46:3, v/v/v) or [bmim]PF_6/MES-NaOH (97:3, v/v).

3.10. Determination of Scavenging Activity of Synthesized Feruloylated Glycosides

The antioxidant potential of the feruloylated glycosides was estimated based on the measurement of their free-radical scavenging activity using the free radical DPPH$^{\bullet}$ according to a modification of the method of Silva et al. (2000). In a 1 mL spectrophotometric cuvette, 0.122 mL of feruloylated glycoside and its corresponding ferulic acid was added to 1.378 mL of a DPPH$^{\bullet}$ ethanolic solution (0.1 mM). The reduction of DPPH$^{\bullet}$ was monitored spectrophotometrically at 517 nm until the reaction reached a plateau against a blank assay containing only DPPH$^{\bullet}$, using a Beckman spectrophotometer. The scavenging activity was obtained from the slope (Ab_{517nm}/min) of the sample reaction divided by the volume of the feruloylated glycoside, while the scavenging yield was calculated as the absorbance of the DPPH$^{\bullet}$ control, at 517 nm, minus the absorbance of the sample divided by that of the DPPH$^{\bullet}$ control, multiplied by 100.

3.11. Optimization of Feruloyl Esterase-Catalyzed Esterification of Feruloylated Raffinose by Response Surface Methodology

3.11.1. Experimental Design

Optimization of the feruloylated raffinose bioconversion yield was achieved by using response surface methodology (RSM), according to a modification of the method described by Yuan et al. (2006). A five level, four variable central composite rotatable design (CCRD) was employed. The full factorial design consisted of 16 factorial points, 8 axial points (2 axial points on the axis of each design variable at a distance of 2.2 from the centre) and 8 centre points, leading to 32 sets of experiments. The variables and their levels selected for this study were X_1 temperature (24.2-45.8ºC), X_2 substrate molar ratio (0.8-5.2:1 mM; ferulic acid to raffinose), X_3 water content (0.8-5.2%, v/v), and X_4 enzyme amount (21-415 enzymatic FAE units (μmol/min)). Table 2 shows the four variable replicate CCRD that was used for developing the second-order mathematical model.

Table 2. Variables and their coded levels employed in a central composite rotatable design for optimization of feruloylated raffinose production by Depol 740L from *Humicola* spp.

Variables	Coded levels				
	-2.2	-1	0	+1	+2.2
X_1: temperature (ºC)	24.2	30	35	40	45.8
X_2: substrate molar ratio (ferulic acid to raffinose)	0.8:1	2:1	3:1	4:1	5.2:1
X_3: water content (%)	0.8	2	3	4	5.2
X_4: enzyme amount (enzymatic FAE units)	21	127	218	309	415

3.11.2. Statistical Analysis

Regression analysis was performed, based on the experimental data, and was fitted into the following empirical second order polynomial equation using the software Design-Expert 8.0.2 (Stat-Ease, Inc. Minneapolis, MN, USA).

$$Y_i = a_0 + a_i X_1 + a_i X_2 + a_i X_3 + a_i X_4 + a_{ij} X_1 X_2 + a_{ii} X_1^2 + a_{ii} X_2^2 + a_{ii} X_3^2 + a_{ii} X_4^2 + \varepsilon \quad (2)$$

where Y_i (i = 1-2) are the predicted responses for μM of feruloylated raffinose (Y_1) and % feruloylated raffinose (Y_2), a_0 is the value of the fitted response at the center point of the design, a_i, a_{ij}, a_{ii} are the linear, cross-product and quadratic terms, respectively, ε is the random error, and X_1-X_4 are the uncoded independent variables. The variability of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and its statistical significance was checked using an F-test.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Feruloyl Esterase-Catalyzed Regioselective Synthesis of Feruloylated Monosaccharides

4.1.1. Enzymatic Isolation of Feruloylated Non-Digestible Oligosaccharides from Selected Plant Cell Wall

Equal amounts of sugars including arabinose, glucose and uronic acid make up sugar-beet pulp (72%, w/w) with almost 1.2% (w/w) of ferulic acid (Ralet et al., 1994b). In contrast, wheat bran (96%, w/w) contains equal amounts of xylose and glucose, followed by lower amounts of arabinose and consists of 0.43% (w/w) of ferulic acid (Ralet et al., 1994a; Barberousse et al., 2009). The well characterized NSPs of these by-products (Saulnier et al., 1995; Levigne et al., 2004) have been identified as potential sources of feruloylated NDOs. In addition, ferulic acid was reported to be ester-linked to *O*-5 of arabinose residues in arabinoxylans from wheat bran (Borneman et al., 1986), while in sugar-beet pulp it is found linked at 50–60% to the *O*-2 of arabinose residues of α-(1→5)-arabinan and at 40–50% to the *O*-6 of galactose residues of β-(1→4)-galactan (Guillon and Thibault 1990). The fungal multi-enzymatic preparation, driselase, was used as biocatalyst to isolate feruloylated NDOs from the selected by-products. Driselase from *Basidiomycetes* spp. is reported to exhibit various glycosyl hydrolases, including laminarinase (0.026 units/mg), xylanase (0.01 units/mg) and cellulase (0.6 units/mg); however, it is almost devoid of FAE (Ralet et al., 1994b). Figure 2 illustrates the time course for the release of feruloylated NDOs from sugar-beet pulp and wheat bran by driselase from *Basidiomycetes* spp. The results show that within the first 18 h of reaction time, there was a large increase in the concentration of the released feruloylated NDOs from sugar-beet pulp to a value of 309 mmol/g; thereafter, the released feruloylated NDOs concentration increased to a lesser extent up to a maximum of 317 mmol/g after 22 h of reaction. Compared with sugar-beet pulp, wheat bran shows biphasic behaviour. Within the first 3 h, a large increase in the concentration of the released feruloylated NDOs from wheat bran was obtained, subsequently there was a slow but continuous increase up to a maximum of 173 mmol/g after 22 h of reaction. The variation in the NSP

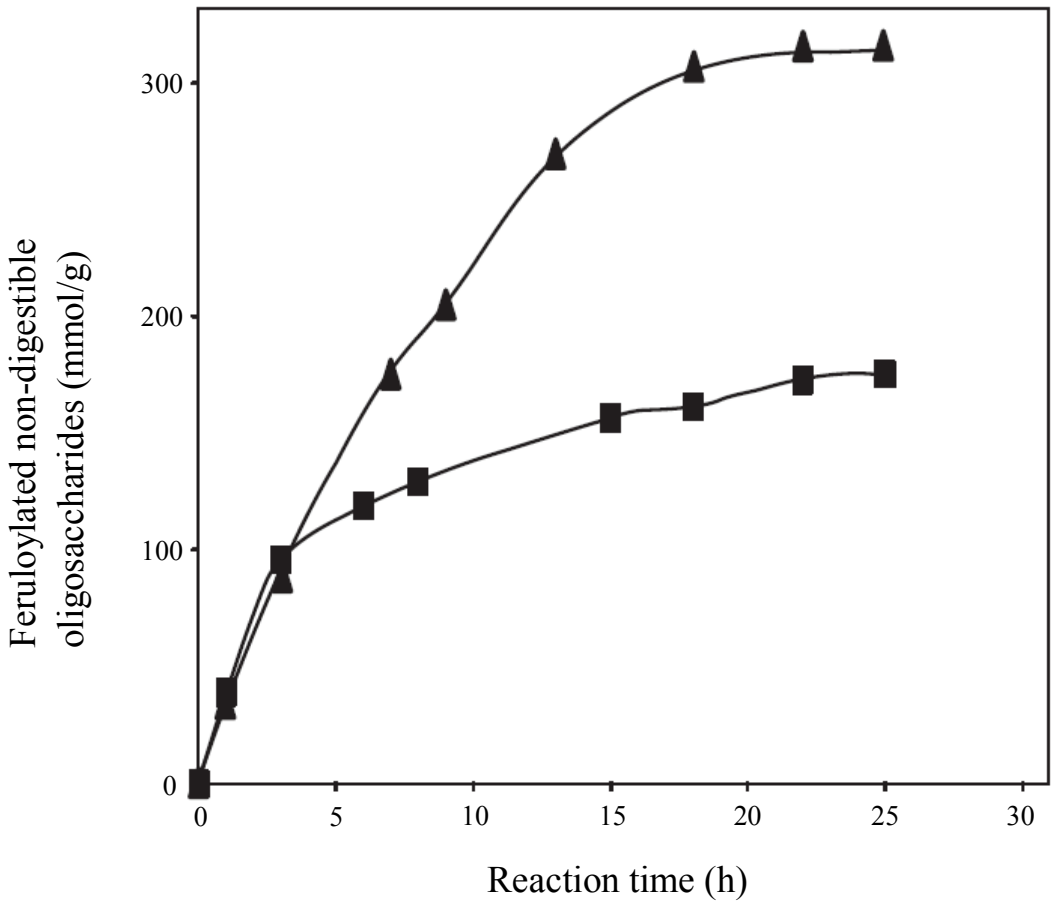


Figure 2. Time course for the release of feruloylated non-digestible oligosaccharides from sugar-beet pulp (▲) and wheat bran (■) by driselase from *Basidiomycetes* spp.

composition and proportion of ferulic acid may account for the difference in the maximum concentration of the released feruloylated NDOs from sugar-beet pulp and wheat bran. Compared with wheat bran, sugar-beet pulp comprising a higher proportion of ferulic acid resulted in a higher concentration of the released feruloylated NDOs.

Figure 3 shows the HPLC elution profiles of the enzymatic hydrolysates, containing feruloylated NDOs, of sugar-beet pulp (a) and wheat bran (b) monitored at 300 nm. Three major peaks numbered 1, 2 and 3, with retention times (E_t) of 16.0, 16.2 and 16.7 min, were characterized as feruloylated NDOs, since they showed a UV-spectral scanning profile comparable to that of methyl ferulate (Figures 3(a) and (a')). Moreover, compared with known oligomers, the isolated feruloylated NDOs from sugar-beet pulp had a DP of about 2 to 7 when qualitatively analyzed by TLC (data not shown). The recovery of four predominant feruloylated NDOs upon the hydrolysis of sugar-beet pulp with driselase has been reported by Ralet and colleagues (1994a,b) and were identified as corresponding to the feruloylated hexa-, hepta- and octasaccharides of arabinose and the feruloylated galactose disaccharide. On the other hand, the presence of four predominant feruloylated NDOs (numbered 1', 2', 3' and 4') in the enzymatic hydrolysate of wheat bran with E_t of 15.3, 15.5, 17.1, and 17.5 min is shown in Figure 3(b). The degrees of polymerization of these isolated feruloylated NDOs from wheat bran were about 1 to 4. Similarly, the release of four predominant feruloylated NDOs, upon the hydrolysis of wheat bran with driselase, have been reported (Ralet et al., 1994a) and characterized as feruloyl arabinose, arabinoxylodisaccharide, arabinoxylotrisaccharide and arabinoxylotetrasaccharide. Upon treatment of wheat flour arabinoxylan with endoxylanase from *Thermoascus aurantiacus,* one predominant feruloylated NDO, identified as feruloyl arabinoxylotrisaccharide, was obtained by Katapodis and colleagues (2003). The UV-spectral scan of the produced feruloylated NDOs (Figures 3(a') and (b')) indicates a bathochromic displacement of the absorption maxima at pH 10 (λ_{max}, 375 nm) compared with pH 6 (λ_{max}, 322 nm). These results reveal that the ferulic acid was linked through its carboxylic group to the NDOs.

