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Production of feruloyl oligosaccharides from wheat bran insoluble dietary fibre by xylanases from *Bacillus subtilis*

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Abstract

Feruloyl oligosaccharides production from wheat bran insoluble dietary fibre was carried out using xylanases from *Bacillus subtilis*. Response surface methodology (RSM), based on a five level, five variable central composite rotatable design (CCRD), was employed to obtain the best possible combination of reaction temperature, pH, time, enzyme concentration and substrate (wheat bran insoluble dietary fibre) concentration for maximum feruloyl oligosaccharides production. The optimum reaction conditions were as follows:temperature, 42 °C; pH, 5.2; reaction time, 35 h; enzyme concentration, 4.8 g/l and substrate concentration, 120 g/l. Under these conditions, the experimental yield was 1.55 mM, which is well matched with the predictive yield.
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Keywords: Central composite rotatable design; Feruloyl oligosaccharides; Insoluble dietary fibre; Xylanases

1. Introduction

Wheat bran, as an important by-product of the cereal industry, is produced worldwide in enormous quantities and recognized as a good source of dietary fibre. Wheat bran, from the outer tissues of wheat kernel, is mainly composed of cell wall polysaccharides, among which xylans represent 40% of dry matter (Thiago & Kellaway, 1982). In addition, ferulic acid constitutes about 0.5% (w/w) of the wheat bran. The main phenolic acid in wheat bran cell wall is covalently bound to the cell wall arabinoxylan via the acetylation of an acidic group with the primary hydroxyl at the C5 position of α-L-arabinofuranosyl residues (Hatfield, Ralph, & Grabber, 1999).

Many studies have been performed on the isolation of feruloyl oligosaccharides from Gramineae by mild acid hydrolysis or by treatment with a mixture of polysaccharide hydrolyzing enzymes, such as the fungal hydrolases Driselase (Ishii, 1997; Rhodes, Sadek, & Stone, 2002). The feruloyl oligosaccharides prepared, so far, from graminaceous cell walls have shown good consistency of structure. The α-L-arabinofuranosyl residues are attached to *O*-3 positions of β-1,4-linked D-xylan and are substituted at position *O*-5 with a feruloyl group (Yoshida-Shimokawa, Yoshida, Kakegawa, & Ishii, 2001). The isolation of these feruloyl oligosaccharides has allowed a better understanding of the plant cell wall structures. Furthermore, interest in these oligosaccharides is motivated by their diverse biological activities and their functional applications. They are potential sources of antioxidants with a capacity to inhibit the peroxidation of low density lipoproteins (Ohta, Nakano, Egashira, & Sanada, 1997a; Ohta, Sembokum, Kuchii, Egashira, & Sanada, 1997b) and to protect normal rat erythrocytes against oxidative damage in vitro (Yuan, Wang, & Yao, 2005). Rondini et al. (2004) reported that bound ferulic acid from bran was more bioavailable than the free compound in rat. In the food industry,

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such oligosaccharides are useful, due to their unique gelating properties, induced by oxidative crosslinking (Ishii, 1997; Kroon & Williamson, 1999).

However, efficient production of feruloyl oligosaccharides has been a bottleneck in all their promising applications. In our previous study, treatment of wheat bran insoluble dietary fibre with xylanases from *Bacillus subtilis* released feruloyl oligosaccharides (Yuan et al., 2005). The objective of this present study was to optimise the process for enzymatic production of feruloyl oligosaccharides, using response surface methodology (RSM), employing a five-level, five-variable central composite rotatable design (CCRD).

2. Materials and methods

2.1. Materials

Wheat bran was obtained from a local milling plant in Wuxi, China. The bran was milled and passed through a 0.5 mm sieve. Xylanases from *Bacillus subtilis* were kindly provided by the Sunhy Biology Company (Wuhan, the Peoples Republic of China). Heat-stable α-amylase, Termamyl 120 L (EC 3.2.1.1 from *Bacillus licheniformis,* 120 KNU/g), protease Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g), and amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger,* 300 AGU/g) were from Novo Nordisk (Bagsvaerd, Denmark). Amberlite XAD-2 was obtained from the Rohm and Haas Company (Philadelphia, USA). Birchwood xylan, ferulic acid, arabinose and xylose were purchased from the Sigma Company. All other chemicals and solvents used were of analytical grade.

