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Optimization of feruloyl esterase-catalyzed synthesis of feruloylated oligosaccharides by response surface methodology

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ABSTRACT

The feruloyl esterase expressed in Depol 740L from *Humicola* spp. exhibited esterifying activity for the feruloylation of selected di- and oligosaccharides in a surfactant-less microemulsion medium composed of n-hexane, 2-butanone and MES–NaOH buffer (51:46:3, v/v/v). As compared to their corresponding ferulic acid, the feruloylated di- and oligosaccharides demonstrated similar or higher potential radical scavenging properties. By varying the media composition, the highest bioconversion yields were obtained in the n-hexane, 2-butanone and MES–NaOH buffer mixture using arabinobiose (8%), xylobiose (9%) and raffinose (11%) as 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. The chemical structure of the feruloylated di- and oligosaccharides was confirmed by APCI-MS. Response surface methodology, based on a 5-level and 4-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.

1. Introduction

Feruloyl esterases (FAEs, EC 3.1.1.73), a subclass of the carboxylic acid esterases, catalyze the cleavage of ester bonds between hydroxycinnamic acids and glycosides in plant cell walls [1,2]. FAEs are expressed in many multi-enzymatic preparations in which they act synergistically for the biodegradation of plant cell wall polysaccharides [3]. Although numerous studies have been reported on FAEs, only limited numbers have investigated the ability of FAEs as biocatalysts for the esterification of glycosides with phenolic acids in non-conventional media [4–7]. Indeed, FAE-catalyzed esterification reaction of oligosaccharides with phenolic acids is a very potential approach for the synthesis of phenolated oligosaccharides. In fact, the feruloylated oligosaccharides from plant cell walls have shown complementary functional properties, both stimulating the growth of *Bifidobacterium* and protecting against oxidative damage [8,9]. These bioactive molecules might open a new effective pathway of delivering phenolic compounds to the colon and thereby reduce the risk of chronic diseases in the distal intestinal region.

The acylation of glycosides with phenolic acids has been little studied and limited to the use of mono- and disaccharides [4–6]. In our previous study, the efficiency of FAEs expressed in different multi-enzymatic products to catalyze the esterification of ferulic acid with various monosaccharides in surfactant-less organic microemulsion systems was investigated [7]. FAEs expressed in Depol 740 from *Humicola insolens*, Multifect P 3000 from *Bacillus amyloliquefaciens* and Depol 670L from *Trichoderma reesei* displayed higher esterification abilities for the feruloylation of arabinose, galactose and xylose in reaction mixtures of n-hexane, 1-butanol or 2-butanone and 3-(*N*-morpholino)ethanesulfonic acid (MES)–sodium hydroxide (NaOH) buffer (51:46:3, v/v/v) [7]. As far as the authors are aware, only one example of chemoenzymatic esterification of arabino-oligosaccharides, containing three to six arabinofuranose units, with ferulic acid has been reported [10].

Finding the most appropriate reaction medium and parameters that favour the modulation of FAE properties towards the synthesis is a key step in the enzymatic acylation of oligosaccharides with phenolic acids. Because the reaction media have the ability to alter the reaction equilibrium, non-conventional systems, with low water activity, are more suited for the synthetic reactions [11]. In addition to the ability of non-conventional media to control thermodynamically the unfavorable hydrolytic reaction, they also increase the solubility of substrates and facilitate the product recovery [12]. Emulsion systems, such as surfactant-less and ionic liquid (IL)-based emulsions, have gained interest because they

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often do not inactivate enzymes, simplifying reactions involving polar substrates [4,13].

The aim of the present work was to investigate the effect of glycoside structures and reaction media on the bioconversion yield of feruloylated glycosides. The free radical scavenging activity of the synthesized feruloylated di- and oligosaccharides were assessed and their chemical structures were confirmed by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The enzymatic synthesis of feruloylated raffinose via FAE-catalyzed esterification was optimized by investigating the relationship between reaction parameters (temperature, substrate ratio, water content, enzyme amount) and bioconversion yield. The optimal conditions were determined using response surface methodology (RSM).

2. Experimental

2.1. Chemicals

Depol 740L commercial multi-enzymatic preparation was obtained from Biocatalysts Limited (Wales, UK). L-Arabinobiose, L-galactobiose and L-xylobiose were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Fructooligosaccharide (FOS) and xylooligosaccharide (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 plate, dialysis tubing, ammonium sulfate ($(NH_4)_2SO_4$), bovine serum albumin, NaOH, and high performance liquid chromatography (HPLC) grade acetic acid, acetonitrile, chloroform, methanol, and n-hexane were purchased from Fisher Scientific (Fair Lawn, NJ). Folin Ciocalteu's Phenol Reagent, sulfuric acid, and all other chemicals were purchased from Sigma Chemical Co. (St-Louis, MO).