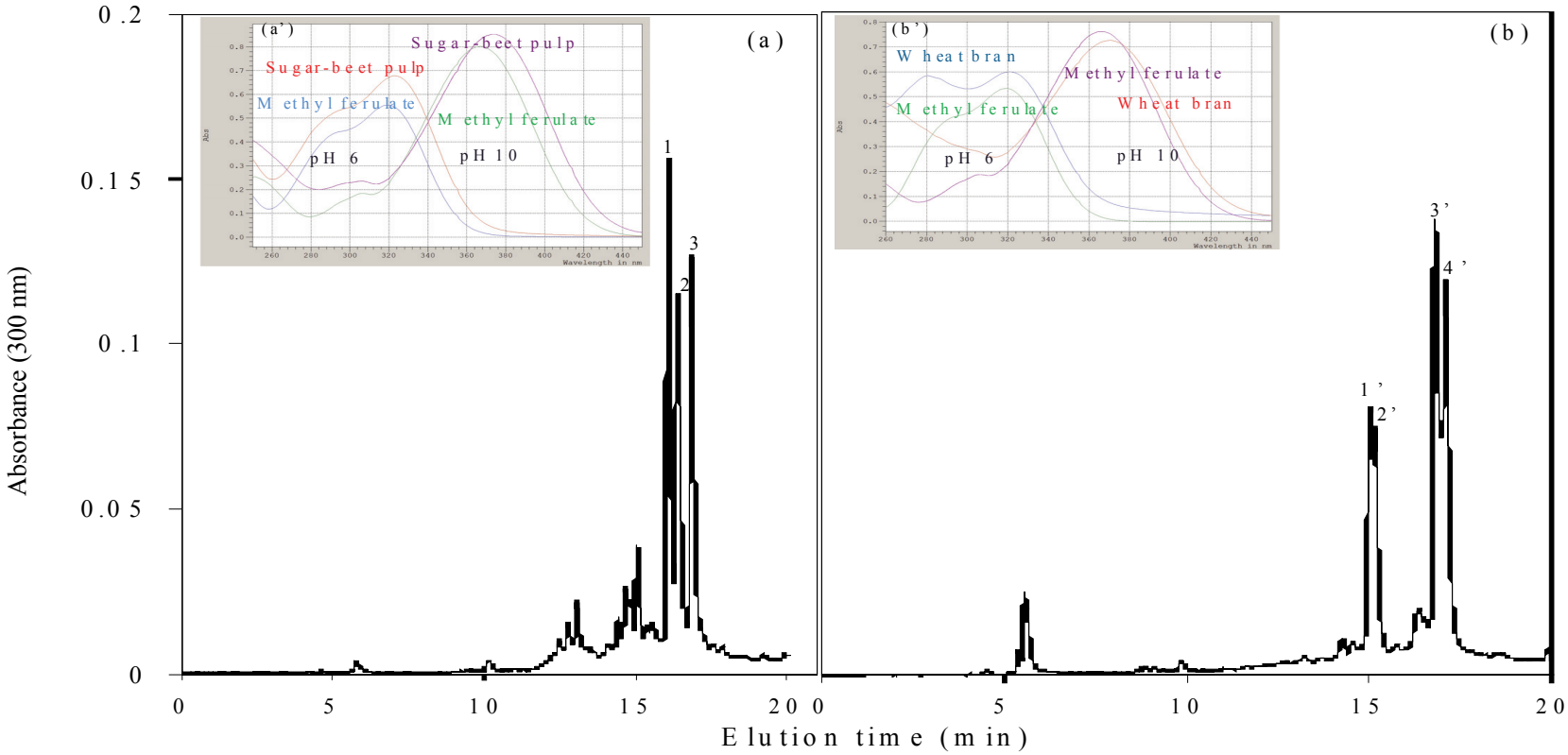


Figure 3. HPLC chromatogram of the enzymatic hydrolysates, containing feruloylated non-digestible oligosaccharides, of sugar-beet pulp (a) and wheat bran (b). Inserts: UV-spectral scan of methyl ferulate and feruloylated non-digestible oligosaccharides at pH 6 and 10 ((a') and (b')).

4.1.2. Feruloyl Esterase Activity Expressed in Selected Multi-Enzymatic Preparations

The FAE activities of 20 multi-enzymatic preparations from different bacterial and fungal sources were investigated (Table 3). Three feruloylated substrates, including methyl ferulate, feruloylated arabino/galacto-NDOs (di to hepta) from sugar-beet pulp as well as feruloylated arabinoxylo-NDOs (mono to tetra) from wheat bran, were used as substrates to assess the substrate specificity of FAEs. In order to remove the polyols and salts added by the manufacturers to stabilize the commercial multi-enzymatic preparations, a pre-purification step of dialysis or ultrafiltration with a cut off of 10 kDa was carried out. The protein contents of the commercial multi-enzymatic preparations varied greatly from almost 0.4% (w/v) for CGT Amano from *Bacillus macerans* to 40.9% (w/w) for Biozyme F10 SD from *B. subtilis* (data not shown).

The experimental findings (Table 3) show that the hydrolytic activity of FAEs on methyl ferulate varied widely from 7.5 to 8522.8 nmol/mg protein.min, whereas that on feruloylated NDOs ranged from 14.3 to 67 825.9 nmol/mg protein.min. Interestingly, most of the multi-enzymatic preparations exhibited higher specificity towards the feruloylated NDOs of cell-wall origin than on synthetic methyl ferulate. Only Lipase A from *A. niger* displayed higher activity on the methyl ferulate substrate than the feruloylated NDOs. This substrate–enzyme interaction may be explained by the hydrophobic nature of methyl ferulate, which may have favoured the opening of the lid covering the active site of Lipase A (Halimi et al., 2005). On the other hand, similar FAE hydrolytic activities towards the three investigated substrates were seen by Flavourzyme from *A. oryzae* and Lipase M from *Mucor javanicus*.

The results (Table 3) also reveal that the highest FAE activities were obtained with RP-1 from *B. subtilis* using methyl ferulate and feruloylated NDOs from sugar-beet pulp and wheat bran as substrates. In contrast, the lowest FAE activities towards the three investigated substrates were exhibited by Laminex BG and Cellulase from *T. reesei* as well as AMG and glucoamylase from *A. niger*. However, Pectinase from *A. niger* hydrolyzed the methyl ferulate and the feruloylated NDOs at higher rates compared with

Table 3. Specific activity of feruloyl esterase, expressed in selected multi-enzymatic preparations, for the hydrolysis of methyl ferulate and sugar-beet pulp- and wheat bran-derived non-digestible oligosaccharides as substrates.

Enzyme preparations	Microbial sources	Specific activity (nmol/mg protein.min)		
		Methyl ferulate	Sugar-beet pulp NDOs	Wheat bran NDOs
Cellulase	*Trichoderma reesei*	7.5 (± 0.4)[a]	14.3 (± 2.3)	55.43 (± 7.7)
Glucoamylase	*Aspergillus niger*	53.6 (± 2.2)	79.2 (± 6.9)	159.3 (± 4.4)
Ceremix	*Bacillus* spp.	133.4 (± 0.6)	179.3 (± 1.1)	1066.2 (± 4.8)
Gamanase	*Aspergillus niger*	313 (± 0.6)	759.1 (± 2.5)	397.9 (± 2.5)
Laminex BG	*Trichoderma reesei*	75.9 (± 0.6)	27.2 (± 1.7)	281.53 (± 6.4)
Diazyme L-200	*Aspergillus niger*	79.7 (± 1.7)	128.5 (± 3.0)	76.8 (± 4.5)
AMG	*Aspergillus* spp.	29.6 (± 5.8)	69.9 (± 2.1)	106.4 (± 6.7)
CGT Amano	*Bacillus macerans*	258.3 (± 0.3)	286 (± 0.6)	1826.9 (± 3.6)
Multifect P 3000	*Bacillus amyloliquefaciens*	61.9 (± 0.5)	139.7 (± 1.0)	3303.9 (± 6.1)
Depol 670L	*Trichoderma reesei*	490 (± 8.6)	496.7 (± 17.1)	200.8 (± 11.4)
Depol 740L	*Humicola* spp.	591.6 (± 9.8)	1674.8 (± 8.8)	296.9 (± 0.9)
Flavourzyme	*Aspergillus oryzae*	2039.4 (± 0.0)	2028.6 (± 0.0)	2382.8 (± 0.0)
YL-15	*Achromobacter lunatus*	1938.8 (± 0.0)	2807.2 (± 0.0)	2563.6 (± 0.0)
Newlase 11	*Rhizopus niveus*	282.3 (± 0.0)	601.1 (± 0.0)	1856.8 (± 0.2)
Biozyme F10 SD	*Bacillus subtilis*	155.7 (± 0.0)	216.8 (± 0.0)	455.8 (± 0.0)
RP-1	*Bacillus subtilis*	8522.8 (± 0.0)	66619.6 (± 0.2)	67825.9 (± 0.2)
Pectinase	*Aspergillus niger*	108.9 (± 0.0)	216.8 (± 0.0)	629.3 (± 0.0)
Lipase M	*Mucor javanicus*	2940.8 (± 0.1)	2804.4 (± 0.0)	2429.3 (± 0.0)
Lipase A	*Aspergillus niger*	3652.6 (± 0.2)	967.7 (± 0.0)	1455.9 (± 0.1)
Lipase PS30	*Pseudomonas* spp.	280.8 (± 0.0)	386.3 (± 0.0)	1620.4 (± 0.0)

[a] Data are presented as the average of three determinations ± standard deviation.

the other multi-enzymatic preparations from the same microbial source. The activity of two forms of FAE expressed in Pectinase from *A. niger* on a synthetic feruloylated substrate and on 11 different feruloylated NDOs from sugar-beet pulp and wheat bran have been identified by Ralet et al. (1994a).

Elevated levels of FAE activity towards the isolated feruloylated NDOs were obtained with selected multi-enzymatic preparations, including Ceremix from *Bacillus* spp., Depol 740L from *Humicola* spp., Depol 670L from *T. reesei*, Flavourzyme from *A. oryzae*, Multifect P 3000 from *B. amyloliquefaciens*, CGT Amano from *B. macerans*, YL-15 from *Achromobacter lunatus*, Newlase 11 from *Rhizopus niveus*, Gamanase from *A. niger*, and RP-1 from *B. subtilis* (Table 3). Interestingly, higher FAE activities towards the wheat bran feruloylated NDOs were obtained with Ceremix, Multifect P 3000, CGT Amano, and Newlase 11 products, while Depol 740L, Depol 670L and Gamanase exhibited higher FAE activities towards the sugar-beet pulp feruloylated NDOs. These results indicate that the FAEs from *T. reesei*, *Humicola* spp. and *A. niger* have higher specificity for the arabinose and galactose residues of sugar-beet arabinan and galactan, respectively, to which the ferulic acid is attached; while FAEs from *Bacillus* spp., *B. amyloliquefaciens*, *B. macerans,* and *R. niveus* have higher specificity towards the arabinoxylose residues of wheat arabinoxylan to which the ferulic acid is attached. On the other hand, Flavourzyme from *A. oryzae*, YL-15 from *A. lunatus* and RP-1 from *B. subtilis* showed similar FAE activities on sugar-beet pulp and wheat bran feruloylated NDOs. These results suggest that the nature of the sugar esterifying the feruloyl group is not of fundamental importance for these FAEs. Based on the specific activity expressed per mg proteins (Table 3) and the enzyme activity expressed per mg or mL preparations (data not shown), Ceremix, Depol 740L, Flavourzyme, Multifect P 3000, RP-1, and Depol 670L multi-enzymatic preparations were selected as sources of FAE biocatalysts.

4.1.3. Synthesis of Feruloylated Monosaccharides via Feruloyl Esterase-Catalyzed Esterification Reaction

The efficiency of FAEs expressed in selected multi-enzymatic preparations to catalyze the production of feruloylated monosaccharides via esterification reaction in non-conventional reaction media was investigated using a molar ratio of ferulic acid to monosaccharide of 3:1. Finding suitable reaction media for the enzymatic esterification of phenolic acid with glycosides is problematic owing to the inherently low solubility of these compounds in most non-aqueous media and the inactivation of most hydrolases in highly polar organic media, in which the two polar substrates exhibit appreciable solubility. As a result, a surfactant-less organic microemulsion medium composed of n-hexane, 1-butanol or 2-butanone and MES–NaOH buffer was used in the present study (Topakas et al., 2005a). After examining three different ratios of the surfactant-less organic microemulsion mixture, the n-hexane, 1-butanol or 2-butanone and MES–NaOH buffer mixture ratio of 51:46:3 (v/v/v) compared with 30:67:3 and 67:30:3 (v/v/v) was determined as the most appropriate reaction medium for the enzymatic feruloylation of monosaccharides (data not shown).