2.2. Preparation of wheat bran insoluble dietary fibre

Wheat bran (100 g) was autoclaved for 45 min at 121 °C in order to destroy endogenous enzymatic activities (Zilliox & Debeire, 1998) and subsequently swollen at 60 °C for 16 h in water (1000 ml) with continuous stirring. Then, α-amylase (7.5 ml) was added to the suspension. Beakers with 1000 ml wheat bran suspension were heated in a boiling water bath for 40 min and shaken gently every 5 min. The pH was adjusted to 7.5 with 275 mM NaOH, and the samples were incubated with protease (3.0 ml) at 60 °C for 30 min with continuous mild agitation. After the pH had been adjusted to 4.5 with 325 mM HCl, amyloglucosidase (3.5 ml) was added and the mixture was incubated at 60 °C for 30 min with continuous mild agitation. The suspension was centrifuged (10,000*g*, 10 min). The residue was stirred in hot distilled water, washed repeatedly by decantation with large volumes of hot water, and then washed with cold distilled water until no cloudiness was evident. Finally, the residue was washed twice with hot distilled water, 95% (v/v) ethanol and acetone, successively, and then dried at 40 °C overnight in a vacuum oven to get wheat bran insoluble dietary fibre (Bunzel, Ralph, Marita, Hatfield, & Steinhart, 2001). The process described was repeated several times to get sufficient wheat bran insoluble dietary fibre for the production of feruloyl oligosaccharides.

2.3. Enzymes assays

One gramme of xylanases from *Bacillus subtilis* was dissolved in 100 ml of acetate buffer (50 mM, pH 5.0) with continuous stirring for 30 min at 28 °C. The precipitate was removed by centrifugation (10,000*g*, 20 min), whereas the resulting supernatant was used as the enzyme solution. Xylanase activity was routinely assayed in a reaction mixture (2 ml) containing boiled birchwood xylan (1%, w/v), 50 mM acetate buffer, pH 5.0 and appropriately diluted enzyme solution. After 30 min incubation at 50 °C, the reducing sugar produced in the reaction mixture was measured by the dinitrosalicylic acid method, with xylose as standard (Miller, 1959). All activity measurements were performed at least three times.

2.4. Enzymatic hydrolysis

Hydrolysis of wheat bran insoluble dietary fibre was performed in a 250 ml stoppered Erlenmeyer flask with a working volume of 100 ml of 50 mM acetate buffer at the required pH values, containing the required amount of xylanases from *Bacillus subtilis*. An appropriate amount of wheat bran insoluble dietary fibre was added to the freshly prepared enzyme solution. The reaction mixture was incubated on a super water bath thermostatic vibrator (HZS-H-Model, Donglian Electronic and Technology Development Co., China) at 150 rpm and at specified temperatures.

2.5. Determination of feruloyl oligosaccharides by spectrophotometry

The concentrations of feruloyl oligosaccharides were determined at 286 and 323 nm for both free ferulic acid and esterified ferulic acid using a UV-1000 UV/VIS Recording Spectrophotometer (Rayleigh Analytical Instruments, Beijing, China) (Katapodis et al., 2003). The following molar absorption coefficients were determined at pH 6 in 100 mM MOPS (3-(*N*-morpholino)-propanesulfonic acid) $\varepsilon_{286} = 14176$ and $\varepsilon_{323} = 10350$l mol^{-1} cm^{-1} for free ferulic acid and $\varepsilon'_{286} = 12465$ and $\varepsilon'_{323} = 19345$ l mol^{-}cm^{-1} for esterified ferulic acid. Estenfied ferulic acid ($[FA]_e$) and free ferulic acid ($[FA]_f$), released by xylanases, were calculated from the absorbances measured at 286 nm (A_{286}) and 323 nm (A_{323}) by the following equations:

$$[\mathrm{FA}]_{\mathrm{e}} = [(A_{323} \times \varepsilon_{286}) - (\varepsilon_{323} \times A_{286})]/[(\varepsilon'_{323} \times \varepsilon_{286}) - (\varepsilon'_{286} \times \varepsilon_{323})] \quad (1)$$

$$[\mathrm{FA}]_{\mathrm{f}} = [A_{286} - (\varepsilon'_{286} \times [FA]_{e})]/\varepsilon_{286} \quad (2)$$

2.6. Experimental design

A five level, five variable central composite rotatable design (Cochran & Cox, 1992) was applied to determine the best combination of enzyme reaction variables for the production of feruloyl oligosaccharides from wheat bran insoluble dietary fibre. The factorial design consisted of 16 factorial points, 10 axial points (two axial points on the axis of each design variable at a distance of 2 from the design centre) and 6 centre points, leading to 32 sets of experiments. The variables X_i were coded as x_i according to the following Eq. (3):

$$x_i = (X_i - \bar{X}_i)/\Delta X_i, \quad (3)$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, $\bar{X}_i$ is the real value of an independent variable at the centre point, and ΔX_i is the step change value. The variables and their levels, with both coded values and natural values investigated in this study, are represented in Table 1. Table 2 lists the actual experimental parameters corresponding to the designed levels that were carried out for developing the model. Each experiment was performed in duplicate and the average of concentration of feruloyl oligosaccharides was taken as the response, Y.

Regression analysis was performed, based on the experimental data, and was fitted into an empirical second order polynomial model as shown below in Eq. (4):

$$Y = b_0 + \sum_{i=1}^{5} b_i x_i + \sum_{i=1}^{5} b_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} b_{ij} x_i x_j, \quad (4)$$

where Y is the response variable, b_0, b_i, b_{ii}, b_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively, and x_i and x_j are independent variables.