2.2. 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 [14]. Prior to both ultrafiltration and ammonium sulfate precipitation (516 g/L), Depol 740L (15 mL) was diluted in a 45 mL MOPS buffer (100 mM, pH 6.0). Depol 740L was ultrafiltrated 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 retentate for 24 h. The ammonium sulfate precipitation was carried out at 8 °C under gently stirring for 2 h. The enriched fraction containing FAE activity was 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 (2 mL, 100 mM, pH 6.0). The enriched multi-enzymatic product was dialysed against MOPS buffer (5 mM, pH 6.0) using a membrane with a cut off of 6–8 kDa for 48 h-period, followed by freeze-drying for 24 h.

2.3. Feruloyl esterase activity assay

The hydrolytic activity of FAE expressed in the enriched multi-enzymatic preparation was assayed using methyl ferulate as substrate, according to a modification of the method outlined by Bunzel et al. [15]. Prior to the enzymatic reaction, a stock solution of methyl ferulate (33 μM) was prepared in MOPS buffer at 100 mM and pH 6.0. 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 enriched Depol 740L at appropriate dilutions (0.44–11.3 mg protein) to 0.7 mL of substrate solution. The concentration of the consumed feruloylated substrate was monitored spectrophotometrically at 335 nm over a reaction period of 10 min using a DU-650 spectrophotometer (Beckman Instruments Inc., San Raman, CA) equipped with a thermocontrolled sample compartment. Reaction rate was calculated from the slope of the curve absorbance versus time using a molar extinction coefficient of 3940 1/M cm for the esterified ferulic acid. 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.

2.4. Esterification reaction

Enzymatic synthesis of feruloylated di- and oligosaccharides was carried out using a surfactant-less organic microemulsion as reaction medium, according to the method reported by Couto et al. [7]. Prior to the enzymatic reaction, a stock solution of ferulic acid (30 mM) was prepared in 2-butanone, while those of di- and oligosaccharides (100 mM) were prepared in MES–NaOH buffer (20 mM, pH 6.0). Defined amounts of substrate stock solutions were diluted with sufficient amount of n-hexane to obtain a final substrate molar ratio of phenolic acid to di- or oligosaccharide 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 8.9–14.1 mg proteins of the enriched Depol 740L. 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 triplicate. At defined time intervals over a 3- and 6-day period, the reaction mixtures were recovered and concentrated 10 times using a speed vacuum evaporator. The concentration of the formed feruloylated di- and oligosaccharides was quantified by HPLC.

2.5. Analysis of the reaction components of di- and oligosaccharide feruloylation

Quantitative analysis of reaction mixtures of di- and oligosaccharide feruloylation was carried out, according to a modification of the modified method of Couto et al. [7]. 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 oligosaccharide standards. The bioconversion yield (%) was calculated from the concentration of the synthesized feruloylated di- and oligosaccharides divided by the initial concentration of di- and oligosaccharides, multiplied by 100%.

The molecular structures of feruloylated di- and oligosaccharides were characterized using HPLC interfaced to 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 200–1500, 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.

2.6. Effect of glycoside structure on bioconversion yield

To study the effect of glycoside structure on the bioconversion yield of the feruloylated glycosides, selected di- and oligosaccharides were investigated as glycoside substrates. The disaccharides, including L-arabinobiose, L-galactobiose, L-xylobiose, α-lactose, and sucrose were investigated as substrates for the synthesis of feruloylated disaccharides. The synthesis of feruloylated oligosaccharides was studied using D-raffinose, FOSs and XOSs as glycoside substrates. The enzymatic synthesis was carried out using a surfactant-less organic microemulsion as reaction medium as described previously (Section 2.3).

2.7. Effect of reaction media on bioconversion yield

Selected surfactant-less organic microemulsions and IL-based emulsions were investigated as reaction media for the enzymatic feruloylation of selected di- and oligosaccharides. 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 oligosaccharides, including: n-hexane/1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF_4)/MES–NaOH (51:46:3), 1-butyl-3-methylimidazolium hexafluoro-phosphate ([bmim]PF_6)/2-butanone/MES–NaOH (51:46:3, v/v/v) or [bmim]PF_6/MES–NaOH (97:3).