High esterifying activity for the feruloylation of arabinose (15–32%) was obtained with Ceremix from *Bacillus* spp.; however, with galactose and xylose monosaccharides, very low bioconversion yields (<0.3%) were obtained (Table 4). The feruloyl esterifying activity of Ceremix is in accordance with its hydrolytic activity, which was more efficient for the hydrolysis of the ester linkage of the feruloyl group attached to arabinoxylose residues of wheat arabinoxylan than that linked to the galactose residues of sugar-beet galactanan (Table 3). Although the FAE hydrolytic activity in RP-1 from *B. subtilis* did not show any preference for sugars esterifying the feruloyl group, higher esterifying activity efficiency was shown for the feruloylation of galactose (10–20%) and xylose (4–16%) compared with arabinose (<0.3%) (Table 4). Among the selected biocatalysts, higher specificity towards the feruloylation of galactose was displayed by FAEs expressed in Flavourzyme from *A. oryzae* (42%) and Depol 670L from *T. reesei* (61%). On the other hand, higher specificity towards the feruloylation of xylose was exhibited by FAEs present in Multifect P 3000 from *B. amyloliquefaciens* (31%), Depol 670L from

Table 4. Bioconversion yield of selected feruloylated monosaccharides obtained through feruloyl esterase-catalyzed esterification reaction in surfactant-less organic microemulsions composed of n-hexane, 1-butanol or 2-butanone and MES-NaOH buffer at a ratio of 51:46:3 (v/v/v).

Enzyme preparations	Bioconversion yield[a]	
	2-Butanone	1-Butanol
	D-Arabinose	
Ceremix from *Bacillus* spp.	15.3 (± 0.0)[b]	32.5 (± 2.0)
Depol 740L from *Humicola* spp.	7.2 (± 0.1)	17.7 (± 0.2)
Flavourzyme from *Aspergillus oryzae*	21.9 (± 8.9)	32.9 (± 1.2)
Multifect P 3000 from *Bacillus amyloliquefaciens*	11.3 (± 0.1)	36.7 (± 4.1)
RP-1 from *Bacillus subtilis*	< 0.3	< 0.3
Depol 670L from *Trichoderma reesei*	9.6 (± 0.4)	< 0.3
	D-Galactose	
Ceremix from *Bacillus* spp.	< 0.3	< 0.3
Depol 740L from *Humicola* spp.	15.8 (± 0.7)	3.4 (± 4.1)
Flavourzyme from *Aspergillus oryzae*	36.2 (± 0.9)	41.9 (± 6.2)
Multifect P 3000 from *Bacillus amyloliquefaciens*	8.6 (± 1.2)	5.6 (± 0.5)
RP-1 from *Bacillus subtilis*	19.8 (± 2.1)	10.2 (± 2.9)
Depol 670L from *Trichoderma reesei*	25.4 (± 1.8)	61.5 (± 7.1)
	D-Xylose	
Ceremix from *Bacillus* spp.	< 0.3	< 0.3
Depol 740L from *Humicola* spp.	20.9 (± 1.2)	26.5 (± 3.2)
Flavourzyme from *Aspergillus oryzae*	20.1 (± 1.5)	21.7 (± 0.3)
Multifect P 3000 from *Bacillus amyloliquefaciens*	12.1 (± 0.4)	30.8 (± 1.5)
RP-1 from *Bacillus subtilis*	4.4 (± 0.1)	16.3 (± 0.2)
Depol 670L from *Trichoderma reesei*	37.3 (± 5.6)	30.3 (± 4.3)

[a]The bioconversion yield (%) was calculated as the concentration of consumed glycosides over the initial concentration multiplied by 100%.
[b]Data are average of two or three determinations ± standard deviation.

T. reesei (37%) and Depol 740L from *Humicola* spp. (26%). Higher bioconversion yields for the feruloylation of arabinose were obtained with Multifect P 3000 from *B. amyloliquefaciens* (37%), Ceremix from *Bacillus* spp. (32%) and Flavourzyme from *A. oryzae* (33%).

Table 4 also shows that the bioconversion yield of the feruloylated monosaccharides is dependent on the nature of the surfactant-less organic microemulsion mixture used as a reaction medium. The water–enzyme interactions, which are essential for the catalytic activity, can be affected by the ability of these media to strip the water from the enzyme microenvironment (Halling, 2002). However, the effect of either 2-butanone or 1-butanol on the esterifying efficiency of FAEs expressed in the selected multi-enzymatic preparations seemed to be dependent on the structure of monosaccharide substrate. Using arabinose as substrate, higher bioconversion yields of 18-37% were obtained using the surfactant-less organic microemulsion mixture containing 1-butanol compared with that containing 2-butanone (7-22%). With galactose as substrate, a higher bioconversion yield of 9, 16 and 20% in organic microemulsion mixture containing 2-butanone compared with that containing 1-butanol was achieved with the FAEs expressed in Multifect P 3000 from *B. amyloliquefaciens*, Depol 740L from *Humicola* spp. and RP-1 from *B. subtilis*, respectively. Alternatively, a higher bioconversion yield of feruloylated galactose of 42% was obtained with the FAEs present in Flavourzyme from *B. subtilis* in the organic microemulsion mixture containing 1-butanol. With xylose, no significant effect of organic solvent on the esterifying efficiency of FAEs expressed in Depol 740L from *Humicola* spp. and Flavourzyme from *B. subtilis* was seen. However, feruloylated xylose resulted in higher bioconversion yields of 31 and 16% in the surfactant-less organic microemulsion mixture containing 1-butanol using FAE activities of Multifect P 3000 from *B. amyloliquefaciens* and RP-1 from *B. subtilis* as biocatalysts, respectively. The overall results (Table 4) suggest that the presence of the organic solvent affected the enzyme itself by binding in or near its active site, resulting in conformational changes (Fitzpatrick et al., 1993). The effect of substrate–solvent interactions on the availability and the partition of the substrate in the enzyme's micro and macro environment may also explain the dependence of the esterifying efficiency of FAEs expressed in the multi-enzymatic preparations on the nature of solvent (Kvittingen, 1994). The bioconversion yield values

obtained by Topakas et al. (2005a) for the transesterification of methyl ferulate with L-arabinose by StFaeC are in the same range as those obtained for the esterification of ferulic acid with monosaccharides (Table 4). However, Mastihubova et al. (2006) reported higher bioconversion yields (up to 95%) for the esterification of vinyl ferulate with arabinose and galactose by Lipolase 100T. For further FAE-catalyzed esterification reactions, the multi-enzymatic preparation Depol 740L was used. According to pre-screening results, Depol 740L was determined as the most appropriate multi-enzymatic preparation for the enzymatic feruloylation of di- and NDOs (data not shown).

4.1.4. Structural Characterization of Feruloylated Monosaccharides

Figure 4 illustrates the TLC chromatogram of monosaccharides and their feruloylated derivatives. Feruloylated xylose ascended the TLC plate the fastest, followed by the feruloylated arabinose and galactose. Further analyses of the synthesized feruloylated monosaccharides were conducted by APCI-MS in the positive-ion mode in order to characterize the molecular structure (Figure 5). The position of the phenolic group on the glycoside backbone of the feruloylated monosaccharides is shown for simplicity as the regioselectivity of the reaction is unknown. The fragmentation pattern of the peak corresponding to the feruloylated arabinose (Figure 5(a)) shows abundant molecular ions at *m*/*z* 309.2 ($[M+H-H_2O]^+$) and 327.2 ($[M+H]^+$) and two fragment ions at *m*/*z* 150.2 and 194.1 ($[M]^+$) representing arabinose and ferulic acid, respectively. Likewise, the peak corresponding to the feruloylated xylose (Figure 5(b)) shows molecular ions at *m*/*z* 309.2 ($[M+H-H_2O]^+$) and 326.3 ($[M+H]^+$) and two fragment ions at *m*/*z* 150.4 and 194.1 ($[M]^+$) corresponding to xylose and ferulic acid, respectively. On the other hand, the fragmentation produced abundant molecular ions at *m*/*z* 339.1 ($[M+H-H_2O]^+$) and 356.2 ($[M+H]^+$), which are characteristics of feruloylated galactose (Figure 5(c)), as well as two fragment ions at *m*/*z* 180.1 and 194.1 ($[M]^+$) representing galactose and ferulic acid, respectively. Overall, the formation of various feruloylated monosaccharides by FAE-catalyzed esterification of ferulic acid with the selected monosaccharides was confirmed by HPLC/APCI-MS analyses. Only Topakas et al. (2005a) has characterized the feruloylated monosaccharide end product of FAE-catalyzed transesterification of methyl ferulate with L-arabinose.

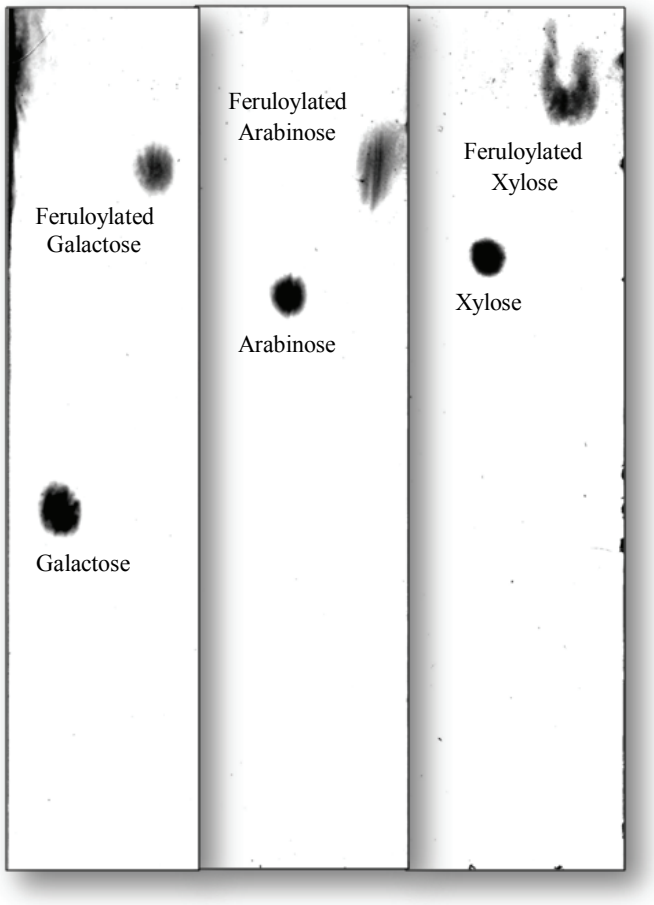


Figure 4. TLC chromatogram of monosaccharides and feruloylated monosaccharides.

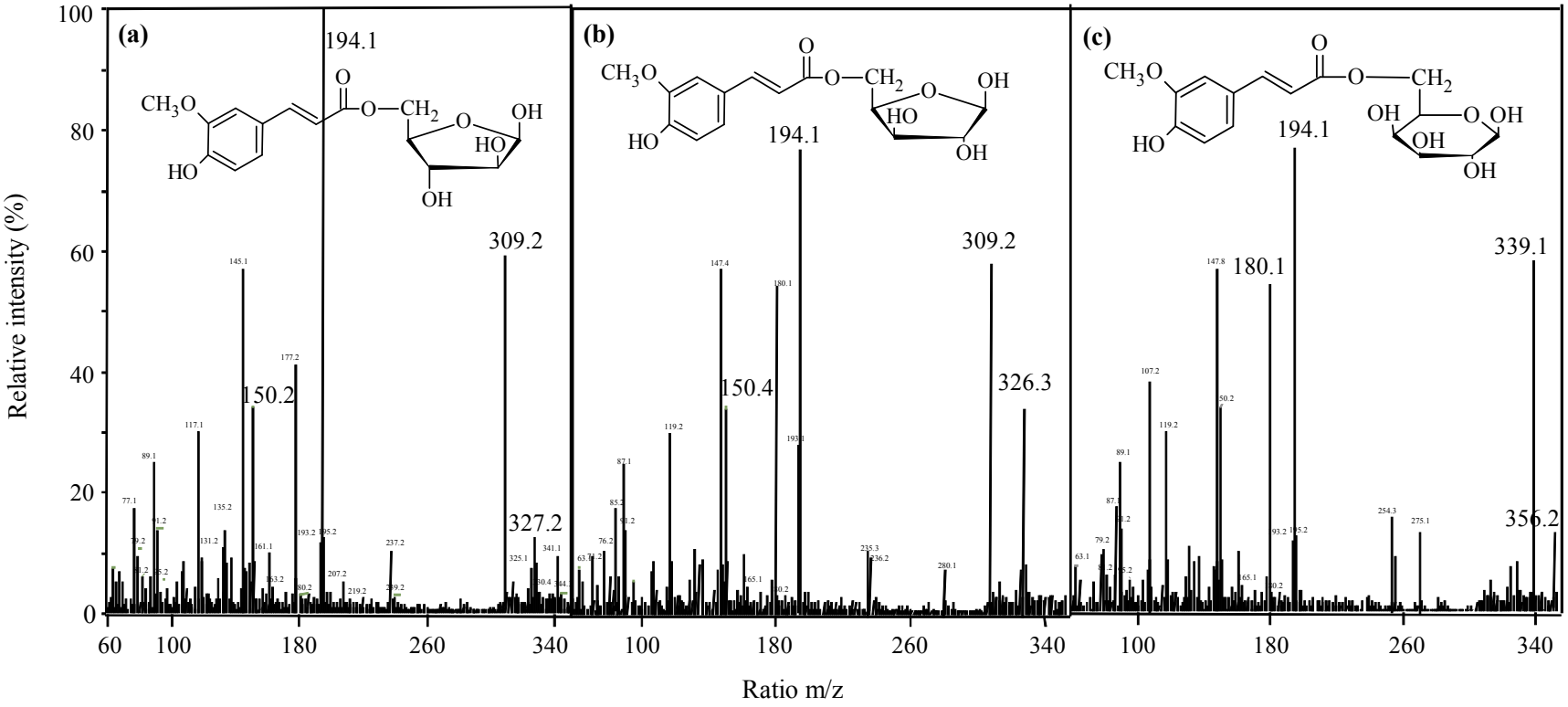


Figure 5. APCI-MS spectrum of the fragmentation pattern of the feruloylated monosaccharides obtained by feruloyl esterase-catalyzed esterification of ferulic acid with D-arabinose (a), D-xylose (b) and D-galactose (c).