Table 1
Variables and their levels employed in a central composite rotatable design for optimization of feruloyl oligosaccharide production by *Bacillus subtilis* xylanases

Variable	Coded levels				
	−α(−2)	−1	0	+1	+α(+2)
Natural levels					
Temperature (°C)	35	40	45	50	55
pH	4.5	5.0	5.5	6.0	6.5
Reaction time (h)	12	24	36	48	60
Enzyme (g/l)	1.0	2.0	3.0	4.0	5.0
Substrate (g/l)	10	30	50	70	90

The responses obtained from each set of experimental design (Table 2) were subjected to multiple nonlinear regression using the software STATISTICA 6.0 to obtain the coefficients of the second polynomial model. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by an F-test. The significances of the regression coefficient were tested by a t-test.

2.7. Analysis of characterization of feruloyl oligosaccharides

The enzymatic hydrolysate of wheat bran insoluble dietary fibre was passed through a 0.45 μm membrane filter and concentrated by rotary evaporation. The concentrated solution was applied to an open column (80 × 2.5 cm i.d.) packed with Amberlite XAD-2 (previously washed with 95% ethanol and then water). Elution was successively carried out with 2 column volumes of distilled water, 3 column volumes of 50% (v/v) methanol/water and 2 column volumes of methanol. The fractions eluted by methanol/water were concentrated and lyophilized with a freeze–dry system (A1PHA1-4, Christ, Germany) to get feruloyl oligosaccharides for further analysis.

Feruloyl oligosaccharides were analyzed by paper chromatography, which was performed on Whatman No. 1 filter paper by the descending method with n-butanol/acetic acid/water (12:3:5) as the mobile phase (Wende & Fry, 1997). The separated feruloyl oligosaccharides were located by UV radiation (before and after exposure to NH_3) (Smith & Harris, 2001), and the spots were visualized with an oxalate/aniline reagent (2 volumes of 2% aniline in ethanol and 3 volumes of 2.5% oxalic acid) by heating in an oven at 105 °C for 10–20 min for sugar detection (Lequart, Nuzillard, Kurek, & Debeire, 1999).

Feruloyl oligosaccharides (100 μl, 1 mg/ml) were saponified with NaOH (100 μl, 0.4 M) for 2 h in the dark at room temperature. The reaction was stopped by adding H_3PO_4 (150 μl, 0.4 M). This solution was analyzed for ferulic acid by HPLC (Waters 600/650 E, USA) using a C18 Symmetry column (150 × 3.9 mm i.d., 5 μm particle size, Waters, USA). The column was maintained at 30 °C. A sample volume of 10 μl was injected into the HPLC column, and the phenolic acid was eluted with methanol/water/acetic acid (50:50:0.5) at a flow rate of 0.8 ml/min in an isocratic programme for 15 min. The absorbance of the eluate was monitored continuously at 320 nm. Ferulic acid released from feruloyl oligosaccharides was identified by comparison of its relative retention time with the standard compound (ferulic acid). Then, the deesterified oligosaccharides were again hydrolyzed in CF_3COOH (2 M, 1 h, 121 °C). The resulting solution was diluted 50-fold, filtered, and analyzed

Table 2
Experimental design of 5-level 5-variable central composite rotatable design[a,b]

Run	X_1, temperature (°C)	X_2, pH	X_3, reaction time (h)	X_4, enzyme (g/l)	X_5, substrate (g/l)	FOs[c] producted (mM)	
						Experimental	Predicted
1	40(−1)	5.0(−1)	24(−l)	2.0(−1)	70(+1)	1.08	1.01
2	50(+1)	5.0(−1)	24(−l)	2.0(−1)	30(−1)	0.384	0.381
3	40(−1)	6.0(+1)	24(−l)	2.0(−1)	30(−1)	0.332	0.381
4	50(+1)	6.0(+1)	24(−l)	2.0(−1)	70(+1)	1.01	1.01
5	40(−1)	5.0(−l)	48(+l)	2.0(−1)	30(−1)	0.438	0.439
6	50(+1)	5.0(−1)	48(+l)	2.0(−1)	70(+1)	1.05	1.07
7	40(−1)	6.0(+1)	48(+l)	2.0(−l)	70(+1)	0.999	1.07
8	50(+1)	6.0(+1)	48(+l)	2.0(−1)	30(−1)	0.391	0.439
9	40(−1)	5.0(−1)	24(−l)	4.0(+1)	30(−1)	0.514	0.490
10	50(+1)	5.0(−1)	24(−l)	4.0(+1)	70(+1)	1.16	1.12
11	40(−1)	6.0(+1)	24(−l)	4.0(+1)	70(+1)	1.11	1.12
12	50(+1)	6.0(+1)	24(−l)	4.0(+1)	30(−1)	0.44	0.490
13	40(−1)	5.0(−1)	48(+l)	4.0(+1)	70(+1)	1.22	1.18
14	50(+1)	5.0(−1)	48(+l)	4.0(+1)	30(−1)	0.559	0.548
15	40(−1)	6.0(+1)	48(+l)	4.0(+1)	30(−1)	0.497	0.548
16	50(+1)	6.0(+1)	48(+l)	4.0(+1)	70(+1)	1.11	1.18
17	35(−2)	5.5(0)	36(0)	3.0(0)	50(0)	0.712	0.896
18	55(+2)	5.5(0)	36(0)	3.0(0)	50(0)	0.921	0.896
19	45(0)	4.5(−2)	36(0)	3.0(0)	50(0)	0.702	0.767
20	45(0)	6.5(+2)	36(0)	3.0(0)	50(0)	0.767	0.767
21	45(0)	5.5(0)	12(−2)	3.0(0)	50(0)	0.588	0.680
22	45(0)	5.5(0)	60(+2)	3.0(0)	50(0)	0.824	0.796
23	45(0)	5.5(0)	36(0)	1.0(−2)	50(0)	0.704	0.787
24	45(0)	5.5(0)	36(0)	5.0(+2)	50(0)	0.896	1.01
25	45(0)	5.5(0)	36(0)	3.0(0)	10(−2)	0.090	0.087
26	45(0)	5.5(0)	36(0)	3.0(0)	90(+2)	1.28	1.35
27	45(0)	5.5(0)	36(0)	3.0(0)	50(0)	0.927	0.896
28	45(0)	5.5(0)	36(0)	3.0(0)	50(0)	0.884	0.896
29	45(0)	5.5(0)	36(0)	3.0(0)	50(0)	0.965	0.896
30	45(0)	5.5(0)	36(0)	3.0(0)	50(0)	0.911	0.896
31	45(0)	5.5(0)	36(0)	3.0(0)	50(0)	0.89	0.896
32	45(0)	5.5(0)	36(0)	3.0(0)	50(0)	0.861	0.896