2.8. Determination of radical scavenging activity

The antioxidant potential of the feruloylated di- and oligosaccharides was estimated by measuring their free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) as free radical according to a modification of the method of Silva et al. [16]. In a 1 mL spectrophotometric cuvette, 0.122 mL of feruloylated di- and oligosaccharides (50 μM) or its corresponding ferulic acid was added to 1.378 mL of a DPPH$^\bullet$ ethanolic solution (0.1 mM). Feruloylated di- and oligosaccharides were isolated according to the HPLC method described in Section 2.5. 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 di- and oligosaccharides, while the scavenging yield was calculated as the absorbance of the DPPH$^\bullet$ control (517 nm), minus the absorbance of the sample divided by that of the DPPH$^\bullet$ control, multiplied by 100.

2.9. Experimental design

Optimization of the feruloylated raffinose bioconversion yield was achieved by using RSM, according to a modification of the method described by Yuan et al. [17]. 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, 30, 35, 40, 45.8 °C), X_2 substrate molar ratio (0.8, 2:1, 3:1, 4:1, 5.2:1; ferulic acid to raffinose), X_3 water content (0.8, 2, 3, 4, 5.2%, v/v), and X_4 enzyme amount (21, 127, 218, 309, 415 enzymatic FAE units (μmol/min)).

2.10. 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).

$$Y_i = a_0 + a_iX_1 + a_iX_2 + a_iX_3 + a_iX_4 + a_{ij}X_1X_2 + a_{ii}X_1^2 + a_{ii}X_2^2 + a_{ii}X_3^2 + a_{ii}X_4^2 + \varepsilon \quad (1)$$

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.

3. Results and discussion

3.1. Enrichment of selected multi-enzymatic preparation with feruloyl esterase activity

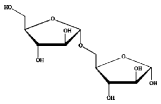
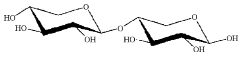
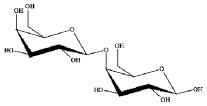
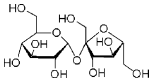
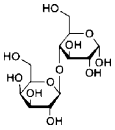
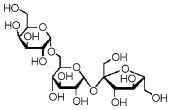
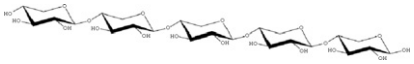
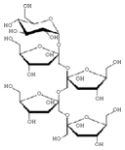
In our previous work [7], FAEs expressed in selected multi-enzymatic products have shown ability to catalyze the feruloylation of arabinose, galactose and xylose in selected surfactant-less organic microemulsion media. However, in a pre-screening study, FAE expressed in Depol 740 from *H. insolens* was determined as the most appropriate multi-enzymatic preparation for the enzymatic feruloylation of di- and oligosaccharides (data not shown). Two different separation techniques, including ammonium sulfate precipitation and ultrafiltration, were investigated to enrich the selected multi-enzymatic preparation Depol 740L with FAE activity. The multi-enzymatic product Depol 740L exhibited a FAE activity of 405.9 μmol/mL min. Lower FAE activity of Depol 740L on methyl ferulate (10.5 μmol/mL min) was reported by Vafiadi et al. [18].

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 [19]. However, the ammonium sulfate precipitation resulted in an enrichment factor and activity yield of 2.5 and 143%, respectively (data not shown). Vafiadi et al. [18] 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. [20] reported that gradual precipitation with $(NH_4)_2SO_4$ resulted in a slightly higher enrichment fold (2.1) of a polyphenol esterase from *Aspergillus niger*.

3.2. 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 oligosaccharides) via esterification reaction was investigated

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

Glycoside substrates	Structures	Bioconversion yield[a]
Arabinobiose		7.9 (±1.1)[b]
Xylobiose		9.4 (±0.0)
Galactobiose		5.4 (±6.0)
Sucrose		13.2 (±0.6)
Lactose		4.4 (±0.6)
Raffinose		11.0 (±2.8)
XOS		2.8 (±0.4)
FOS		9.6 (±0.6)

[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 determinations ± standard deviation.