4.1.5. Radical Scavenging Activity of Feruloylated Monosaccharides

The free radical DPPH$^{\bullet}$ was used to investigate the free-radical scavenging activity of the synthesized feruloylated monosaccharides as well as of their corresponding ferulic acid. The results, in Table 5, show that a higher time (540 s) was required for the feruloylated arabinose to reach the steady state compared with the free ferulic acid and other ferulates (240–300 s). The most potent scavenger was found to be the feruloylated galactose, with a radical scavenging activity and yield of 6.3 Ab at 517 nm/min.mL and 93%, respectively. Although the scavenging activity of the feruloylated xylose (2.1 Ab at 517 nm/min.mL) was two times lower than that of the ferulic acid, both esterified and free ferulic acid led to a similar maximum radical scavenging yield of 91–93% at steady state. In contrast, the feruloylated arabinose compared with the free ferulic acid resulted in a lower scavenging activity and maximum yield of 2.3 Ab at 517 nm/min.mL and 70%, respectively. The results indicate that the scavenging activity was not affected by the esterification of ferulic acid with hexose (galactose), whereas that with pentose (arabinose and xylose) resulted in a slight decrease of the antiradical potency. Katapodis et al. (2003) stated that compared with the ferulic acid, the isolated feruloylated arabinoxylo-NDOs exhibited a reduced scavenging activity towards DPPH$^{\bullet}$. Despite advances in our understanding of the molecular mechanisms underlying the radical scavenging activity, the effect of the molecular structure of phenolic compounds on their reactivity as radical scavengers stirs up some controversy. The effect of the esterification of the phenolic acid's carboxyl group on its radical scavenging activity has been previously reported (Chen and Ho 1997; Safari et al., 2006) and ascribed mainly to the steric hindrance and the H-donating effect of the acyl groups on the interaction with the DPPH$^{\bullet}$ free radical and the subsequent resonance stabilization and the formation of quinone.

Table 5. Scavenging activity of ferulic acid and the feruloylated monosaccharides using DPPH$^{\bullet}$ as stable free radical.

Components	Scavenging activity[a]	T_{sd}[b]	Scavenging yield[c]
Ferulic acid	4.5 (± 0.2)[d]	300	93
Feruloylated arabinose	2.3 (± 0.1)	540	70
Feruloylated galactose	6.3 (± 0.4)	240	93
Feruloylated xylose	2.1 (± 0.2)	300	91

[a] The scavenging activity is expressed as the decreased absorbance at 517 nm per min of reaction per mL of feruloylated glycosides of ferulic acid.

[b] T_{sd} is the time (in s) required to reach the steady state.

[c] Radical scavenging yield (%) at the steady state was calculated as the absorbance of the DPPH$^{\bullet}$ control, at 517 nm, minus the absorbance of the sample divided by that of the DPPH$^{\bullet}$ control, multiplied by 100.

[d] Data are presented as the average of two or three determinations ± standard deviation.

4.2. Optimization of Feruloyl Esterase-Catalyzed Esterification of Feruloylated Di- and Non-Digestible Oligosaccharides

4.2.1. Enrichment of Selected Multi-Enzymatic Preparation with Feruloyl Esterase Activity

Two different separation techniques, including ammonium sulfate precipitation and ultrafiltration, were investigated to enrich the selected multi-enzymatic preparation Depol 740L from *Humicola* spp. with FAE activity. $(NH_4)_2SO_4$ was used as a precipitant due to its low cost and ability to chill aqueous solutions (Cao et al., 2000; Schoevaart et al., 2004). The FAE activity of the multi-enzymatic preparation Depol 740L was investigated before and after the enrichment step. The results (Table 6) show that the multi-enzymatic product Depol 740L exhibited a FAE specific activity on methyl ferulate of 9.5 μmol/mg protein.min and a FAE activity of 405.9 μmol/mL.min (data not shown). Lower FAE activity of Depol 740L on methyl ferulate (10.5 μmol/mL.min) was reported by Vafiadi et al. (2008).

The ultrafiltration of Depol 740L did not succeed in the enrichment of FAE activity (data not shown). This may be explained by the formation of large protein aggregates that may have clogged the ultrafiltration membrane, making it difficult to concentrate/buffer exchange by ultrafiltration (Doyle, 2009). However, the ammonium sulfate precipitation resulted in an enrichment factor and activity yield of 1.4 and 112%, respectively. The experimental findings (Table 6) also indicate that the FAE activity was not affected by freeze-drying (23.7 μmol/mg protein.min) displaying a final purification factor and activity yield of 2.5 and 143%, respectively. Vafiadi et al. (2008) showed that out of 10 different precipitants, saturated $(NH_4)_2SO_4$ was found to be the best precipitant, retaining 73% of Depol 740L's initial activity. Madani et al. (1996) reported that gradual precipitation with $(NH_4)_2SO_4$ resulted in a slightly higher enrichment fold (2.1) of a polyphenol esterase from *A. niger*. On the other hand, Vafiadi et al. (2008) similarly reported a FAE activity yield of 146% after freeze-drying.

Table 6. Enrichment of feruloyl esterase expressed in multi-enzymatic preparation Depol 740L from *Humicola* spp.

Enrichment step	Specific activity (μmol/mg protein.min) Methyl ferulate	Enrichment fold[a]	Activity yield[b]
Depol 740L	9.5 (± 3.3)[c]	-	-
Ammonium sulfate precipitation	12.9 (± 3.6)	1.4	111.9
Freeze-drying	23.7 (± 6.3)	2.5	143.3

[a] The enrichment fold was calculated as the number of times the specific activity increased at each enrichment step over the initial specific activity of Depol 740L.

[b] The activity yield (%) was calculated as the number of times the feruloyl esterase activity increased at each enrichment step over the initial feruloyl esterase activity in Depol 740L multiplied by 100%.

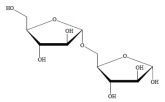
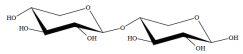
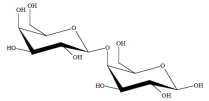
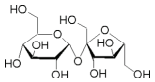
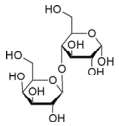
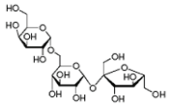
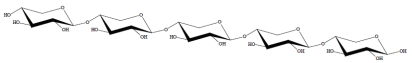
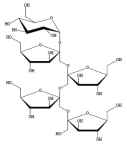
[c] Data are average of seventeen determinations ± standard deviation.

4.2.2. Effect of Glycoside Structures on the Bioconversion Yield

The efficiency of the enriched FAE expressed in Depol 740L to catalyze the synthesis of selected feruloylated glycosides (di- and NDOs) via esterification reaction was investigated using a ferulic acid to glycoside molar ratio of 3:1 and the mixture composed of n-hexane, 2-butanone and MES-NaOH buffer (51:46:3, v/v/v) as reaction medium. In addition to being a non-reactive and non-toxic environment, the selected surfactant-less organic microemulsion mixture was determined in our previous study to be the most appropriate reaction medium for the enzymatic feruloylation of monosaccharides (Couto et al., 2010). The results (Table 7) show that the bioconversion yield of feruloylated di- and NDOs was low and varied from 3 to 13%. These values are lower than those reported in our previous study (Couto et al., 2010) for the feruloylation of monosaccharides (3 to 61 %). These results indicate that FAE from *Humicola* spp. exhibited higher substrate specificity towards the esterification of monosaccharides as compared to their corresponding disaccharides. However, a similar bioconversion yield of 13% was obtained by Topakas et al. (2003b) for the transesterification of methyl ferulate with 1-butanol catalyzed by a *F. oxysporum* FAE. Only one study has carried out the transferuloylation of the disaccharide L-arabinobiose by FAE from *S. thermophile* and reported a similar bioconversion yield of 10 to 18% (Vafiadi et al., 2006).

The overall results reveal that the bioconversion yield of ferulated glycosides is dependent on the structural characteristics of di- and NDOs, in particular the type of hexose/pentose moiety, the length and the glycosidic linkages. Interestingly, higher bioconversion yields of 11 to 13% were obtained with the di- (sucrose) and trisaccharide (raffinose) composed of hexose (fructose, glucose) moieties (Table 7). Increasing the chain length of fructose units in FOSs resulted in a decrease of bioconversion yield from 13 to 10%. These results may be due to the substrate steric hindrance affecting the binding of FOSs on the FAE's active site. The decrease in the substrate availability due to the increase of its hydrophilicity may also be an explanation of the low bioconversion yield of FOSs as compared to sucrose and raffinose. However, a higher inhibitory steric effect of the chain length was obtained with the xylose moiety showing a decrease in the bioconversion yield from 9% with xylobiose to 3% with XOSs.

Table 7. Bioconversion yield of selected feruloylated di- and non-digestible oligosaccharides obtained through feruloyl esterase-catalyzed esterification reaction in a surfactant-less organic microemulsion composed of n-hexane, 2-butanone and MES-NaOH buffer at a ratio of 51:46:3 (v/v/v).

Glycoside Substrates	Structures	Glycosides esterified[a]	Bioconversion yield[b]
Arabinobiose		79.0	7.9 (± 0.7)[c]
Xylobiose		94.5	9.4 (± 0.0)
Galactobiose		53.6	5.4 (± 0.6)
Sucrose		131.8	13.2 (± 0.6)
Lactose		44.1	4.4 (± 0.4)
Raffinose		110.3	11.0 (± 1.1)
XOS		27.8	2.8 (± 0.2)
FOS		95.7	9.6 (± 0.6)

[a] The glycosides esterified (μM) was calculated as the sum of the HPLC peak areas of formed feruloylated glycosides over the ferulic acid standard curve slope.

[b] The bioconversion yield (%) was calculated as the concentration of consumed glycosides over the initial concentration multiplied by 100%.

[c] Data are average of two determinations ± standard deviation.

Among the investigated disaccharides, galactobiose and lactose containing galactose showed the lowest bioconversion yields of 4 and 5%, respectively (Table 7). On the other hand, sucrose containing glucose and fructose revealed the highest bioconversion yield of 13% followed by xylobiose (9%) containing xylose and arabinobiose (8%) containing arabinose. Dissimilarly, in our previous study (Couto et al., 2010) with monosaccharides, galactose led to the highest bioconversion yield (61%) followed by xylose and arabinose (37%). These results suggest that the electronic distribution and the steric hindrance of the enzyme/substrate complex may be different for the di- and NDOs.