[a] Experimental feruloyl oligosaccharide productions are averages of duplicates within ±5% error.
[b] Average absolute relative deviation (%) = 5.75.
[c] Feruloyl oligosaccharides.

by high performance anion-exchange chromatography with a pulse amperometric detector (HPAEC-PAD) on a Dionex BioLC system using a Carbopac™ PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA, USA). The column was maintained at 25 °C and eluted with 100 mM NaOH at a flow rate of 0.7 ml/min. Monosaccharide composition was identified by comparison to relative retention times of authentic standards (arabinose, xylose).

3. Results and discussion

3.1. Effect of temperature and pH on xylanase activity and stability

Thermostability and thermoactivity of the xylanases from *Bacillus subtilis*, determined at pH 5.0, are shown in Fig. 1. The enzyme in 50 mM acetate buffer (pH

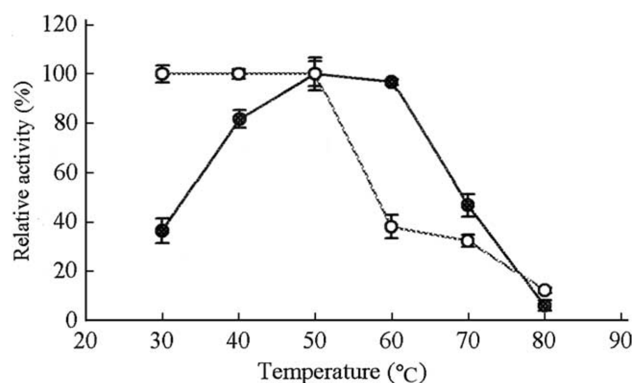

Fig. 1. Effect of temperature on stability (○) and activity (●) of xylanases from *Bacillus subtilis*. For stability, the enzyme solution in acetate buffer (50 mM, pH 5.0) was incubated for 1 h at various temperatures, and then the residual enzyme activities were assayed. For activity, the enzyme activity was assayed at various temperatures by the standard assay method.

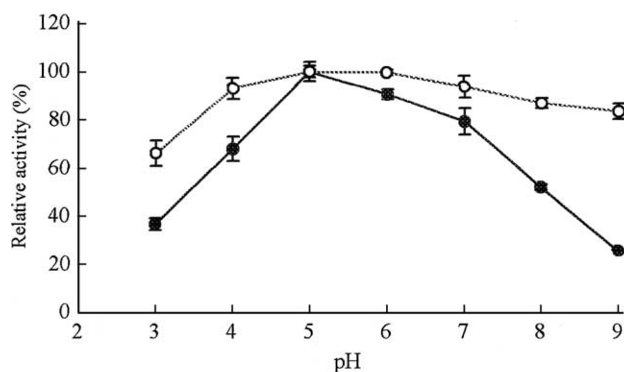

Fig. 2. Effect of pH on stability (○) and activity (●) of xylanases from *Bacillus subtilis*. For stability, the enzyme solutions in 50 mM appropriate buffer, at various pH values, were incubated for 1 h at 40 °C. After adjustment of pH, the residual activity was assayed by the standard method. The enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pH. The following buffers were used: citrate-phosphate (pH 3.0–8.0) and glycine–NaOH (pH 9.0).