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 [7] to be an appropriate reaction medium for the enzymatic feruloylation of monosaccharides. The results (Table 1) show that the bioconversion yield of feruloylated di- and oligosaccharides was low and varied from 3 to 13%. These values are lower than those reported in our previous study [7] for the feruloylation of monosaccharides (3–61%). These results indicate that FAE from *Humicola* spp. exhibited higher substrate specificity towards the esterification of monosaccharides as compared to their corresponding disaccharides. In addition, the electronic distribution and the steric hindrance of the enzyme/substrate complex may have been different for the mono- and disaccharides. However, a similar bioconversion yield of 13% was obtained by Topakas et al. [11] for the transesterification of methyl ferulate with 1-butanol catalyzed by a *Fusarium oxysporum* FAE. Only one study has carried out the transferuloylation of the disaccharide L-arabinobiose by FAE from *Sporotrichum thermophile* and reported a similar bioconversion yield of 10–18% [6].

The overall results (Table 1) reveal that the bioconversion yield of feruloylated glycosides is dependent on the structural characteristics of di- and oligosaccharides, in particular the type of hexose/pentose moiety, the length and the glycosidic linkages. Interestingly, higher bioconversion yields of 11–13% were obtained with the di- (sucrose) and trisaccharide (raffinose) composed of hexose (galactose, fructose, glucose) moieties. Increasing the chain length of fructose units in FOSs resulted in a decrease of bioconversion yield from 13.2 to 9.6%. 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 9.4% with xylobiose to 2.8% with XOSs.

3.3. Determination of radical scavenging activity of feruloylated di- and oligosaccharides

The free radical scavenging activity of the synthesized feruloylated di- and oligosaccharides as well as that of their corresponding ferulic acid were investigated using DPPH$^{\bullet}$ as free radical. Table 2 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 towards the scavenging of 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 oligosaccharides 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 ability of ferulic acid to donate hydrogen to DPPH$^{\bullet}$ and to stabilize the phenoxy radical upon its conformational modification with di- and oligosaccharides. In contrast, Katapodis et al. [21] reported that the isolated feruloylated arabinoxylo-oligosaccharides displayed a reduced specificity towards DPPH$^{\bullet}$ as compared to ferulic acid.

Table 2
Scavenging activity of the ferulic acid and the feruloylated di- and oligosaccharides using DPPH• 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] 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• control, at 517 nm, minus the absorbance of the sample divided by that of the DPPH• control, multiplied by 100.
[d] Data are average of three determinations ± standard deviation.

Table 3
Effect of media on bioconversion yield of selected feruloylated di- and oligosaccharides obtained through feruloyl esterase-catalyzed esterification reaction using Depol 740L from *Humicola* spp.

Media mixture[a]	Log *P* value[b]	Substrates[c]			
		Arabinobiose	Xylobiose	Galactobiose	Raffinose
n-Hexane/2-butanone/MES–NaOH	1.8	7.9 (±1.1)[d]	9.4 (±0.0)	5.4 (±6.0)	11.0 (±2.8)
n-Hexane/1,4-dioxane/MES–NaOH	1.6	<0.01	4.2 (±7.3)	26.8 (±3.7)	3.1 (±3.1)
n-Hexane/[bmim]BF_4[e]/MES–NaOH	1.3	0.2 (±0.0)	0.8 (±0.0)	0.4 (±0.0)	-[g]
[bmim]PF_6[f]/2-butanone/MES–NaOH	−0.3	0.3 (±0.1)	0.1 (±0.1)	0.1 (±0.1)	0.5 (±0.1)
[bmim]PF_6/MES–NaOH, 97:3 (v/v)	−0.8	2.8 (±0.4)	4.2 (±4.0)	1.0 (±0.2)	2.0 (±0.7)

[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 = X_1 log *P* + X_2 log P_2 + X_3 log P_3, in which X_1, X_2 and X_3 are the mole fractions of media 1, 2 and 3.
[c] Maximum bioconversion yield (%) after 144 h.
[d] Data are average of two determinations ± standard deviation.
[e] 1-Butyl-3-methylimidazolium tetrafluoroborate.
[f] 1-Butyl-3-methylimidazolium hexafluorophosphate.
[g] Not determined.

