Aureobasidium pullulans Fermented Feruloyl Oligosaccharide: Optimization of Production, Preliminary Characterization, and Antioxidant Activity

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Wheat bran (WB) was subjected to processing with *Aureobasidium pullulans* (*A. pullulans)* under selected conditions to partially break down the xylan into soluble products (mainly feruloyl oligosaccharides, FOs). The objective of this study was to investigate the technology for one-step fermentation of WB by *A. pullulans* without melanin secretion to produce FOs as well as to determine their structural features and antioxidant activity. Initial pH, inoculation quantity, and fermentation temperature were found to be efficient for releasing FOs according to the Plackett-Burman design (PBD). Based on the D-Optimal design, a yield of 904 nmol of FOs / L of fermentation broth was obtained under optimal conditions of initial pH 6.0, inoculation quantity 4.50%, and fermentation temperature 29 ^{o}C. Purification of FOs was performed with alcohol precipitation and Amberlite XAD-2. GC, IR, and ESI-MS demonstrated that FOs consist of feruloyl arabinosyl xylopentose (FAX5, Mw986), feruloyl arabinosyl xylotetraose (FAX4, Mw854), feruloyl arabinosyl xylotriose (FAX3, Mw722), and feruloyl arabinosyl xylobiose (FAX2, Mw590). Increasing the FO dose led to increased activity of SOD and GSH-Px in serum of S_{180} tumor-bearing mice, while the level of MDA was reduced, thus improving its *in vivo* antioxidant activity.

Keywords: Wheat bran (WB); A. pullulans; Feruloyl oligosaccharides (FOs); Antioxidant activity; One-step fermentation

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INTRODUCTION

Ferulic oligosaccharides (FOs), which are present in a wide variety of gramineous plants, are a type of functional oligosaccharide formed through the carboxyl esterification of ferulic acid (FA) and sugar hydroxyl groups. Wheat bran (WB) from the production of flour is an important source of FOs. WB is a rich source of dietary fiber that contains 34% arabinoxylan and 11% cellulose (Escarnot *et al*. 2011). The antioxidant activity of FOs is higher than that of FA and vitamin C, exhibiting a strong inhibition effect on the hemolysis of mouse red blood cells as well as eliminating Fe^{2+}, H_2O_2, and hydroxyl radicals (Wang *et al*. 2010, 2011). FOs have also been reported to present significant antioxidant capacity in DPPH and lipid peroxidation systems (Wang *et al*. 2008, 2009). Hence, FOs are a natural antioxidant with high research and application value (Wang *et al*. 2008, 2009, 2010).

At present, the methods of producing FOs include physical methods, chemical methods, and biological enzymes. Rose *et al*. (2010) used microwave radiation to treat corn bran, aimed at opening the main chain of xylose units connected by β-1,4-glycosidic

bonds to release FOs. However, with increments in microwave temperature and extension of the treatment time, the FOs gradually degraded to FA, xylose, and arabinose. Physical methods are used less frequently due to their complicated and strict processing conditions. It was reported that corn bran was hydrolyzed using 0.05 M trifluoroacetic acid by Allerdings *et al*. (2005) and Saulnier *et al*. (1995), and FOs were separated using Amberlite XAD-2 and Sephadex LH-20, obtaining three major oligosaccharides identified by ^{13}C NMR, including F7 of 5-O-trans-feruloyl-L-arabinose, F6 of O-feruloyl-D-xylosyl-(1, 2)-[5-O-(trans-feruloyl)-L-arabinose], and F3 of O-L-galactopyranosyl-(1-4)-O-D-xylosyl-(1, 2)-[5-O-(trans- feruloyl)-L-arabinose]. However, this method could not be applied in the food and medicine industries because of the detection of chemical residues and the environmental pollution caused by the by-products. The biological enzyme method of FO production is currently commonly used due to its mild reaction conditions. Yuan *et al*. (2006) produced two FOs from hydrolyzed wheat bran (WB) insoluble dietary fiber using *Bacillus subtilis* xylanase, O-xylosyl-[5-O-(feruloyl)- α-arabinoseyl-(1, 3)]-xylosyl-(1, 4)-β-xylose, and O-xylosyl-[5-O-(feruloyl)-α-arabinosyl-(1, 3)]-xylosyl-(1, 4)-β-xylosyltaxol-(1, 4)-β-xylose, as confirmed by ESI-MS. Lequart *et al*. (1999) also produced the FO *O*-b-D-xylosyl-(1, 4)-*O*-[5-*O*-(feruloyl)-a-L-arabinosyl-(1, 3)]-*O*-b-D-xylosyl-(1, 4)-*O*-b-D-xylosyl-(1, 4)-D-xylose, as demonstrated by NMR, by hydrolysis of WB and straw utilizing endogenous xylanase. Feruloyl arabinosyl xylan disaccharide was isolated from the enzymolysis hydrolyzate of WB as well (Beaugrand *et al*. 2004; Ko *et al*. 2013). The FA in WB mostly exists in insoluble dietary fiber, which is covalently cross-linked with xylan by an ester bond (Mazzaferro *et al*. 2011; Schooneveld-Bergmans *et al*. 1998; Yuan *et al.* 2005; Zhang *et al*. 2011). Therefore, using the biological enzyme method to produce FOs requires extraction of the insoluble dietary fiber from the raw materials, which not only increases production costs, but also produces a large quantity of wastewater.