5.0) was highly stable up to 50 °C for 1 h. It lost its stability at 60 °C with 38% residual activity. The enzyme was most active at 50 °C with 97% relative activity at 60 °C and 6% activity at 80 °C under the assay conditions used.

The enzyme was fully stable at pH 4.0–6.0 (1 h at 40 °C) (Fig. 2). It displayed an optimum activity at pH 5.0 with 37% relative activity at pH 3.0 and 26% relative activity at pH 9.0. However, the enzyme showed excellent activity at pH 6.0 (91%).

3.2. Effects of *Bacillus subtilis* xylanases, at different concentrations, on the production of feruloyl oligosaccharides

The effects of *Bacillus subtilis* xylanases, at different concentrations, on the production of feruloyl oligosaccharides were studied Fig. 3 shows the effect of various levels of xylanases on wheat bran insoluble dietary fibre. The concentration of feruloyl oligosaccharides reached 0.658 mM at low enzyme dosage (1.0 g/l). Their concentration increased with increasing enzyme dosage. When the enzyme level was up to 4.0 g/l, the feruloyl oligosaccharide concentration increased very slowly.

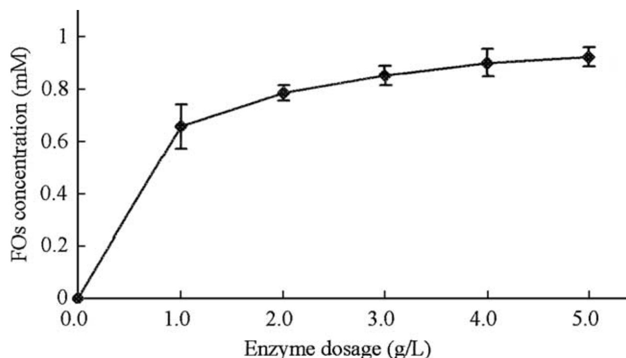

Fig. 3. Effect of *Bacillus subtilis* xylanases, at different concentrations, on the hydrolysis of wheat bran insoluble dietary fibre. reaction conditions: reaction temperature, 50 °C; the enzyme solution (100 ml) in acetate buffer (50 mM, pH 5.0); wheat bran insoluble dietary fibre, 50 g/l; reaction time, 36 h. FOs: feruloyl oligosaccharides.

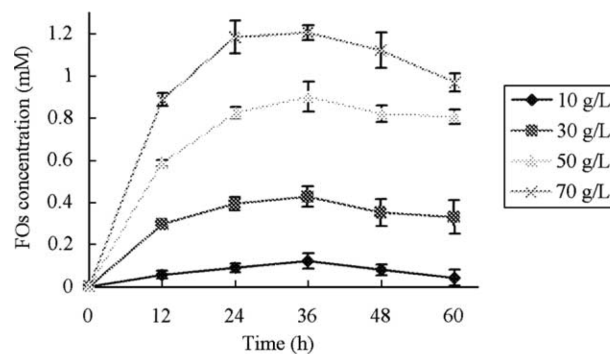

Fig. 4. Time course of hydrolysis of wheat bran insoluble dietary fibre at different concentrations. Reaction conditions: reaction temperature, 50 °C; xylanase (0.4 g) was, dissolved in 100 ml acetate buffer (50 mM, pH 5.0). FOs: feruloyl oligosaccharides.

3.3. Effects of substrate, different concentrations, on the production of feruloyl oligosaccharides

The effects of varying substrate (wheat bran insoluble dietary fibre) over-α range of concentrations (10–70 g/l) on the production of feruloyl oligosaccharides are shown in Fig. 4. The feruloyl oligosaccharide production increased up to the first 36 h and then decreased slowly. It is speculated that the decrease is due to the possible feruloyl esterase in xylanases from *Bacillus subtilis*, as there existed some free ferulic acid in the hydrolyzate (data not shown). At a low substrate concentration, of 10 g/l the maximum feruloyl oligosaccharide yield was relatively low (0.121 mM). However, at high substrate concentrations, over 50 g/l, the maximum concentration of feruloyl oligosaccharides increased relatively fast. The concentration of feruloyl oligosaccharides reached 1.205 mM at 70 g/l of wheat bran insoluble dietary fibre.

3.4. Optimization of enzyme reaction conditions

Based on the investigations on *Bacillus subtilis* xylanase characterization, the effects of enzyme and substrate (wheat bran insoluble dietary fibre) concentration on the production of feruloyl oligosaccharides, the variables considered are substrate concentration, enzyme concentration, reaction time, temperature, and pH in the experimental design. To optimize the production process of feruloyl oligosaccharides using xylanases from *Bacillus subtilis*, reaction temperature 45 °C, pH 5.5, reaction time 36 h, enzyme concentration 3.0 g/l, and substrate

concentration 50 g/l were chosen as the central condition of the CCRD.