4.2.3. Determination of Radical Scavenging Activity of Feruloylated Di- and Non-Digestible Oligosaccharides

The free radical scavenging activity of the synthesized feruloylated di- and NDOs as well as of their corresponding ferulic acid was investigated using $DPPH^{\bullet}$ as free radical. Table 8 shows that the feruloylated FOSs required a higher time (750 s) to reach the steady state as compared to the free ferulic acid and other ferulates (405-585 s). The feruloylated raffinose showed the highest affinity toward the scavenging of the free radical $DPPH^{\bullet}$ with an activity of 4.3 Ab at 517 nm/min.mL. In fact, as compared to their corresponding free ferulic acid, all feruloylated di- and NDOs led to higher scavenging activity with the exception of feruloylated XOSs (3.1 Ab at 517 nm/min.mL). These results reveal an increase in the affinity of ferulic acid to donate hydrogen to $DPPH^{\bullet}$ in order to stabilize the phenoxy radical upon its conformational modification with di- and NDOs. In contrast, Katapodis et al. (2003) reported that the isolated feruloylated arabinoxylo-NDOs displayed a reduced specificity towards $DPPH^{\bullet}$ as compared to ferulic acid. However, the scavenging activity of ferulates seems to be dependent on the chemical structure of the glycosides. The acylation of ferulic acid with hexoses (galactobiose, sucrose, lactose, raffinose, and FOS) resulted in higher scavenging activity compared with pentoses (arabinobiose, xylobiose and XOS). These results could be explained by the effect of the glycosidic substituents and the steric hindrance of their functional groups on the rotation degree of the phenyl moiety (Silva et al., 2000). Similarly, in our previous study (Couto et al., 2010), the esterification of ferulic acid with the pentoses xylose and arabinose reported to have the lowest scavenging activity of 2.1 and 2.3 Ab at 517 nm/min.mL, respectively.

Table 8. Scavenging activity of the ferulic acid and the feruloylated di- and non-digestible oligosaccharides using $DPPH^{\bullet}$ as stable free radical.

Components	Scavenging activity [a]	T_{sd} [b]	Scavenging yield [c]
Ferulic acid	3.4 (± 0.1) [d]	405	92.1
Feruloylated arabinobiose	3.5 (± 0.2)	570	83.7
Feruloylated xylobiose	3.6 (± 0.2)	525	87.7
Feruloylated galactobiose	3.8 (± 0.2)	555	85.6
Feruloylated sucrose	3.8 (± 0.2)	450	93.3
Feruloylated lactose	4.0 (± 0.2)	540	86.5
Feruloylated raffinose	4.3 (± 0.2)	540	87.0
Feruloylated XOS	3.1 (± 0.2)	585	94.4
Feruloylated FOS	3.7 (± 0.1)	750	93.5

[a] The scavenging activity is expressed in the decreased Ab at 517 nm per min of reaction per mL of feruloylated glycosides of ferulic acid.

[b] Tsd is the time (in sec) required to reach the steady state.

[c] Radical scavenging yield (%) at the steady state was calculated as the absorbance of the $DPPH^{\bullet}$ control, at 517 nm, minus the absorbance of the sample divided by that of the $DPPH^{\bullet}$ control, multiplied by 100.

[d] Data are average of three determinations ± standard deviation.

Although feruloylated XOSs exhibited the lowest scavenging activity, it resulted in the most potent scavenger with a yield of 94% (Table 8). On the other hand, feruloylated arabinobiose displayed the lowest ability to delocalize the phenoxy radical across the entire molecule and to stabilize the phenoxy radicals leading to a scavenging yield of 84%. Likewise, feruloylated arabinose revealed to have the lowest scavenging yield (70%) in our previous study with monosaccharides (Couto et al., 2010). A higher scavenging yield of 93% was obtained with the hexose galactose compared with the pentoses arabinose and xylose, which had a lower scavenging yield of 70 and 91%, respectively (Couto et al., 2010).

4.2.4. Effect of Reaction Media on Bioconversion Yield of Selected Feruloylated Di- and Non-Digestible Oligosaccharides

The FAE-catalyzed esterification of selected feruloylated di- and NDOs was carried out using selected surfactant-less organic microemulsions (51:46:3, v/v/v) and IL-based emulsions (51:46:3, v/v/v or 97:3, v/v) as reaction media. The amount of esterified di- and NDOs and bioconversion yield are shown in Table 9. No significant FAE-catalyzed esterification could be obtained in n-hexane/[bmim]BF_4/MES-NaOH, [bmim]PF_6/2-butanone/MES-NaOH and [bmim]PF_6/1,4-dioxane/MES-NaOH (51:46:3, v/v/v), with log *P* of 1.3, -0.3 and -0.5, respectively. The highest bioconversion yield of 27% was obtained with galactobiose in the n-hexane, 1,4-dioxane and MES-NaOH buffer mixture (51:46:3, v/v/v) (log *P* of 1.6) followed by 11% with raffinose in the n-hexane, 2-butanone and MES-NaOH buffer mixture (51:46:3, v/v/v) (log *P* of 1.8).

The results (Table 9) show that as compared to other reaction media, the n-hexane, 2-butanone and MES-NaOH buffer mixture (log *P* of 1.8) resulted in the highest bioconversion yields using arabinobiose (8%), xylobiose (9%) and raffinose (11%) as glycoside substrates. However, using galactobiose as substrate, the highest bioconversion yield (27%) was obtained in the n-hexane, 1,4-dioxane and MES-NaOH buffer mixture (log *P* of 1.6).

The substitution of 2-butanone in the reaction mixture with dioxane or the IL [bmim]BF_4 resulted in a significant decrease of the bioconversion yield of feruloylated arabinobiose, xylobiose and raffinose from 8-11% to 0.2-4% (Table 9). Such results may be due to the

Table 9. Effect of media on bioconversion yield of selected feruloylated di- and non-digestible oligosaccharides obtained through feruloyl esterase-catalyzed esterification reaction using Depol 740L from *Humicola* spp.

Media mixture[a]	Log *P* value[b]	Substrates							
		Arabinobiose		Xylobiose		Galactobiose		Raffinose	
		A[c]	B[d]	A	B	A	B	A	B
n-hexane/2-butanone/MES-NaOH	1.8	79.0	7.9 (± 1.1)[e]	94.5	9.4 (± 0.0)	53.6	5.4 (± 0.4)	110.3	11.0 (± 0.8)
n-hexane/1,4-dioxane/MES-NaOH	1.6	<0.01	<0.01	42.4	4.2 (± 0.3)	267.5	26.8 (± 2.7)	31.2	3.1 (± 0.1)
n-hexane/[bmim]BF_4[f]/MES-NaOH	1.3	1.6	0.2 (± 0.0)	7.6	0.8 (± 0.0)	3.7	0.4 (± 0.0)	-[h]	-
[bmim]PF_6[g]/2-butanone/MES-NaOH	-0.3	2.9	0.3 (± 0.1)	1.2	0.1 (± 0.1)	0.6	0.1 (± 0.01)	5.5	0.5 (± 0.01)
[bmim]PF6/MES-NaOH, 97:3 (v/v)	-0.8	28.1	2.8 (± 0.2)	42.1	4.2 (± 0.1)	10.1	1.0 (± 0.2)	19.6	2.0 (± 0.2)

[a] Media mixture at a ratio of 51:46:3 (v/v/v).

[b] Log *P* value defined as the partition coefficient of the media mixture between water and 1-octanol. Log *P* of the media mixture was calculated according to the empirical formula log *P* mixture = X1 log *P* + X2 log *P*2 + X3 log *P*3, in which X1, X2 and X3 are the mole fractions of media 1, 2 and 3.

[c] Esterified glycosides (μM).

[d] Maximum bioconversion yield (%) after 144h.

[e] Data are average of two determinations ± standard deviation.

[f] 1-butyl-3-methylimidazolium tetrafluoroborate.

[g] 1-butyl-3-methylimidazolium hexafluorophosphate.

[h] Not determined.

stripping water effect of 1,4-dioxane and IL [bmim]BF_4, denoted by their lower log *P*, thus affecting the water-enzyme interactions, which are essential for the catalytic activity (Halling, 2002). Similarly, the use of the IL [bmim]PF_6 instead of n-hexane in the selected reaction mixture composed of 2-butanone and MES-NaOH led to a significant decrease in the bioconversion yield from 3-27% to 0.1-0.5%. While higher bioconversion yields were obtained when the IL was used in excess in the reaction mixture composed of [bmim]PF_6 and MES-NaOH (log *P* of -0.8). Vafiadi et al. (2009) reported that AnFaeA was not able to catalyze the transesterification and esterification of glycerol with sinapic acid in [bmim]PF_6. However, higher bioconversion yields (up to 55%) were reported by Martín et al. (2008) for the esterification of phthalic acids with ethanol using *Bacillus thermocatenulatus* lipase in [bmim]BF_4 and [bmim]PF_6.

The effect of reaction media mixture on the esterifying efficiency of FAE expressed in Depol 740L is dependent on the structure of glycoside substrate. Nevertheless, the results (Table 9) confirm higher maximum bioconversion yields with the di- and trisaccharide composed of hexoses (galactobiose and raffinose) as compared to the pentose-based disaccharides (arabinobiose and xylobiose). Similar results for the feruloylation of monosaccharides were obtained in our previous work (Couto et al., 2010).

The overall findings (Table 9) indicate clearly that there was no correlation between the esterifying efficiency of FAEs and the Log *P* value of the organic solvent mixture. Likewise, Arriagada-Strodthoff et al. (2007) reported no correlation between chlorophyllase activity and the Log *P* value of the organic solvent media. These results may indicate the direct effect of the organic solvent on the enzyme itself by binding in or near its active site (Fitzpatrick et al., 1993). Such effect seems also to affect the substrate specificity of FAEs showing different solvent effect patterns (Kvittingen, 1994). The effect of substrate-solvent interactions on the availability of substrate to the enzyme may also be an explanation of the effect of organic solvents. For further optimization studies, the trisaccharide raffinose and the surfactant-less organic microemulsion mixture containing n-hexane, 2-butanone and MES-NaOH were used. Hexane and 2-butanone are suitable solvents since they are non-toxic and permitted for use in the production of edible foods (Yan et al., 2002).

4.2.5. Structural Characterization of Selected Feruloylated Di- and Non-Digestible Oligosaccharides

In order to characterize the molecular structure of the synthesized feruloylated di- and NDOs, further analysis of the eluting peaks by APCI-MS spectrometry in the positive ion mode was conducted (Figure 6). The position of the phenolic group on the glycoside backbone of the feruloylated di- and NDOs is shown for simplicity as the regioselectivity of the reaction is unknown. The fragmentation pattern in Figure 6(a) corresponding to diferuloylated arabinobiose shows abundant molecular ions at *m/z* 441.0 $[M+H-H_2O]^+$ and 636.0 $[M+H]^+$ corresponding to mono- and diferuloylated arabinobiose, respectively, and two fragment ions at *m/z* 182.0 and 262.7 $[M+H-H_2O]^+$ representing ferulic acid and arabinobiose, respectively. On the other hand, the fragmentation (Figure 6(b)) of the product obtained with galactobiose produced abundant molecular ions at *m/z* 501.1 $[M+H-H_2O]^+$ representing monoferuloylated galactobiose and at *m/z* 660.1 $[M+2H-2H_2O]^+$ and 694.0 $[M]^+$, which are characteristics of diferuloylated galactobiose, as well as two fragment ions at *m/z* 194.1 $[M]^+$ and 313.0 $[M+2H-2H_2O]^+$ corresponding to ferulic acid and galactobiose, respectively. The fragmentation pattern of the peak corresponding to the feruloylated raffinose (Figure 6(c)) shows abundant molecular ions at *m/z* 662.7 $[M+H-H_2O]^+$ and 680.5 $[M+H]^+$ and two fragment ions at *m/z* 190.8 $[M]^+$ and 486.6 $[M+H-H_2O]^+$ representing ferulic acid and raffinose, respectively.