Because of these issues, a method of directly using microorganisms that can produce xylanase to produce FOs in a one-step process seems attractive and worth investigating. *A. pullulans* is one kind of food-safe fungus that can produce endo-xylanases (xylanase or 1-4-β-D-xylanxylanohydrolase, EC3.2.1.8) of high activity and specificity (Navarini *et al*. 1996). Endo-xylanases can selectively hydrolyze hemicellulose while not affecting cellulose (Nagar *et al*. 2012; Christov *et al*. 1997), which may help improve the purity of FOs. Here, we creatively used *A. pullulans* previously bred by mutation with no melanin production during fermentation to hydrolyze WB for the purpose of producing FOs in a one-step process. Therefore, the production technology of FOs through fermentation and its structure-activity relationship was acquired as the foundation for future research concerning FOs as functional biological materials.

EXPERIMENTAL

Microorganism and Medium

A. pullulans was isolated from the soil surrounding a flour factory. Stock cultures were maintained on potato dextrose agar at 4 ^{o}C and subcultured every 2 weeks. WB was supplied by the flour mill of Qinda Co., Ltd of Jiangsu, China. The inoculum medium contained 50 g of glucose, 2.0 g of yeast extract, 5.0 g of K_2HPO_4, 0.6 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1.0 g of NaCl in 1 L of distilled water. The pH was adjusted to 6.0, and the medium was autoclaved at 121 ^{o}C for 20 min. Dried WB was crushed into

flour and passed through a 40-mesh sieve to remove some starch. The WB obtained was then dissolved in distilled water. The pH of the mixture was adjusted to 5.5 with a 2% (v/v) sulfuric acid solution to a concentration of 60 g/L WB. Then, 10 g/L oat xylan and 1 g/L peptone were added, and the mixture was incubated at 50 ^{o}C for 2 h. The resulting WB solutions were prepared as the fermentation medium.

Seed Culture Methods

The seed culture was prepared by inoculating a full loop of *A. pullulans* from a fresh slant tube into an Erlenmeyer flask (500 mL) containing 100 mL of fresh medium and was cultivated by agitation using a reciprocal shaker (180 rpm) at 28 ^{o}C for 72 h.

Fermentation Conditions Optimization for FOs

PBD experiment: The PBD was used to screen influential fermentation conditions, with FO yield as the response value. Each independent variable had both high and low levels in the test. The examined factors and their codes with the levels are presented in Table 1(A).

D-optimal test: The results of the PBD experiment were analyzed with Design-expert 7.0 software to obtain significant impact factors on FO production. It was found that positive factors included initial pH, inoculum size, and temperature, which were further optimized by response surface methodology, as listed in Table 2(A). Thus, the optimum fermentation conditions were determined by evaluation of the interaction of each variable through D-optimal design.

The model equation for the response value (Y) and the three process parameters (X_1, X_2, X_3) is

$$Y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j} a_{ij} X_i X_j \quad (1)$$

where a_0 is a constant term, a_i is the first-order coefficient, a_{ii} is the quadratic coefficient, a_{ij} is the coefficient of the interaction term, X_i and X_j represent the level of independent variables, and 3 is the number of factors.

The first-order coefficient, quadratic coefficient, and coefficient of the interaction term were obtained through analysis of the model equations. The significance each factor had in the model was determined by the calculated F value and probability level (P value) with unremarkable factors of $P > 0.05$ removed through statistical analysis. The corresponding response surface diagram of the regression model was drawn using the regression coefficient.

Separation of FOs

The fermentation broth was centrifugated to collect the supernatant, which was then precipitated by 50% ethanol to remove non-FO precipitates. The supernatant was then precipitated by 80% ethanol, and the precipitate was collected Then, the collected precipitate was dissolved with Sevage reagent to erase proteins. After that, it was again treated with alcohol precipitation and redissolved with hot water (60 to 70 ^{o}C) and concentrated in a vacuum rotary evaporator at 40 ^{o}C. Then, it was purified using Amberlite XAD-2 resin. The column was pre-activated with 95% ethanol and washed with distilled water until there was no alcohol present in the outflow. After the sample was filled, it was eluted successively with 2 column volumes of distilled water, 3 column

volumes of 50% aqueous methanol solution, and 2 column volumes of anhydrous methanol. The eluted component with 50% aqueous methanol solution was collected and concentrated in the vacuum rotary evaporator at 40 ^{o}C. Afterwards, FOs were obtained by freeze-drying the concentrated component.