Table 2 shows the experimental conditions and the results of feruloyl oligosaccharide production according to the factorial design. Maximum concentration of feruloyl oligosaccharides (1.28 mM) was recorded under the experimental conditions of temperature 45 °C, pH 5.5, reaction time 36 h, enzyme concentration 3.0 g/l and substrate concentration 90 g/l. The lowest concentration of feruloyl oligosaccharides (0. 090 mM) was observed in the test set No. 25 (compared to all others). Statistical analysis revealed that the most relevant variable ($P < 0.001$) for the feruloyl oligosaccharides production was the concentration of substrate (wheat bran insoluble dietary fibre) (Table 3). The feruloyl oligosaccharide production was also high (1.22 mM) with an enzyme concentration of 4.0 g/l, corresponding to the test set No. 13. Not much variation in feruloyl oligosaccharide production was observed between pH 4.5 and pH 6.5. This might be due to similar stabilities of the enzyme within the tested pH ranges. However, a higher concentration of feruloyl oligosaccharides from the test set No. 22 than from from the test set No. 21 could imply that increase in reaction time was more favourable for feruloyl oligosaccharides production.

Multiple regression analysis was performed on the experimental data and the coefficients of the model were evaluated for significance with the Student *t*-test. The linear coefficients except temperature and pH terms, and three quadratic terms (pH, reaction time and substrate concentration) were significant ($P < 0.05$). All the cross-product coefficients were eliminated in the refined equation as the *P* values of these coefficients are very insignificant ($P > 0.5$). The values of the coefficients are presented in Table 3. The analysis of variance (ANOVA) for the CCRD is shown in Table 4. The coefficient of determination (R^2) of the model is 0.975, which indicates that the model adequately represented the real relationship among the parameters chosen. The average absolute relative deviation of the reduced model is 5.75%. Neglecting the insignificant terms, the final predictive equation obtained is as given below:

Table 3
Estimated coefficients of the fitted second-order polynomial model for feruloyl oligosaccharides production[a]

Term	Coefficients estimated	Standard error	*t* value	*P* value
b_0	0.895602	0.031072	28.82	<0.0001
b_3	0.028958	0.015902	1.82	0.0459
b_4	0.054542	0.015902	3.43	0.0056
b_5	0.314875	0.015902	19.8	<0.0001
b_{22}	−0.032227	0.014384	−2.24	0.0467
b_{33}	−0.039352	0.014384	−2.74	0.0194
b_{55}	−0.044602	0.014384	−3.1	0.0101

[a] Only terms with $P < 0.05$ were included.

Table 4
Analysis of variance of the second-order feruloyl oligosaccharide production model[a]

	Degree of freedom	Sum of squares	Mean square	*F* value	*P* value
Total model	20	2.603988	0.130199	21.45	<0.0001
Linear	5	2.481394	0.496279	81.78	<0.0001
Quadratic	5	0.117558	0.023512	3.87	0.0286
Cross product	10	0.005037	0.000504	0.42	0.9998
Total error	11	0.066755	0.006069		
Lack of fit	6	0.060044	0.010007	7.46	0.0218
Pure error	5	0.006711	0.001342		

[a] Coefficient of determination (R^2) = 0.975.

$$Y = 0.896 + 0.029x_3 + 0.0545x_4 + 0.315x_5 - 0.0322x_2^2 - 0.0394x_3^2 - 0.0446x_5^2 \quad (5)$$

To determine optimal levels of the variables for feruloyl oligosaccharide production, three dimension surface plots were constructed according to Eq. (5). Fig. 5 shows the effect of substrate (wheat bran insoluble dietary fibre) concentration and pH on feruloyl oligosaccharide production. While the feruloyl oligosaccharide concentration reached a maximum near the central condition of pH at a fixed substrate concentration, increase in substrate concentration at a fixed pH value led to an increase in feruloyl oligosaccharide concentration. The effect of substrate concentration and reaction time was similar to that of substrate concentration and pH (Figure not shown). The effect of pH and reaction time

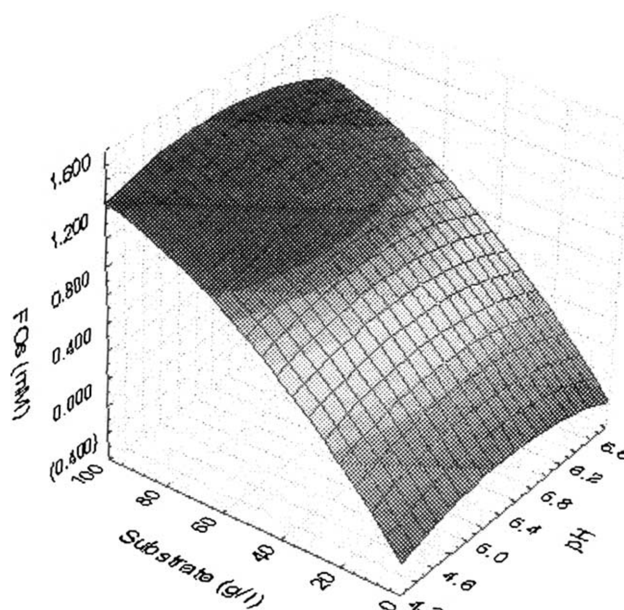

Fig. 5. Response surface plot showing the effect of pH and substrate concentration (g/l) on the production of feruloyl oligosaccharides. Other variables are constant at their zero levels as follows: reaction time, 36 h; enzyme concentration, 3.0 g/l; and reaction temperature, 45 °C. FOs: feruloyl oligosaccharides.