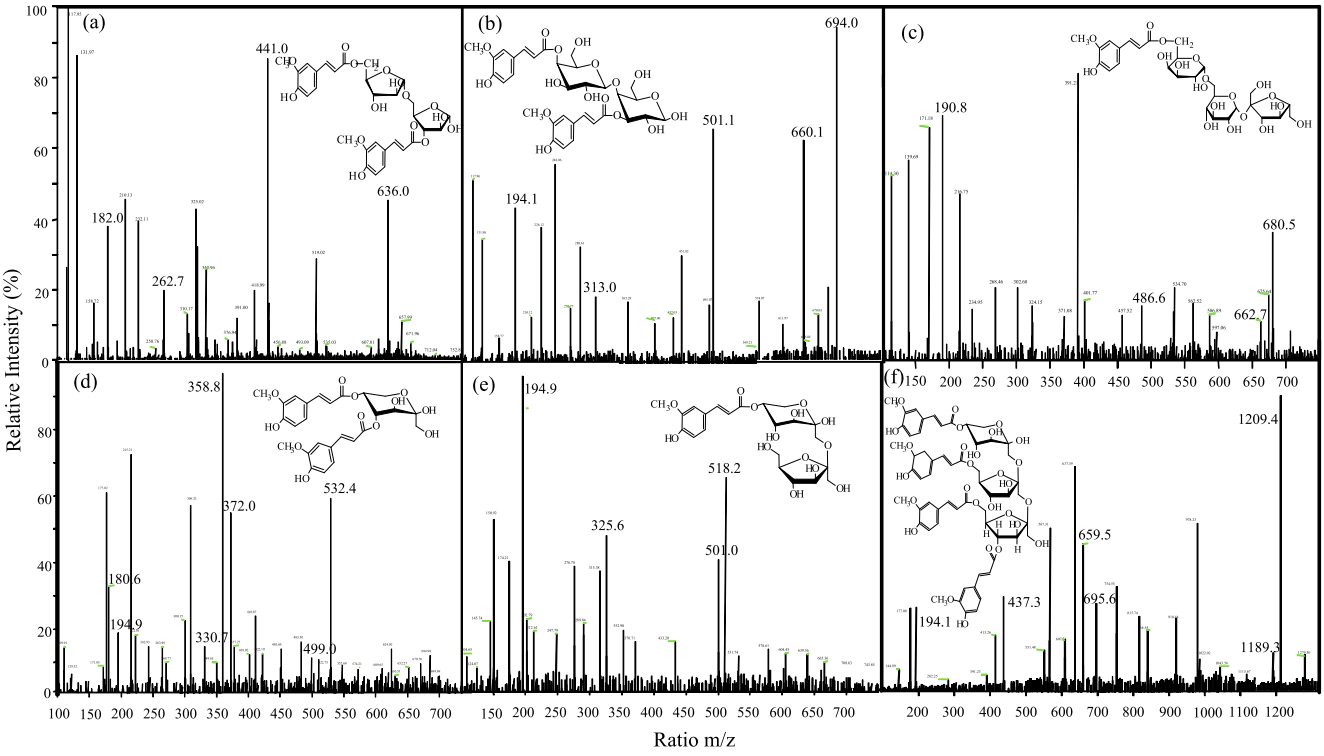


Fig. 1. APCI-MS spectra of the fragmentation patterns of the feruloylated glycosides obtained by feruloyl esterase-catalyzed esterification of ferulic acid with arabinobiose (a), galactobiose (b), raffinose (c), and FOSs (d, e, and f).

The scavenging activity of ferulates seems to be dependent on the chemical structure of the glycosides (Table 2). The acylation of ferulic acid with hexoses (galactobiose, sucrose, lactose, raffinose, and FOS) resulted in higher scavenging activity as compared with pentoses (arabinobiose, xylobiose and XOS). These results could be explained by the effect of the steric hindrance of the glycosidic substituents on the rotation degree of the phenyl moiety [16]. Similarly, in our previous study [7], the esterification of ferulic acid with the pentoses, xylose and arabinose, showed the lowest scavenging activity of 2.1 and 2.3 Ab at 517 nm/min mL, respectively.

Although feruloylated XOSs exhibited the lowest scavenging activity, it resulted in the most potent scavenger with a yield of 94% (Table 2). 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 [7]. 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 [7].

3.4. Effect of reaction media on the bioconversion yield

The FAE-catalyzed esterification of selected feruloylated di- and oligosaccharides 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 oligosaccharides and bioconversion yield are shown in Table 3. 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 26.8% 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 11.0% 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 3) 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 (7.9%), xylobiose (9.4%) and raffinose (11.0%) as glycoside substrates. However, using galactobiose as substrate, the highest bioconversion yield (26.8%) 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 1,4-dioxane or the IL [bmim]BF_4 resulted in a significant decrease of the bioconversion yield of feruloylated arabinobiose, xylobiose and raffinose from 7.9–11.0% to 0.01–3.1% (Table 3). Such results may be due to the 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 [22]. 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 5.4–11.0% 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. [23] reported that a type A FAE from *A. niger* 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. [24] 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 3) 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 [7]. The overall findings (Table 3) 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. [25] 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 [26]. Such effect seems also to affect the substrate specificity of FAEs showing different solvent effect patterns [27]. 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.