FO Assays

FOs content was estimated by the method of Yu and Gu (2013) and Xie *et al.* (2010).

Composition Analysis of FOs

Determination of sugar composition

After the addition of 5 mg of FOs to 2 mL of trifluoroacetic acid solution (2 M), the mixture was hydrolyzed at 120 ^{o}C for 2 h. After cooling to room temperature, it was evaporated in a vacuum rotary evaporator and then dried at 60 ^{o}C. The dry product was stirred with 50 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine for 2 min. Then, the reaction was conducted in a 90 ^{o}C water bath for 15 min. The reaction system was cooled to room temperature before the addition of 0.5 mL of acetic anhydride, followed by a 20-min reaction at 90 ^{o}C.

The conditions of the gas chromatograph (Agilent 6890) include (1) column: HP-5 fused silica capillary column (30 m×0.32 mm×0.25 mm), (2) carrier gas and its flow rate: H_2 40 mL/min, air 450 mL/min, and N_2 25 mL/min, (3) column temperature: 100 ^{o}C, (4) inlet temperature: 200 ^{o}C, (5) detector and its temperature: hydrogen flame ionization detector, 250 ^{o}C, and (6) injection volume: 1 μL (Xie 2010).

IR analysis of FOs

A mixture of 1 mg of dried FOs and 100 mg of KBr was ground. This ground sample was pressed into a transparent sheet under vacuum and then placed in the sample holder for IR analysis, with a scanning range of 500 to 4000 cm^{-1}.

HPLC-ESI-MS analysis of FOs

The structure of FOs was analyzed by high performance liquid chromatography-tandem mass spectrometry with a LCQ DECAXP from American Finnigan Co.

The conditions of the liquid chromatography were as follows: C18 stainless steel column (250×4.6, 5 μm, Dikma Technologies DiamonsilTM), 9% (v%) acetonitrile mobile phase, isocratic elution strength, 1 mL/min flow rate, 25 ^{o}C column temperature, 325-nm detection wavelength, and 20-μL injection volume. The system was shunted in LC-MS to have 150 μL/min liquid injected into the ESI source.

The conditions for mass spectrometry were as follows: ESI source, 4.0-kV source voltage, positive and negative ion detection, m/z 100 to 1600 quality scan range, sheath gas (N_2) flow rate of 20 Arb, auxiliary gas (N_2) flow rate of 10 Arb, 300 ^{o}C capillary temperature, and -15-V capillary voltage. The Mass Lynx V4.1 data processing system was used to obtain and resolve the mass spectrum.

In Vivo Antioxidant Activity of FOs

Animal grouping and drug test

ICR (Institute of Cancer Research) mice with no special conditions (normal mice) were used as the control group (n = 8), and S_{180} tumor-bearing mice were divided into a

model control group, a positive control group, and a FO group (n = 8). For the normal control and model control groups, 0.4 mL/20 g/d normal saline was dosed by lavage administration. For the positive control group, 0.4 mL/20 g 5-FU was dosed with intravenous administration every other day for 10 days. For the FO group, after inoculation of the S_{180} tumor for 24 h, 50, 100, or 250 mg/kg/d were dosed once daily by lavage administration for 10 days (d_1-d_{10}), and blood was collected from the eye socket on the 11th day (d_{11}) after inoculation.

Detection of in vivo antioxidant activity

The blood was centrifugated at 2000 rpm for 10 min to obtain serum. The activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the serum as well as the content of lipid peroxidation products (MDA) were measured according to the kit method.

Statistical Analysis

All data are expressed as means ± SD of triplicates. SPSS 18.0 software was used for statistical analysis. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

One-step Process Study for FO Production from Wheat Bran

The FO yield in fermentation broth of *A. pullulans* was considered the response value, and the PBD and results are shown in Table 1(A). Design-Expert software was employed to conduct a stepwise regression analysis for FO production, and the optimal regression equation using FO yield as the response value is as follows:

$$Y = 521.92 + 30.75A - 137.75B + 69.75C + 71.08D + 132.08E + 101.08F + 89.25G \quad (2)$$

From Eq. (2), it can be seen that light, fermentation temperature, fermentation time, liquid medium volume, inoculum size, and initial pH had positive effects on the FO yield, whereas shaking speed had a negative effect.