shown in Fig. 6 for the production of feruloyl oligosaccharides, demonstrated that the feruloyl oligosacchairde concentration could reach a maximum value near their zero levels. Fig. 7 shows the response surface plot at various substrate and enzyme concentrations. A linear increase in the concentration of feruloyl oligosaccharides with increase in enzyme concentration and substrate concentration was observed. Particularly, increase in substrate concentration at a fixed enzyme concentration led to a marked increase in feruloyl oligosaccharide concentration (0.135–1.39 mM at 4.5 g/l of enzyme concentration, and 0.056–1.32 mM at 2.5 g/l of enzyme concentration). As shown in Fig. 8, a quadratic effect of reaction time and a linear effect of enzyme concentration on the response were observed, and similar effects were observed between pH and enzyme concentration (Figure not shown). Therefore, an increase of enzyme concentration results in higher feruloyl oligosaccharide production at a fixed reaction time or pH value.

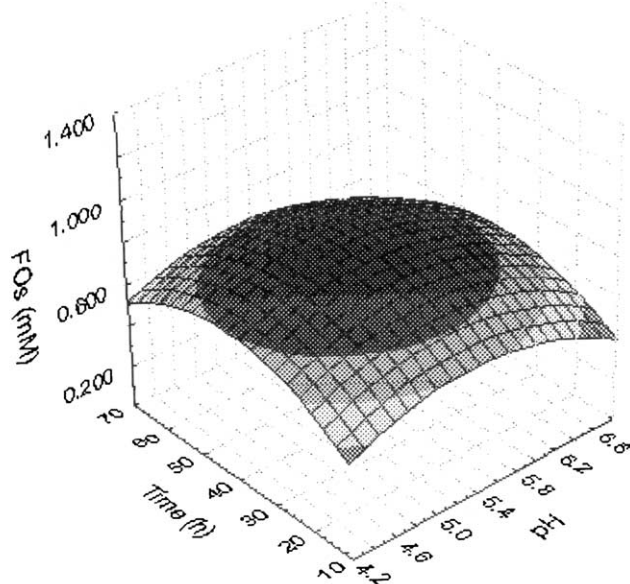

Fig. 6. Response surface plot showing the effect of pH and reaction time (h) on the production of feruloyl oligosaccharides. Other variables are constant at their zero levels as follows: enzyme concentration, 3.0 g/l and reaction temperature, 45 °C; substrate concentration, 50 g/l. FOs: feruloyl oligosaccharides.

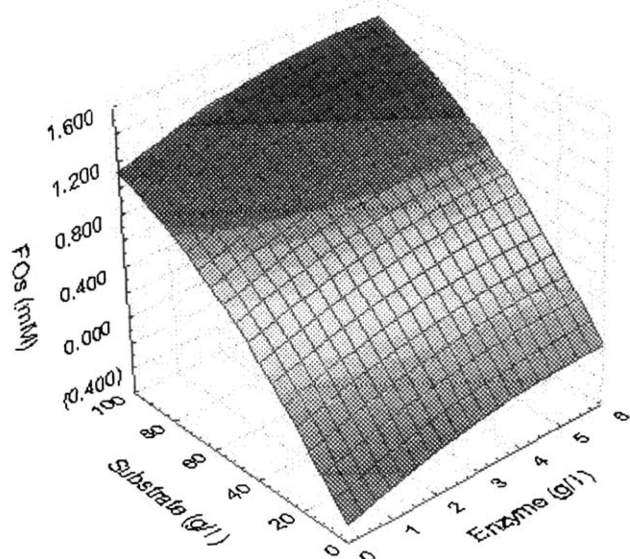

Fig. 7. Response surface plot showing the effect of enzyme concentration (g/l) and substrate concentration (g/l) on the production of feruloyl oligosaccharides. Other variables are constant at their zero levels as follows: reaction time, 36 h; pH, 5.5; and reaction temperature, 45 °C. FOs: feruloyl oligosaccharides.