3.5. Structural characterization of selected feruloylated di- and oligosaccharides

In order to characterize the molecular structure of the synthesized feruloylated di- and oligosaccharides, further analysis of the eluting peaks by APCI-MS spectrometry in the positive ion mode was conducted (Fig. 1). The positions of the phenolic group on the glycoside backbone of the feruloylated di- and oligosaccharides are unknown and arbitrarily depicted. The fragmentation pattern in Fig. 1(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 (Fig. 1(b)) of the product obtained with galactobiose produced abundant molecular ions at *m*/*z* 660.1 $[M+2H-2H_2O]^+$ and 694.0 $[M]^+$, which are characteristics of diferuloylated galactobiose, as well as three fragment ions at *m*/*z* 501.1 $[M+H-H_2O]^+$ representing monoferuloylated galactobiose and 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 (Fig. 1(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 (Fig. 1(d–f)) also confirmed the enzymatic synthesis of selected feruloylated oligosaccharides upon reaction with FOSs, including diferuloylated fructose, feruloylated difructose and tetraferuloylated trifructose. The fragmentation pattern in Fig. 1(d) shows abundant molecular ions at *m*/*z* 499.0 $[M+H-H_2O]^+$ and 532.4 $[M]^+$ representing diferuloylated fructose and at *m*/*z* 330.7 $[M+H-H_2O]^+$ and 358.8 $[M]^+$ corresponding to monoferuloylated fructose as well as two fragment ions at *m*/*z* 180.6 and 194.9, which correspond to fructose and ferulic acid, respectively. On the other hand, the fragmentation pattern of feruloylated difructose (Fig. 1(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 Fig. 1(f) shows abundant molecular ions at *m*/*z* 659.5 $[M+2H-2H_2O]^+$ and 695.6 $[M]^+$ corresponding to diferuloylated difructose and 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 oligosaccharides by the enriched FAE-catalyzed esterification of ferulic acid with the selected glycosides. Only Vafiadi et al. [10] has characterized the feruloylated glycoside

Table 4
Experimental design of a 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.

end product of FAE-catalyzed transesterification of methyl ferulate with linear arabino-oligosaccharides, containing three to six arabinofuranose units.

3.6. Model fitting and analysis of variance

In order to better understand the relationships between the parameters of the FAE-catalyzed esterification of oligosaccharide with ferulic acid, and to optimize these parameters, the highly converted trisaccharide raffinose in the surfactant-less organic microemulsion mixture containing n-hexane, 2-butanone and MES–NaOH was used as glycoside substrate. Hexane and 2-butanone are suitable solvents since they are non-toxic and are permitted for use in the production of edible foods [28]. 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 4).

Table 4 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 bioconversion yield of feruloylated raffinose (114.5 μM, 11.4%) was obtained at 35 °C, ferulic acid to raffinose molar ratio of 3:1 and water content of 3% (v/v) (treatment no. 28). Decreasing the temperature to 24.2 °C resulted in a decrease in the bioconversion yield of feruloylated raffinose (4.2 μM, 0.4%) (treatment no. 19).

To determine the best-fitting model, multiple regression analysis was investigated 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 (data not shown) 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. [17] showed that the production of feruloyl oligosaccharides from wheat bran by xylanases from *Bacillus subtilus* was most suitably described with a quadratic polynomial model. Moreover, Panagiotou et al. [29] demonstrated the essential role of FAEs for the hydrolysis of the plant cell wall using a quadratic model. While, Barberousse et al. [30] 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 5. The highest F value of 102.9 implies that the model was significant ($P \leq 0.0001$). 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

Table 5
The analysis of variance for response surface quadratic model.[a,b]

Source	Feruloylated raffinose (μM)			Bioconversion yield (%)			*F* value	*P* value[d]
	Sum of squares	df[c]	Mean square	Sum of squares	df	Mean square		
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.

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. Neglecting the insignificant terms, the final predictive equations obtained are as given below:

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

$$Y_2\ (\text{Bioconversion yield}\ (\%) = +10.9 + 0.2X_1 + 0.5X_2 - 0.1X_3 + 2.1X_4 + 0.3X_1X_2 - 0.01X_1X_3 - 0.05X_1X_4 + 0.02X_2X_3 - 0.02X_2X_4 + 0.1X_3X_4 - 2.1X_1^2 - 1.6X_2^2 - 1.1X_3^2 - 1.0X_4^2 \quad (3)$$

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. Fig. 2 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.