It can be seen from the variance analysis in Table 1(B) that the obtained regression equation reached a significant level ($P < 0.05$), indicating an extremely good fitting degree within the entire studied regression range. The determination coefficient (R^2) of 0.9440 and adjusted R^2 of 0.8459 suggest that 94.40% of the experimental data variability could be explained by this regression model. In particular, the four fermentation factors of shaking speed, initial pH, inoculum size, and fermentation temperature had significant effects on the the FO yield ($P < 0.05$). Thus, through PBD, the significant positive factors for FO production were determined to be initial pH, inoculum size, and fermentation temperature. D-optimal experimental design was used to investigate the factors that had positive effects on the FO yield determined in the PBD test, including initial pH, inoculum size, and temperature. Table 2(A) exhibits the experimental design and results. Design-Expert software was utilized to fit the data in Table 2(A) using the Scheffe incomplete cubic polynomial, obtaining the incomplete cubic polynomial model between the FO yield Y (nmol/L) and the coding variables X_1 (initial pH), X_2 (inoculum size), and X_3 (fermentation temperature) (Eq. 3).

Table 1(A). PBD for Screening of Significant Variables Affecting the FO Yield

Trials	Variable code and experimental values							Yield of FOs (nmol/L)
	A (lx)	B (rpm)	C (mL)	D (d)	E	F (%)	G(degrees C)	
1	-1 (0)	-1 (0)	-1 (50)	1 (6)	1 (9.0)	1 (10)	-1 (25)	80±2
2	1 (1000)	1 (250)	-1	1	-1 (2.5)	-1 (2.5)	-1	379±4
3	1	-1	-1	-1 (3)	1	1	1 (35)	680±9
4	-1	1	1 (150)	-1	1	-1	-1	850±11
5	-1	1	1	1	-1	1	1	159±3
6	-1	-1	1	1	1	-1	1	766±8
7	1	-1	1	-1	-1	-1	1	287±4
8	1	1	-1	1	1	-1	1	728±9
9	1	1	1	-1	1	1	-1	808±10
10	-1	1	-1	-1	-1	1	1	235±4
11	-1	-1	-1	-1	-1	-1	-1	634±8
12	1	-1	1	1	-1	1	-1	657±9
A- Illumination intensity; B- Shaking speed; C- Culture volume; D- Fermentation time; E- Initial pH; F- Inoculation quantity; G- Fermentation temperature.								

Table 1(B). Analysis of Variance (ANOVA) for the FO Yield in the PBD Experiment

Variation source	Sum of squares	Degree of freedom	Mean square	F Value	P Value
Model	7.856E+005	7	1.122E+005	9.62	0.0225*
A	11346.75	1	11346.75	0.97	0.3798
B	2.277E+005	1	2.277E+005	19.53	0.0115*
C	58380.75	1	58380.75	5.01	0.0889
D	60634.08	1	60634.08	5.20	0.0848
E	2.094E+005	1	2.094E+005	17.95	0.0133*
F	1.226E+005	1	1.226E+005	10.51	0.0316*
G	95586.75	1	95586.75	8.20	0.0458*
Residuals	46645.67	4	11661.42		
Total variation	8.323E+005	11			
R^2 = 0.9440, adj R^2 =0.8459, * Significant effect at P<0.05.					

$$Y=892.09+51.44X_1-29.05X_2-78.07X_3+60.21X_1X_2-126.03X_1X_3$$

$$-49.20X_2X_3-312.37X_1^2-18.12X_2^2-199.05X_3^2 \quad (3)$$

The variance analysis was calculated for the above model (Table 2(B)). It can be seen that the established model for the response value of the FO yield reached an exceedingly significant level ($P < 0.001$). The correlation coefficient was up to 0.9132, and the determination coefficient was 0.8351, indicating that 83.51% of the experiments' data variability can be explained by this regression model.

The significance tests for the regression coefficients showed that the interaction between the initial pH and fermentation temperature affected FO production strikingly. Particularly, the influence on the FO yield of the square of initial pH reached a highly significant level ($P < 0.001$), while that of the square of temperature reached a notable level ($P < 0.05$).

Table 2(A). Fermentation Data Set of D-optimal Design and Corresponding Observed Values

Trials	X1	X2	X3	FOs (nmol/L)
1	2.50	10.00	25.00	263±3
2	3.11	9.41	30.00	345±4
3	9.00	2.50	35.00	221±3
4	4.75	2.50	35.00	641±7
5	4.94	7.35	35.00	543±6
6	9.00	2.50	28.58	588±7
7	6.39	5.49	25.00	751±8
8	2.50	10.00	25.00	267±3
9	2.50	10.00	35.00	268±2
10	2.50	2.50	25.00	322±4
11	2.50	5.58	30.90	683±7
12	2.50	10.00	35.00	250±3
13	6.46	5.49	31.25	942±13
14	9.00	7.33	35.00	223±3
15	2.50	5.58	30.90	465±5
16	4.92	2.50	28.75	958±12
17	9.00	10.00	25.00	717±8
18	9.00	10.00	25.00	679±8
19	2.50	2.50	25.00	242±4
20	6.38	10.00	30.99	845±10

X1: Initial pH, X2: Inoculation quantity (%), X3: Fermentation temperature (degrees C), same as below.