The APCI-MS analysis (Figure 6(d), (e), (f)) also confirmed the enzymatic synthesis of selected feruloylated NDOs upon reaction with FOSs, including diferuloylated fructose, feruloylated difructose and tetraferuloylated trifructose. The fragmentation pattern in Figure 6(d) shows abundant molecular ions at *m/z* 330.7 $[M+H-H_2O]^+$ and 358.8 $[M]^+$ corresponding to monoferuloylated fructose and at *m/z* 499.0 $[M+H-H_2O]^+$ and 532.4 $[M]^+$ representing diferuloylated fructose, as well as three fragment ions at *m/z* 180.6, 194.9 and 372.0, which corresponds to fructose, ferulic acid and diferulic acid, respectively. On the other hand, the fragmentation pattern of feruloylated difructose (Figure 6(e)) exhibited two molecular ions at *m/z* 501.0 $[M+H-H_2O]^+$ and 518.2 $[M]^+$ and fragment ions at *m/z* at 194.9 $[M]^+$ and 325.6 $[M+H-H_2O]^+$ of ferulic acid and difructose, respectively. The fragmentation pattern in Figure 6(f) shows abundant molecular ions at *m/z* 659.5 $[M+2H-2H_2O]^+$ and. 695.6 $[M]^+$ corresponding to diferuloylated difructose and

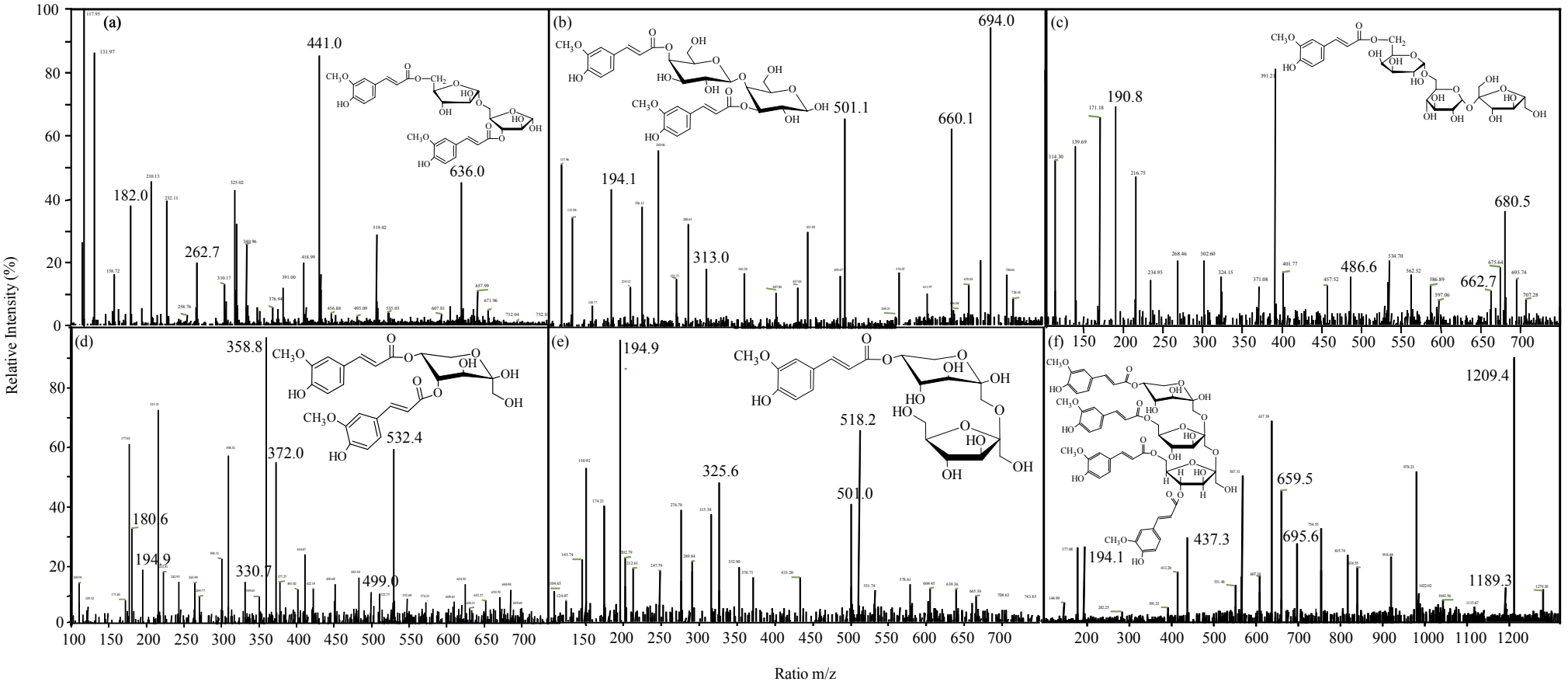


Figure 6. APCI-MS spectrum of the fragmentation pattern of the feruloylated raffinose obtained by feruloyl esterase-catalyzed esterification of ferulic acid with arabinobiose (a), galactobiose (b), raffinose (c), and FOSs (d, e, f).

at *m/z* 1189.3 $[M+H-H_2O]^+$ and 1209.4 $[M]^+$, which are characteristics of tetraferuloylated trifructose, as well as two fragment ions at 194.1 $[M]^+$ and 437.3 $[M+4H-4H_2O]^+$ corresponding to ferulic acid and trifructose, respectively.

Overall, the HPLC/APCI-MS analysis confirmed the formation of feruloylated di- and NDOs by the enriched FAE-catalyzed esterification of ferulic acid with the selected glycosides. Only Vafiadi et al. (2007) has characterized the feruloylated glycoside end product of FAE-catalyzed transesterification of methyl ferulate with linear arabino-oligosaccharides, containing three to six arabinofuranose units.

4.2.6. Model Fitting and Analysis of Variance

In order to optimize and to better understand the relationships between the reaction parameters of FAE-catalyzed esterification of raffinose with ferulic acid, RSM has been used. CCRD was selected for the optimization of the feruloylation of raffinose using five levels and four factors, including temperature, substrate molar ratio, water content as well as enzyme amount. Reaction temperature of 35°C, ferulic acid to raffinose molar ratio of 3:1, water content of 3% (v/v), and enzyme amount of 218 enzymatic FAE units were chosen as the central condition of the CCRD (Table 10).

Table 10 shows the experimental conditions, the actual experimental amounts and the results of the bioconversion yield and the concentration of the produced feruloylated raffinose according to the factorial design. Among the various treatments, the maximum concentration and bioconversion yield of feruloylated raffinose (114.5 μM, 11.4%) was obtained with the treatment No. 28 (temperature of 35°C; ferulic acid to raffinose molar ratio of 3:1; water content of 3% (v/v); and enzyme amount of 218 enzymatic FAE units). The lowest concentration and bioconversion yield of feruloylated raffinose (4.2 μM, 0.4%) was observed with treatment No. 19 (temperature of 24.2°C; ferulic acid to raffinose molar ratio of 3:1; water content of 3% (v/v); and enzyme amount of 218 enzymatic FAE units).

Table 10. Experimental design of 5-level 4-variable central composite rotatable design[a].

Run No.[b]	Coded values				Feruloylated raffinose (μM)		Bioconversion yield (%)	
	X_1	X_2	X_3	X_4	Experimental	Predicted	Experimental	Predicted
1	0 (35)[c]	0 (3:1)	0 (3)	2.2 (415)	100.1	109.6	10.0	11.0
2	0 (35)	0 (3:1)	0 (3)	0 (218)	110.3	108.9	11.0	10.9
3	0 (35)	0 (3:1)	2.2 (5.2)	0 (218)	55.1	56.0	5.5	5.6
4	0 (35)	2.2 (5.2:1)	0 (3)	0 (218)	42.0	42.6	4.2	4.3
5	1 (40)	1 (4:1)	1 (4)	-1 (127)	39.0	39.9	3.9	4.0
6	0 (35)	0 (3:1)	0 (3)	0 (218)	102.5	108.9	10.2	10.9
7	2.2 (45.8)	0 (3:1)	0 (3)	0 (218)	14.1	15.7	1.4	1.6
8	1 (40)	1 (4:1)	1 (4)	1 (309)	84.5	82.0	8.5	8.2
9	0 (35)	-2.2 (0.8:1)	0 (3)	0 (218)	18.6	22.4	1.9	2.2
10	-1 (30)	-1 (2:1)	-1 (2)	1 (309)	75.0	70.7	7.5	7.1
11	1 (40)	-1 (2:1)	1 (4)	-1 (127)	24.6	24.4	2.5	2.4
12	1 (40)	-1 (2:1)	-1 (2)	-1 (127)	28.6	26.1	2.9	2.6
13	1 (40)	1 (4:1)	-1 (2)	-1 (127)	38.2	41.6	3.8	4.2
14	-1 (30)	1 (4:1)	-1 (2)	1 (309)	73.5	73.6	7.4	7.4
15	1 (40)	1 (4:1)	-1 (2)	1 (309)	88.3	83.8	8.8	8.4
16	-1 (30)	1 (4:1)	1 (4)	-1 (127)	27.0	29.7	2.7	3.0
17	1 (40)	-1 (2:1)	1 (4)	1 (309)	67.4	66.5	6.7	6.6
18	1 (40)	-1 (2:1)	-1 (2)	1 (309)	71.0	68.3	7.1	6.8
19	-2.2 (24.2)	0 (3:1)	0 (3)	0 (218)	4.2	7.2	0.4	0.7
20	0 (35)	0 (3:1)	-2.2 (0.8)	0 (218)	56.3	59.9	5.6	6.0
21	0 (35)	0 (3:1)	0 (3)	0 (218)	103.5	108.9	10.4	10.9
22	0 (35)	0 (3:1)	0 (3)	0 (218)	110.2	108.9	11.0	10.9
23	-1 (30)	1 (4:1)	-1 (2)	-1 (127)	34.8	31.4	3.5	3.1
24	-1 (30)	-1 (2:1)	1 (4)	-1 (127)	23.0	26.8	2.3	2.7
25	0 (35)	0 (3:1)	0 (3)	0 (218)	112.7	108.9	11.3	10.9
26	0 (35)	0 (3:1)	0 (3)	0 (218)	108.0	108.9	10.8	10.9
27	-1 (30)	-1 (2:1)	-1 (2)	-1 (127)	28.0	28.5	2.8	2.9
28	0 (35)	0 (3:1)	0 (3)	0 (218)	114.5	108.9	11.4	10.9
29	0 (35)	0 (3:1)	0 (3)	-2.2 (21)	23.3	18.3	2.3	1.8
30	-1 (30)	1 (4:1)	1 (4)	1 (309)	75.5	71.8	7.6	7.2
31	0 (35)	0 (3:1)	0 (3)	0 (218)	106.2	108.9	10.6	10.9
32	-1 (30)	-1 (2:1)	1 (4)	1 (309)	76.8	68.9	7.7	6.9

[a] Experimental feruloyl NDO productions are averages of duplicates within ±5% error.

[b] Numbers were run in random order.

[c] Number in parenthesis represent actual experimental amounts.

The best-fitting model was determined by multiple regression analysis using the software Design-Expert version 8.0.2. The models were compared and evaluated for significance (*F* values, *P* values, lack of fit, and R^2 values). The results (Table 11) show that the quadratic model was statistically more suitable for the description of the FAE-catalyzed feruloylation reaction of raffinose with $P < 0.0001$, no lack of fit ($P = 0.2287$) and coefficient R^2 of 0.9787. Similarly, Yuan et al. (2006) showed that the production of feruloyl oligosaccharides from wheat bran by xylanases from *B. subtilus* was most suitably described with a quadratic polynomial model. Moreover, Panagiotou et al. (2007) demonstrated the essential role of FAEs for the hydrolysis of the plant cell wall using a quadratic model. While, Barberousse et al. (2009) used a Box–Behnken design to evaluate the extraction yield of ferulic acid from wheat bran by FAE and xylanase.

The analysis of variance (ANOVA) for the CCRD is shown in Table 12. The highest *F* value of 102.9 implies that the model was significant ($P = < 0.0001$). The regression analysis revealed a coefficient of determination (R^2) value of 0.9883, which indicates that the model has a high significance to present the relationship between the responses and the variables. In addition, the non-significant "lack of fit *F* value" of 1.8 ($P > 0.05$) indicates that the quadratic polynomial model satisfied all of the design points. As a result, the computed well fitting models for the bioconversion yield and the concentration of the produced feruloylated raffinose were considered for further analysis.

According to the established models, the substrate molar ratio (*F* value of 20.7) and the enzyme amount (*F* value of 426.1) were the most significant model linear terms, affecting importantly the feruloylation of raffinose by FAE; while the water content (*F* value of 0.8) and temperature (*F* value of 3.6) had no significant effect on the investigated feruloylation reaction. However, all quadratic terms of variables (X_1^2, X_2^2, X_3^2, X_4^2) ($P \leq 0.05$) were highly significant. In addition, the positive signs of all interactive term coefficients (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4) indicate the synergistic effects of the variables. However, among the four variables, only reaction temperature (X_1) and substrate molar ratio (X_2) showed a significant interaction effect.

Table 11. Sequential model sum of squares [Type I].