3.7. Effect of reaction parameters and optimal conditions

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 (Eq. (3)). 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 Fig. 3 by holding constant the temperature (30, 35, 40 °C) and water content (2, 3, 4%, v/v). Fig. 3(a)–(c) represents the same water content (2%, v/v), while Fig. 3(a), (d) and (g) denotes the same temperature (30 °C). All nine contour plots (Fig. 3) 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. Ferulic acid may have lowered the pH of the aqueous phase and thereby reduced enzyme activity. 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 [31]. In fact, a ferulic acid to raffinose stoichiometric molar ratio of 3:1 was also employed by Vafiadi et al. [6] for the feruloylation of L-arabinobiose by a FAE from *S. ther-*

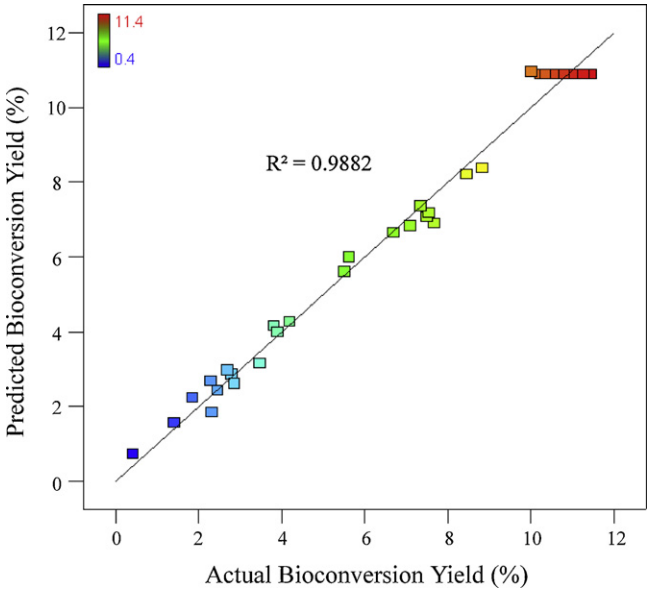


Fig. 2. Model diagnostic plot of predicted vs actual bioconversion yield (%) values.