Table 2(B). Results of Regression Analysis for the D-optimal Design

Variation source	Sum of squares	Degree of freedom	Mean square	F Value	P Value
Model	1.125E+006	9	1.250E+005	11.69	0.0003 **
X1	26343.95	1	26343.95	2.46	0.1476
X2	7871.69	1	7871.69	0.74	0.4110
X3	52968.50	1	52968.50	4.95	0.0502
X1 X2	25938.36	1	25938.36	2.43	0.1504
X1 X3	1.148E+005	1	1.148E+005	10.74	0.0083*
X2 X3	18505.89	1	18505.89	1.73	0.2177
X1^2	2.908E+005	1	2.908E+005	27.20	0.0004**
X2^2	953.21	1	953.21	0.089	0.7714
X3^2	1.337E+005	1	1.337E+005	12.50	0.0054*
Residuals	1.069E+005	10	10692.21		
Total variation	1.232E+006	19	R^2= 0.9132	Adj R^2= 0.8351	

*Significant effect at $P < 0.05$, **Significant effect at $P < 0.001$.

The response surface (Fig. 1) of the influence of interaction between initial pH and temperature on the FO yield was plotted from the model. The interaction of initial pH and temperature influenced the FO yield significantly. When the initial pH remained unchanged, the FO yield first increased and then dropped with increasing temperature, and the trend of the initial pH was similar. When the initial pH and temperature were controlled at 5.75 to 7.38 and 27.5 to 30 ^{o}C, respectively, the greatest FO yield was obtained by fermentation with *A. pullulans*. This can be attributed to the fact that pH and temperature play key roles in the growth and metabolism of *A. pullulans* (Kang *et al*. 2011; McNeil and Kristiansen 1990; Wu *et al*. 2010). Singh (Lin *et al*. 2007; Singh *et al.* 2008) found that the pH of the fermentation medium can alter the morphology of *A. pullulans* and thus affect the bacterial cell growth and polysaccharide production. Wu *et al*. (2010) studied the impact of the two-stage regulation of pH and temperature on the pullulan production by *A. pullulans* and found that a pH of 2.5 and a temperature of 32 ^{o}C benefited *A. pullulans* cell growth, while a higher pH of 5.5 and a lower temperature of 26 ^{o}C boosted the pullulan yield. The optimum pH for *A. pullulans* to produce pullulan was ascertained to be 5.0 by Seo *et al*. (2004) and 6.0 by Lee and Yoo (1993). Furthermore, Xie (2010) confirmed that a medium pH of 5.5 availed Agrocybe fermentation for FO preparation.

Design-Expert software was used to optimize the initial pH, inoculum size, and temperature, which affected the FO yield, obtaining 5.98, 4.50%, and 29.20 ^{o}C for the highest FO yield, respectively. Considering the need for experimental operation, the initial pH, inoculum size, and temperature were separately rounded to 6.0, 4.50%, and 29 ^{o}C.