Source	Feruloylated raffinose (μM)			Bioconversion yield (%)						
	Sum of squares	df[a]	Mean square	Sum of squares	df	Mean square	*F* value	*P* value[b]	Lack of fit *P* value	R^2
Mean vs Total	129665.4	1	129665.4	1296.2	1	1296.2				
Linear vs Mean	11943.0	4	2985.8	119.3	4	29.8	3.0	0.0347	< 0.0001	0.2076
2FI vs Linear	172.7	6	28.8	1.7	6	0.3	0.0	0.9999	< 0.0001	-0.0121
Quadratic vs 2FI	25974.8	4	6493.7	259.8	4	65.0	245.4	< 0.0001	0.2287	0.9787
Residual	201.8	9	22.4	2.0	9	0.2				
Total	168205.8	32	5256.4	1681.5	32	52.5				

[a] Degree of freedom.
[b] $P < 0.05$ indicates statistical significance.

Table 12. The analysis of variance for response surface quadratic model[ab].

Source	Feruloylated raffinose (μM)			Bioconversion yield (%)				
	Sum of squares	df[c]	Mean square	Sum of squares	df	Mean square	*F* value	*P* value[d]
Model	38090.6	14	2720.8	380.9	14	27.2	102.9	< 0.0001
X_1, Temperature	96.8	1	96.8	1.0	1	1.0	3.6	0.0747
X_2, Substrate molar ratio	543.1	1	543.1	5.5	1	5.5	20.7	0.0003
X_3, Water content	19.4	1	19.4	0.2	1	0.2	0.8	0.3956
X_4, Enzyme amount	11283.7	1	11283.7	112.7	1	112.7	426.1	< 0.0001
X_1X_2	158.9	1	158.9	1.6	1	1.6	6.1	0.0244
X_1X_3	0.1	1	0.1	0.0	1	0.0	0.0	0.9276
X_1X_4	3.2	1	3.2	0.0	1	0.0	0.1	0.7183
X_2X_3	0.3	1	0.3	0.0	1	0.0	0.0	0.9038
X_2X_4	0.6	1	0.6	0.0	1	0.0	0.0	0.8956
X_3X_4	9.6	1	9.6	0.1	1	0.1	0.3	0.5670
X_1^2	16501.3	1	16501.3	165.1	1	165.1	624.2	< 0.0001
X_2^2	10072.6	1	10072.6	100.8	1	100.8	381.1	< 0.0001
X_3^2	4481.5	1	4481.5	44.8	1	44.8	169.6	< 0.0001
X_4^2	3506.3	1	3506.3	35.1	1	35.1	132.7	< 0.0001
Residual	449.9	17	26.5	4.5	17	0.3		
Lack of Fit	323.0	10	32.3	3.2	10	0.3	1.8	0.2287
Pure Error	126.9	7	18.1	1.3	7	0.2		
Cor Total	38540.4	31		385.4	31			

[a] Coefficient of variation = 8.08%.
[b] $R^2 = 0.9883$.
[c] Degree of freedom.
[d] $P < 0.05$ indicates statistical significance.

Neglecting the insignificant terms, the final predictive equations (3 and 4) obtained are as given below:

$$Y_1 \text{ (Esterified glycoside (μM))} = +108.9 + 2.0X_1 + 4.6X_2 - 0.9X_3 + 21.1X_4 + 3.2X_1X_2 - 0.1X_1X_3 - 0.4X_1X_4 + 0.1X_2X_3 - 0.2X_2X_4 + 0.8X_3X_4 - 20.9X_1^2 - 15.8X_2^2 - 10.5X_3^2 - 9.6X_4^2 \quad (3)$$

$$Y_2 \text{ (Bioconversion yield (\%))} = +10.9 + 0.2X_1 + 0.5X_2 - 0.1X_3 + 2.1X_4 + 0.3X_1X_2 - 0.01X_1X_3 - 0.05X_1X_4 + 0.02X_2X_3 - 0.02X_2X_4 + 0.1X_3X_4 - 2.1X_1^2 - 1.6X_2^2 - 1.1X_3^2 - 1.0X_4^2 \quad (4)$$

The model diagnostic plot for the predicted versus actual response values (Y_2) helps to detect a value, or group of values, that are not easily predicted by the model. Figure 7 shows that the data points for the bioconversion yield (0.4-11.4%) were split evenly along the 45° line with a R^2 of 0.9882, which confirmed a good model fit for the FAE-catalyzed feruloylation of raffinose.

4.2.7. Effect of Reaction Parameters

The relationships between reaction parameters and bioconversion yield can be better understood by studying the planned series of contour plots generated from the predicted model (Equation 4). The effects of the most important variables, substrate molar ratio (X_2) and enzyme amount (X_4), for the bioconversion yield of feruloylated raffinose are illustrated in Figure 8 by holding constant the temperature (30, 35, 40°C) and water content (2, 3, 4%, v/v). Figures 8(a), 8(b) and 8(c) represent the same water content (2%, v/v), while Figures 8(a), 8(d) and 8(g) denote the same temperature (30°C). All nine contour plots (Figure 8) display similar trends in that the predicted bioconversion yield of feruloylated raffinose increased with an increase in the ferulic acid to raffinose molar ratio from 1:1 to 3:1; however, above the ferulic acid to raffinose molar ratio of 3:1, the bioconversion yield decreased. The inhibitory effect of an excess of ferulic acid may be due to the enzyme denaturation and/or to an increased probability of substrate-enzyme collision (Rodrigues et al., 2008).

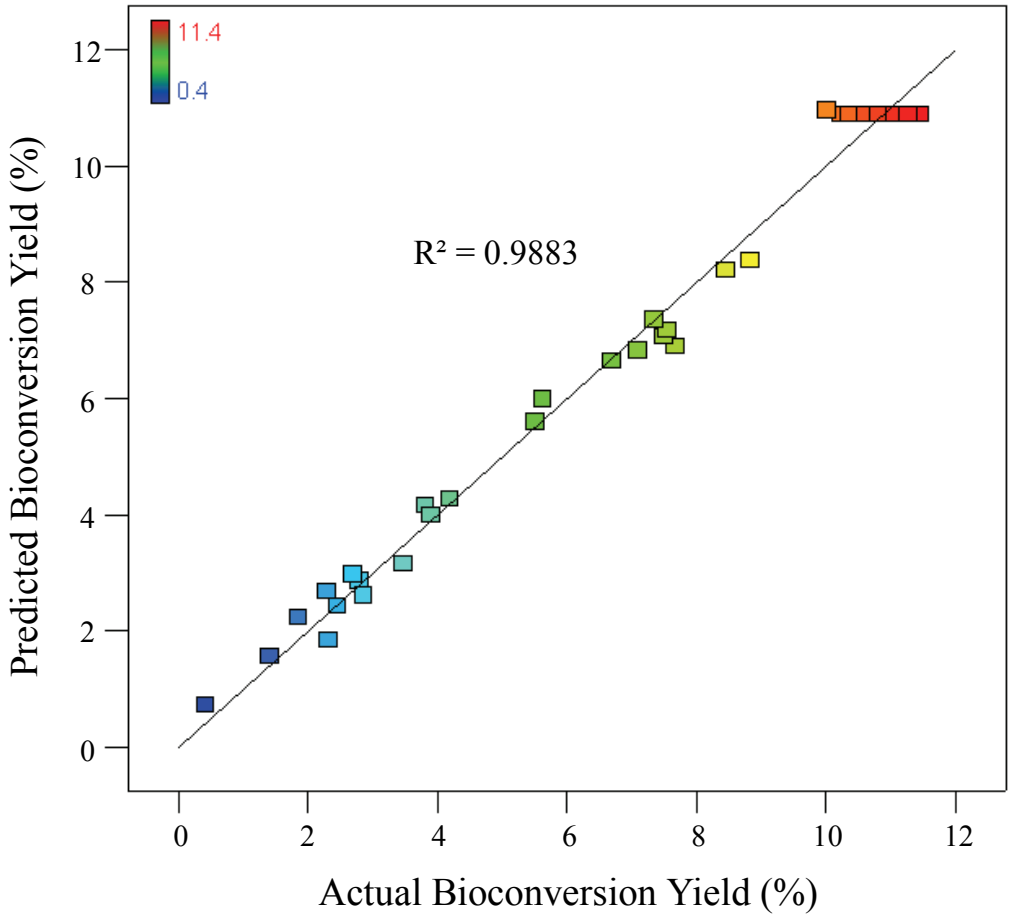


Figure 7. Model diagnostic plot of predicted vs actual bioconversion yield (%) values.

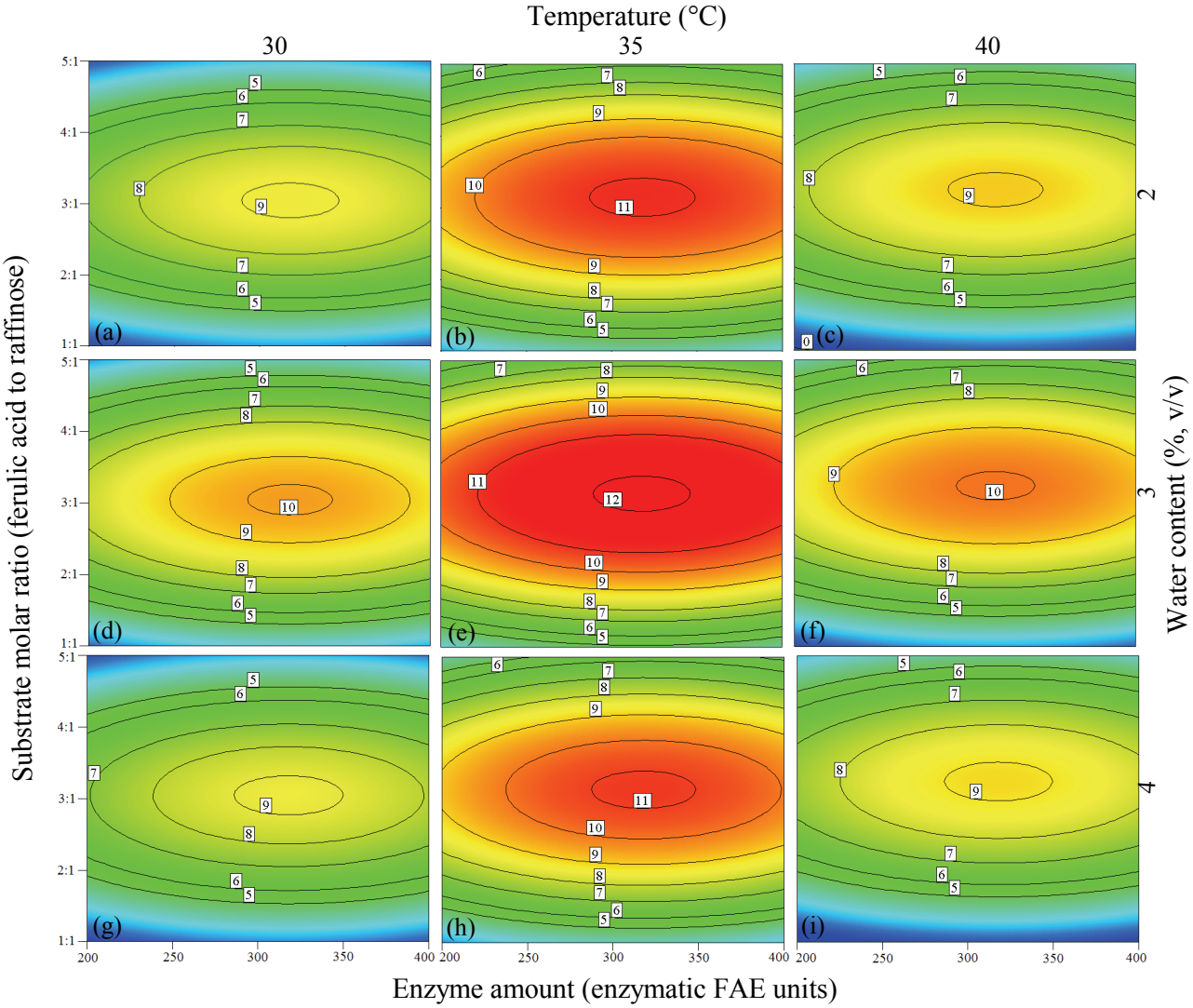


Figure 8. Contour plots of bioconversion yield of feruloylated raffinose by Depol 740L in a surfactant-less organic microemulsion mixture. The numbers inside the contour plots indicate bioconversion yields (%) under given reaction conditions.