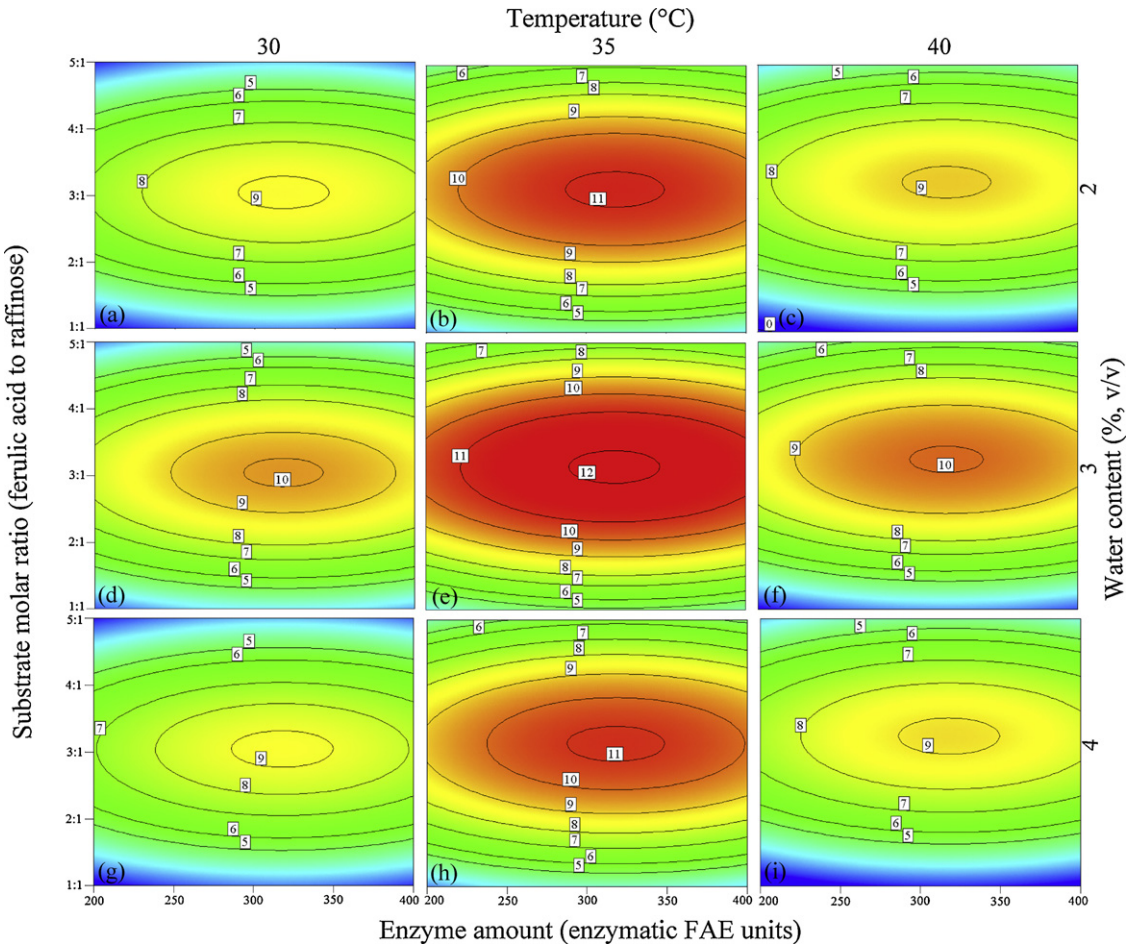


Fig. 3. Contour plots of bioconversion yield of feruloylated raffinose obtained via feruloyl esterase-catalyzed esterification in a surfactant-less organic microemulsion mixture. The numbers inside the contour plots indicate bioconversion yields (%) under given reaction conditions.

mophile. Likewise, an increase in enzyme amount resulted in a higher bioconversion yield (Fig. 3). 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) (Fig. 3(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 [32,33]. Therefore, the optimum enzyme amount was very important in the synthesis of FAE-catalyzed esterification of feruloylated raffinose.

Fig. 3(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 [31]. A similar reaction temperature (37 °C) was employed by Vafiadi et al. [18] for the transesterification of methyl ferulate with 1-butanol by the free and immobilized Depol 740L.

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 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 bioconversion yield of feruloylated raffinose was 12%. The 3D response surface plot (Fig. 4) 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.

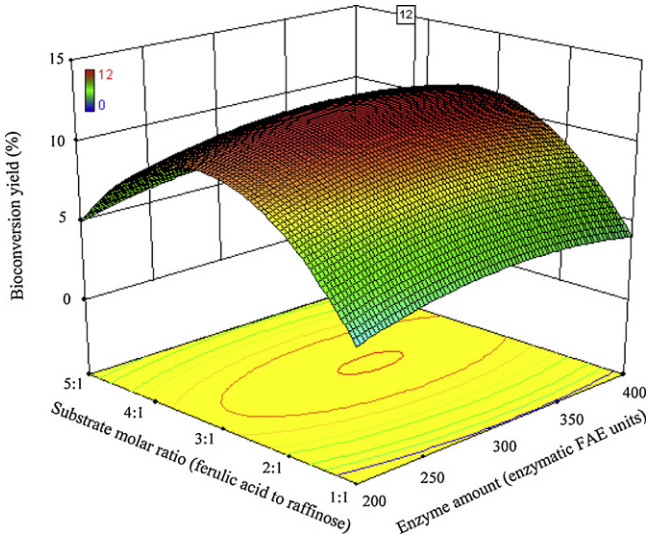


Fig. 4. Response surface plot showing the effect of enzyme amount (enzymatic FAE units) and substrate molar ratio on the bioconversion yield of feruloylated raffinose. Other variables are constant at their zero levels as follows: temperature, 35 °C and water content, 3% (v/v).

The time course results (data not shown) of FAE-catalyzed esterification of feruloylated raffinose under the estimated optimal conditions show an increase in the bioconversion yield of the esterified feruloylated raffinose 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 [4–6]. The overall results obtained from real experiments demonstrated the validation of the RSM model.

4. Conclusions

This study has demonstrated the potential of the enriched FAE expressed in Depol 740L to catalyze the feruloylation of di- and oligosaccharides. Nevertheless, higher bioconversion yields were obtained with the di- and oligosaccharides composed of hexoses as compared to the pentose-based ones. No correlation between the Log *P* value of the reaction media and the esterifying efficiency of FAEs was observed. According to the RSM optimization studies, substrate molar ratio and enzyme amount were the most important parameters for the feruloylation of raffinose. 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 bioconversion yield.

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