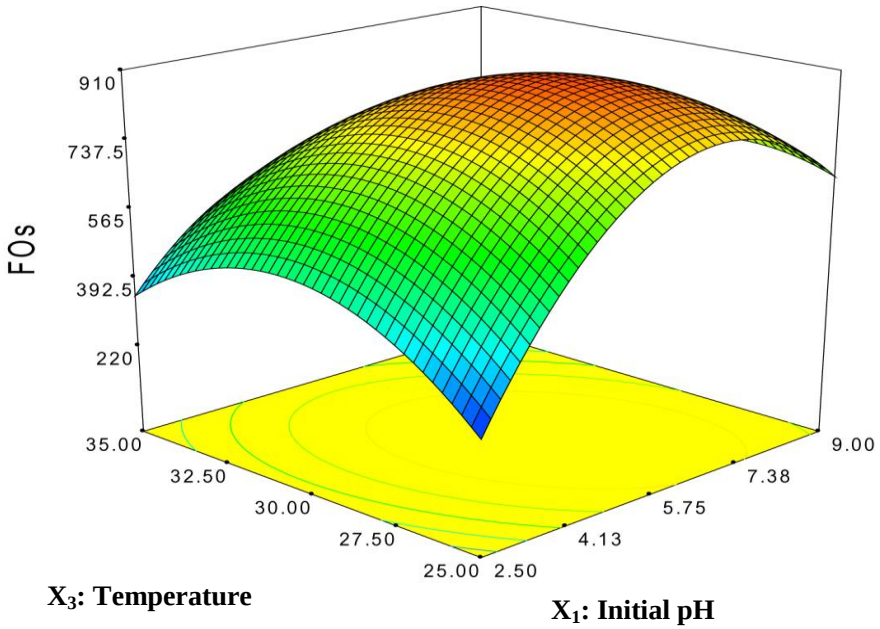

Fig. 1. The effect of initial pH and temperature on yields of FOs

It was verified in Table 3 that the actual and predicted values of the FO yield with *A. pullulans* agreed well, implying the obtained model is valid and reliable and can guide practice effectively. Under the optimal experimental conditions of initial pH 6.0, inoculum size 4.50%, and fermentation temperature 29 ^{o}C, the FO content in the fermentation broth reached 904 nM after fermentation for 96 h. For the ferulic acid content of wheat bran was 0.41%, so 60.98% of ferulic acid was recovered after fermentation with 60 g/L wheat bran liquid using *A. pullulans*. Compared with the study of Xie (2010), the fermentation time was reduced by 2 d and the yield had a 112% increase in this work.

Table 3. Arrangement and Result of Confirmatory Trials

Experiment	Initial pH	Inoculation quantity (%)	Temperature (degrees C)	Yield of FOs (nmol/L)	
				Measured value	Predicted value
Optimal combination	6.0	4.50	29	904 ± 12	907
Random combination 1	4.75	2.50	35	641± 7	687
Random combination 2	6.5	10.00	31	845 ± 8	817

Isolation and Identification of FOs

Because Amberlite XAD-2 possesses the characteristics of an adsorbing aromatic compound and FOs contain a feruloyl group, FOs can be adsorbed on the column (Yuan *et al*. 2006), but the unesterified oligosaccharides cannot be adsorbed and can be eluted with distilled water. The adsorbed substances can be eluted with 50% methanol, and thus FOs can be isolated. Because the above method has the merits of simplicity, rapidity, and efficient isolation, it has been used for FO extraction and purification from wheat bran

(Yuan *et al.* 2006), corn bran (Allerdings *et al.* 2006), beet syrup (Ralet *et al.* 1994), and flour (Sørensen *et al*. 2007). After purification by Amberilite XAD-2 column chromatography, the FOs eluted by 50% methanol were analyzed by gas chromatography (Figures were not shown). Through the GC analysis of standard xylose, arabinose, and FA, it can be seen that the peak times of xylose and arabinose were both at about 17.9 min, with the appearance of arabinose slightly earlier than that of xylose, and the peak time of FA is at 23 min. Therefore, the GC analysis reveals that the FOs were composed of three components: xylose, arabinose, and FA.

The prepared FOs were analyzed with IR, as shown in Fig. 2. The broad peak at 3371 cm^{-1} is classified as O-H stretching vibration absorption. The absorption peak at 2931 cm^{-1} is classified as saccharide C-H stretching vibration adsorption. The absorption peaks at 1200 to 1400 cm^{-1} indicate the presence of C-H deformation vibration. The above absorption peaks are characteristic absorption peaks of saccharides (Yuan *et al*. 2006). The absorption peak at 1657 cm^{-1} is classified as C=O stretching vibration adsorption, which is the characteristic absorption peak of ester bonds, suggesting the presence of an ester bond (Xie 2010). The strong absorption peak at 1037 cm^{-1} points to the emergence of an arabinosyl group connected with the 3rd site of pyranose xylose (Xie 2010). The absorption peak at 1550 cm^{-1} is identified as adsorption by the skeleton structure of an aromatic ring (Yuan *et al.* 2006), which indicates the presence of a mononuclear aromatic ring structure and thus demonstrates the presence of characteristic groups in glycolipids.