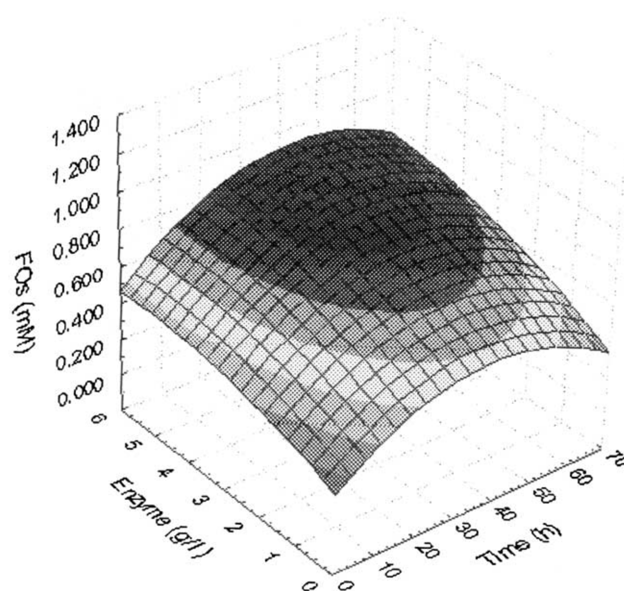

Fig. 8. Response surface plot showing the effect of reaction time (h) and enzyme concentration (g/l) on the production of feruloyl oligosaccharides. Other variables are constant at their zero levels as follows: pH, 5.5; reaction temperature, 45 °C; substrate concentration, 50 g/l. FOs: feruloyl oligosaccharides.

The optimal conditions were thus approached using the model as follows: reaction temperature, 42 °C; pH, 5.2; reaction time, 35 h; enzyme concentration, 4.8 g/l and substrate concentration, 120 g/l. Under the optimal conditions, the model predicted a maximum response of 1.50 mM. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. A mean value of 1.55 ± 0.037 mM ($N = 4$), 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 5).

3.5. Characterization of feruloyl oligosaccharides

In order to get feruloyl oligosaccharides, the hydrolytic products of wheat bran insoluble dietary fibre

Table 5
Optimum conditions, predicted and experimental value of response at that condition

Optimum condition					FO_S[a] producted (mM)	
Temperature (°C)	pH	Reaction time (h)	Enzyme (g/l)	Substrate (g/l)	Experimental[b]	Predicted
42	5.2	35	4.8	120	1.55 ± 0.037	1.50

[a] Feruloyl oligosaccharides.
[b] Mean ± standard deviation ($N = 4$).

incubated with xylanases were applied to Amberlite XAD-2, which is a polymeric adsorbent capable of binding aromatic compounds (Saulnier, Vigouroux, & Thibault, 1995). Initially, all of the oligosaccharides were retained on the Amberlite XAD-2 column. Application of distilled water to the column led to the elution of oligosaccharides that did not contain ester-linked ferulic acid. Four compounds were isolated from the fraction eluted by methanol/water that was subjected to paper chromatography. These separated compounds fluoresced in blue but changed into green on exposure to NH_3 under UV radiation (325 nm). These spots with fluorescence turned into a reddish colour when they were stained with an oxalate/aniline reagent, indicating that they were feruloyl oligosaccharides (Harris & Hartley, 1976; Smith & Harris, 2001). Lequart et al. (1999) reported that hydrolysis of wheat bran by endoxylanase from a thermophilic *Bacillus* sp. released seven feruloyl oligosaccharides. The digestion of wheat aleurone walls with a mixture of polysaccharide hydrolases (Driselase) resulted in at least five feruloyl oligosaccharides (Rhodes et al., 2002).

The hydrolyzed product of the water-soluble feruloyl oligosaccharides from wheat bran insoluble dietary fibre (by treatment with 0.4 M NaOH) was analysed by HPLC. Comparison of its relative retention time with standard compound (ferulic acid) could confirm the presence of ferulic acid. The glycosyl residue composition of the deesterifted products of feruloyl oligosaccharides were analysed by HPAEC-PAD. The monosaccharide composition was characterized as arabinose and xylose by comparison with relative retention times of authentic standards (arabinose, xylose). Smith and Hartley (1983) reported a feruloyl arabinoxylan disaccharide, 2-*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-D-xylopyranose, isolated from the *Oxyporus* cellulase digest of cell walls of wheat bran. An *O*-[5-*O*-feruloyl-α-L-arabinofuranosyl]- (1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose, an *O*-β-D-xylopyranosyl-(1 → 4)-*O*-[5-*O*-feruloyl-α-L-arabinofuranosyl-(1 → 3)]-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose, and an *O*-β-D-xylopyranosyl-(1 → 4)-*O*-[5-*O*-feruloyl-α-L-arabinofuraribsyl-(1 → 3)]-*O*-β-D-xylopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose were isolated from the enzymatic hydrolyzate of cell walls of wheat bran (Lequart et al., 1999; McCallum, Taylor, & Neil Towers, 1991; Rhodes et al., 2002). The composition of the water-soluble feruloyl oligosaccharides from wheat bran insoluble dietary fibre in this study showed good consistency with that of the above feruloylated arabinoxylan oligosaccharides.

4. Conclusions

This present study indicates that the xylanases from *Bacillus subtilis* are able to hydrolyze wheat bran insoluble dietary fibre to produce feruloyl oligosaccharides, which consist of esterified ferulic acid, arabinose and xylose. The optimal conditions obtained by RSM for enzymatic production of feruloyl oligosaccharides from wheat bran insoluble dietary fibre include the following parameters: reaction temperature 42 °C, pH 5.2, reaction time 35 h, enzyme concentration 4.8 g/l, substrate concentration 120 g/l.

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