The formation of a hydrophobic layer in the enzyme's microenvironment that may have limited the access of the hydrophilic raffinose substrate to the enzyme active site can also be an explanation of the inhibitory effect of high ferulic acid to raffinose molar ratio. Nonetheless, the excess ferulic acid to raffinose stoichiometric molar ratio of 3:1 was required to ensure higher reaction rates and minimize the diffusion limitations (Rodrigues et al., 2008). In fact, a ferulic acid to raffinose stoichiometric molar ratio of 3:1 was also employed by Vafiadi et al. (2006) for the feruloylation of L-arabinobiose by a FAE from *S. thermophile*. Likewise, an increase in enzyme amount resulted in a higher bioconversion yield (Figure 8). The maximum feruloylation yield was obtained in the range of enzymatic FAE units from 289 to 347 at 35°C and using a water content of 3% (v/v) (Figure 8(e)). Beyond this point, additional enzyme amount may have caused diffusion and mass transfer limitation by being present within the reaction mixture without taking part in the esterification process (Gunawan et al., 2005; Radzi et al., 2005). Therefore, the optimum enzyme amount was very important in the synthesis of FAE-catalyzed esterification of feruloylated raffinose.

Figure 8(e) also indicates that the predicted bioconversion yield increased with increasing temperature and water content up to 35°C and 3% (v/v), respectively; however, beyond that, the bioconversion yield decreased. Higher temperatures may have probably denatured Depol 740L, while elevated water contents may have altered the reaction equilibrium by mass action effect and promoted the hydrolysis (Rodrigues et al., 2008). A similar reaction temperature (37°C) was employed by Vafiadi et al. (2008) for the transesterification of methyl ferulate with 1-butanol by the free and immobilized Depol 740L.

4.2.8. Optimal Conditions

The optimal conditions of *Humicola* spp. FAE-catalyzed esterification of feruloylated raffinose were estimated via the numerical optimization of the Design-Expert 8.0.2 software. The coded optimum values were: $X_1 = 0$; $X_2 = 0$; $X_3 = 0$; $X_4 = 1.1$. The uncoded 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 3D response surface plot (Figure 9) shows the effect of enzyme amount and substrate molar ratio on the bioconversion yield of feruloylated raffinose, keeping fixed the other variables at the optimum level. The flag (12%) represents the optimal bioconversion yield using the parameters represented on the axis.

4.2.9. Time Course and Model Verification

Figure 10 illustrates the time course of FAE-catalyzed esterification of feruloylated raffinose under the estimated optimal conditions. The results show an increase in the bioconversion yield of the esterified feruloylated raffinose within the first 4 days of reaction time to a value of 11.5% (115 μM); thereafter, the bioconversion yield of the esterified feruloylated raffinose increased to a lesser extent up to a maximum of 11.9% (119.4 μM) after 7 days of reaction. These results are in agreement with the reaction progress for the FAE-catalyzed transesterification of methyl ferulate with L-arabinose and L-arabinobiose (Topakas et al., 2005a; Vafiadi et al., 2005, 2006). Moreover, the results obtained from real experiments demonstrated the validation of the RSM model. The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization (Table 13).

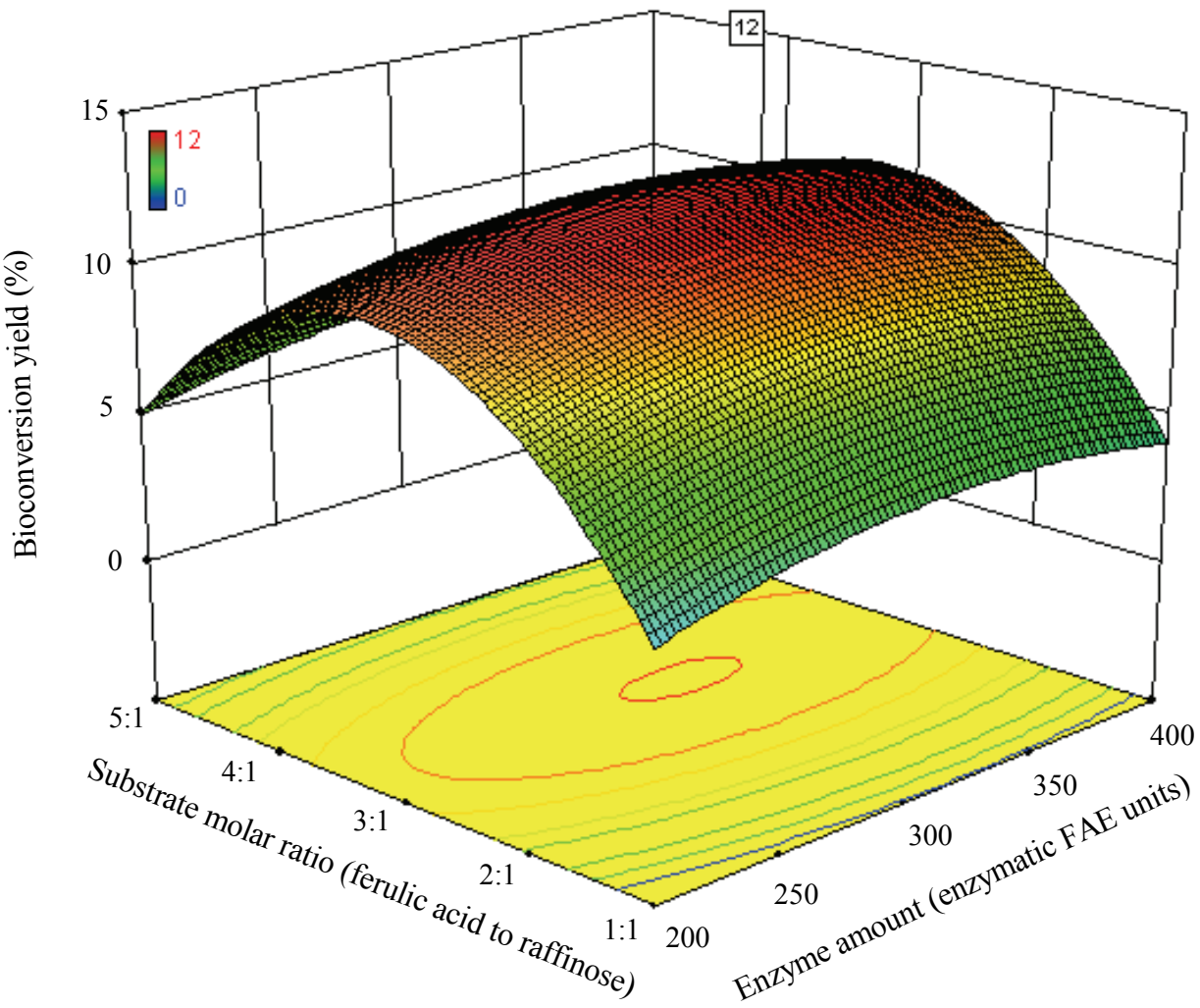


Figure 9. Response surface plot showing the effect of enzyme amount (enzymatic FAE units) and ferulic acid to raffinose molar ratio on the bioconversion yield of feruloylated raffinose. Other variables are constant at their zero levels as follows: temperature, 35°C; and water content, 3% (v/v).

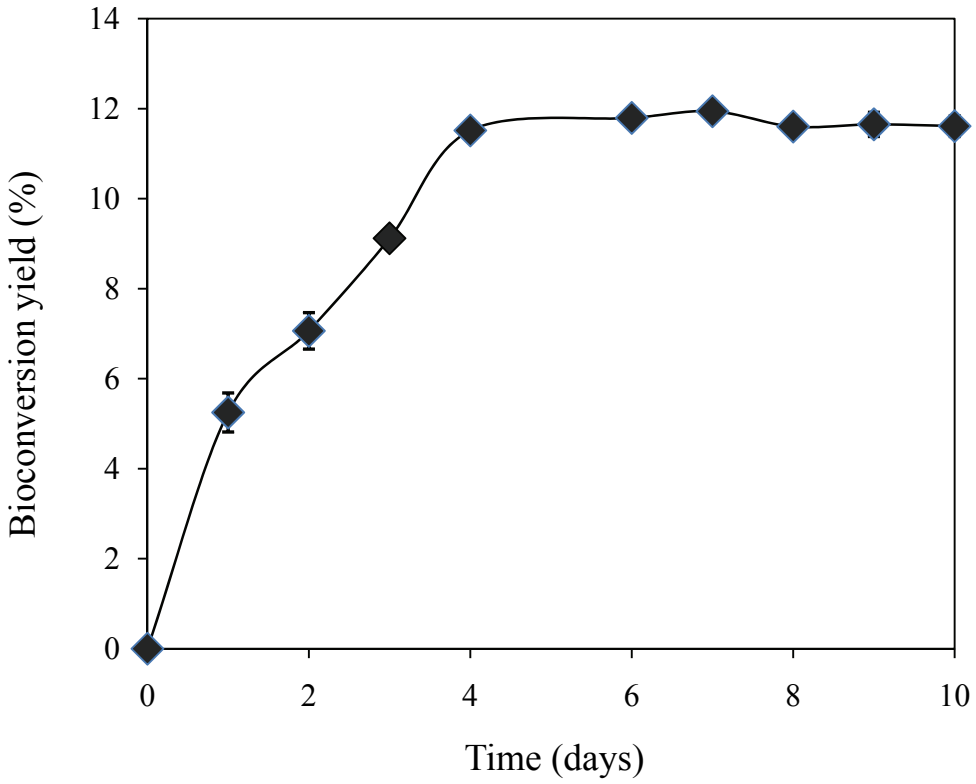


Figure 10. Time course for feruloyl esterase-catalyzed esterification of feruloylated raffinose under optimum conditions.

Table 13. Optimum conditions, predicted and experimental values of responses at that condition.

Optimum condition			Feruloylated raffinose (μM)		Bioconversion yield (%)	
	Coded	Uncoded	Experimental	Predicted	Experimental	Predicted
Temperature (ºC)	0	35	119.4	119.7	11.9 (± 0.2)[a]	12.0
Substrate molar ratio (ferulic acid to raffinose)	0	3:1				
Water content (%, v/v)	0	3				
Enzyme amount (enzymatic FAE units)	1.1	345				

[a] Data are average of two determinations ± standard deviation.

CHAPTER 5
CONCLUSION

The present study has demonstrated the potential of selected FAEs expressed in the multi-enzymatic preparations from different microbial sources for the feruloylation of various monosaccharides in a surfactant-less organic microemulsion system composed of n-hexane, 2-butanone or 1-butanol and MES-NaOH buffer mixture (51:46:3, v/v/v). The highest bioconversion yields of feruloylated galactose and xylose were obtained using FAEs expressed in Depol 670L from *T. reesei*, while that of Multifect P 3000 from *B. amyloliquefaciens* led to the highest bioconversion yield of feruloylated arabinose. The evaluation of free-radical scavenging activity of feruloylated monosaccharides revealed that feruloylated galactose and feruloylated arabinose were the most and the least potent scavengers, respectively.

The enriched FAE expressed in the multi-enzymatic preparation Depol 740L from *Humicola* spp. proved to be effective for the feruloylation of various di- and NDOs. Nevertheless, higher maximum bioconversion yields were obtained with the di- and trisaccharide composed of hexoses (sucrose, raffinose and galactobiose) as compared to the pentose-based disaccharides (arabinobiose, xylobiose and XOS). The effect of substrates and reaction media on the bioconversion yield revealed that raffinose and the surfactant-less organic microemulsion medium composed of n-hexane, 2-butanone and MES-NaOH buffer mixture (51:46:3, v/v/v) were the most appropriate. In addition, the evaluation of free-radical scavenging activity of feruloylated glycosides revealed that the feruloylated raffinose showed the highest scavenging affinity towards the free radical $DPPH^{\bullet}$. Moreover, the chemical structure of the feruloylated raffinose was confirmed by APCI-MS.

According to the numerical RSM optimization studies, substrate molar ratio and enzyme amount were the most important parameters for the bioconversion yield of feruloylated raffinose. However, among the four variables, only reaction temperature and substrate molar ratio showed a significant interaction effect. Comparison of predicted and experimental values showed good correspondence, implying that the empirical model derived from RSM can be used to effectively describe the relationship between the

reaction parameters and the responses in the FAE-catalyzed esterification of feruloylated raffinose.

In conclusion, the overall experimental findings could be considered as an important step in the development of the biocatalytic approach for the synthesis of a wider selection of phenolated NDOs. Future work should look into using different NDOs as substrates and optimizing the immobilization of FAEs present in Depol 740L in order to provide increased robustness and substantially increase its esterifying activity.

CHAPTER 6
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