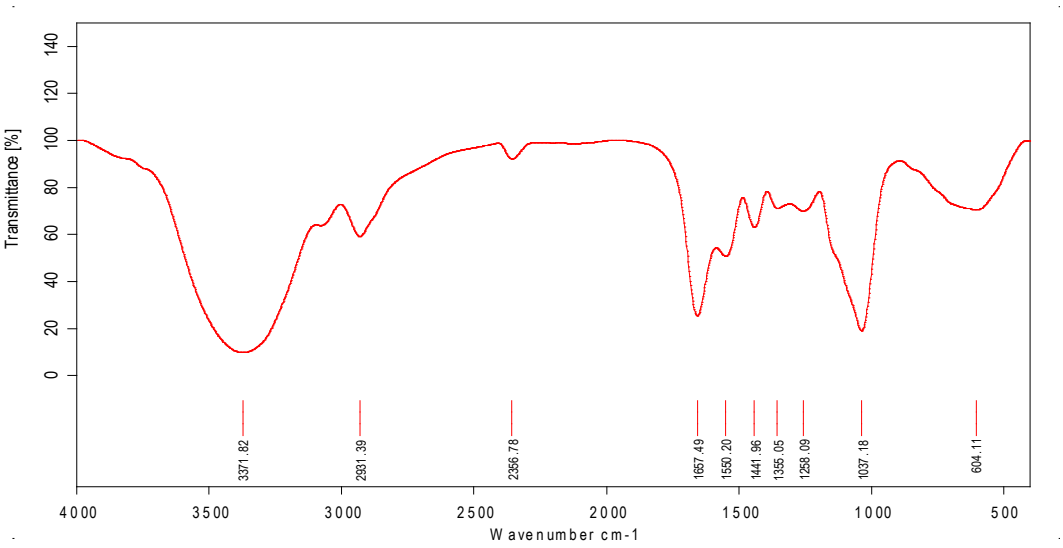

Fig. 2. IR spectrum of FOs

The separated FOs produced through fermentation by one-stage regulation of temperature and pH were analyzed with HPLC-ESI-MS. Because FOs have a maximum UV absorption at 325 nm, the HPLC measurement of FOs was implemented with a UV detector, as shown in Fig. 3(a). The total ion current was obtained using a mass spectrometry detector (Fig. 3(b)).

As can be seen from Fig. 3, the FOs were separated thoroughly, and four main peaks were obtained with retention times of 1.18, 6.23, 9.29, and 10.44 min, correspondingly labeled as 1 through 4. From the positive and negative ion mass spectrum of these four peaks (not shown), it could be seen for peak 1 that the m/z of the

quasi-molecular ion $[M+NH_4]^+$ was 1004 and that of $[M-H]^-$ was 985, suggesting the corresponding component had a molecular weight of 986. Likewise, it is inferred that the molecular weight of the relevant component was 854 for peak 2 due to the 872 m/z of $[M+NH4]^+$ and 853 m/z of $[M-H]^-$; the molecular weight for peak 3 was 722 due to the 740 m/z of $[M+NH4]^+$ and 721 m/z of $[M-H]^-$; and the molecular weight for peak 4 was 590 due to the 608 m/z of $[M+NH4]^+$ and 589 m/z of $[M-H]^-$. It is therefore believed that a molecular weight difference of 132 exists among the four components. Coincidently, 132 is the molecular weight of xylose after the loss of 1 molecule of water. That is to say, the four components differed by 1 molecule of xylose from each other, sequentially. In other words, the polymerization degree of the components of peaks 1 through 4 decreased successively, and the peak of the substance with a high polymerization degree appeared before that of the substance with a low polymerization degree.

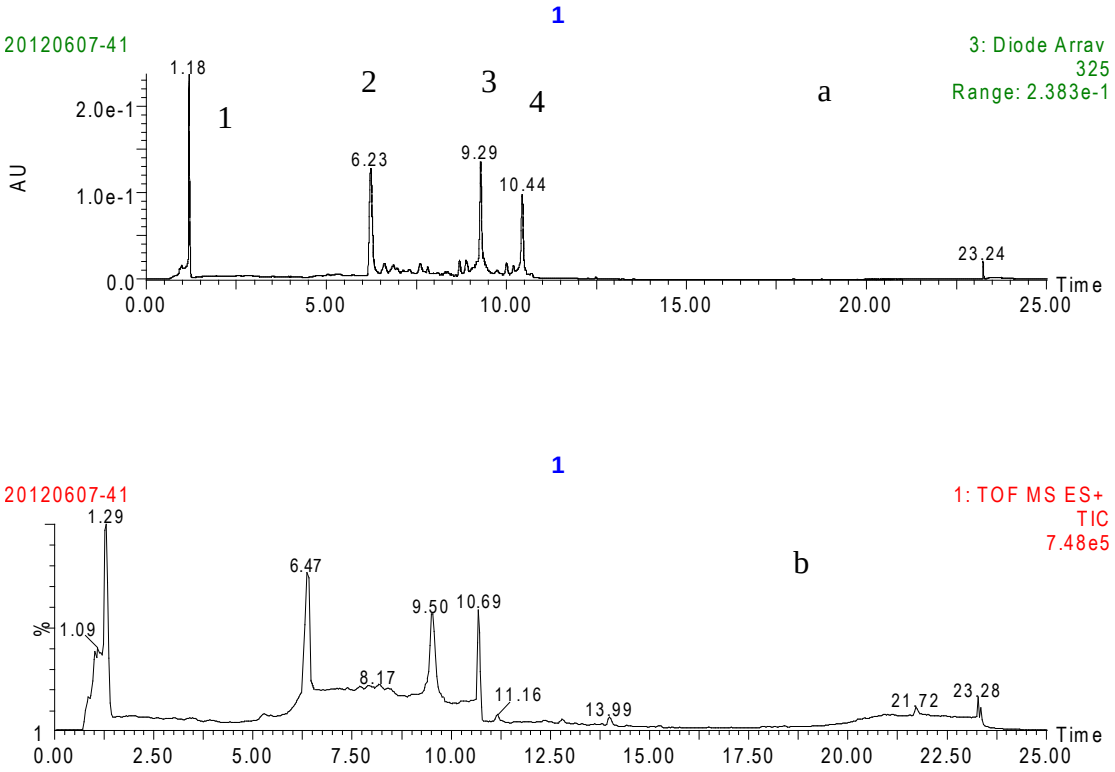

Fig. 3. HPLC-UV chromatogram (a) and total ion current chromatogram (b) of FOs

The aforementioned GC and IR findings for FOs indicated that FOs prepared by our process were composed of xylose, arabinose, and esterified FA. Moreover, the current research suggests that the FOs isolated from the hydrolyzate of Gramineae cell walls were α-L-furanoid arabinose residues connected to the *O*-3 site of xylose residues on the D-xylan skeleton chain with β-1, 4-glycosidic linkage, while FA was linked with the *O*-5 site of arabinose residues (Debeire *et al*. 2012), having a similar structure. Similarly, the main chain of WB arabinoxylan was formed by connection of β-D-furanoid xylose residues through β-1,4-glycosidic linkage, and the substituent group on the side chain was constructed by connection of α-L-furanoid arabinose to the *O*-2 and *O*-3 sites of the xylose residues, while FA was linked to the *O*-3 site of xylose residues after esterification with arabinose (Debeire *et al.* 2012). Thus, based on the results of previous

studies (Xie 2010; Yuan *et al*. 2006) and basic composition and mass spectrometry analysis of FOs, it can be deduced that peaks 1 through 4 sequentially correspond to feruloyl arabinosyl xylo-pentose (FAX5), feruloyl arabinosyl xylotetraose (FAX4), feruloyl arabinosyl xylotriose (FAX3), and feruloyl arabinosyl xylobiose (FAX2).

Impact of FOs on *In Vivo* Antioxidant Activity in S_{180}-bearing Mice

As presented in Table 4, FOs could improve the activity of SOD and GSH-Px in tumor S_{180}-bearing mice serum and reduce the content of MDA with an increased dose, thereby enhancing the antioxidant activity of tumor-bearing mice. When the dose was up to 250 mg/kg/d, the difference between the enzyme activity of the FOs group and that of the 5-FU positive control group was not significant, revealing that FOs had excellent *in vivo* antioxidant effects. It has been reported that the polymerization degree has a significant effect on the antioxidant activity of oligosaccharides (Cao *et al.* 2011; Chen and Yan 2005). Chen *et al*. (2005) found that agarooligosaccharides with a polymerization degree of 6 possessed strong antioxidant activity. According to the literature, hydrogen atoms may be directly involved in the scavenging of free radicals, and thus the antioxidant capacity depends on the capacity for hydrogen atoms. The existence of an FA structure in FOs gives a phenolic hydroxyl group in the parent nucleus, enhancing the capacity for hydrogen atoms as well as the free radical scavenging trend (Wang *et al*. 2010). Thereby, the result in our work is similar to the results of other studies (Wang *et al*. 2008, 2011).

Table 4. Effects of FOs on SOD, GSH-Px, and MDA of S_{180} Tumor-bearing Mice Serum *In Vivo*

Group	Dose (mg/kg/d)	MDA (nmol/mL)	SOD (U/mL)	GSH-Px (U/mL)
Normal control	—	2.85 ±0.49 d	237.68 ±6.07 a	577.39 ±8.97 a
Model control	—	5.62 ±0.37 a	210.10 ±6.10 d	415.51± 5.52 e
5-FU	20	4.73 ±0.28 b	223.11 ±3.85 b	524.08 ±9.73 b
FOs	50	4.99 ±0.11 b	213.40 ±3.55 cd	468.00 ±9.88 d
	100	4.12 ±0.11 c	216.49 ±3.98 bcd	496.24±6.74 c
	250	3.94 ±0.13 c	221.71 ±3.02 bc	517.09 ±5.64 b

CONCLUSIONS

1. The yield of FOs can reach 904 nM after fermentation with 60 g/L wheat bran liquid for 96 h using *A. pullulans* under the optimized conditions of initial pH 6.0, inoculum size 4.50%, and fermentation temperature 29 ^{o}C.
2. The FOs were separated and found to consist of feruloyl arabinosyl xylopentose (FAX5, Mw986), feruloyl arabinosyl xylotetraose (FAX4, Mw854), feruloyl arabinosyl xylotriose (FAX3, Mw722), and feruloyl arabinosyl xylobiose (FAX2, Mw590).
3. As the FO dose increased, the activities of SOD and GSH-Px in tumor S_{180}-bearing mice serum was promoted with decreasing MDA content, thus enhancing the *in vivo* antioxidant activity of tumor-bearing mice.

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