Optimization of Nutrition Constituents for Feruloyl Oligosaccharides Production by a New Isolate of *Aureobasidium pullulans* 2012 Under Fermentation on Wheat Bran

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One-step fermentation of wheat bran (WB) by *Aureobasidium pullulans* 2012 to produce ferulic oligosaccharides (FOs) was developed. As the WB concentration was increased, the xylanase activity and yield of FOs increased; the optimum concentration of WB was 50 to 60 g/L, which enhanced xylanase synthesis and the preparation of FOs. A moderate amount of xylan and peptone promoted xylanase synthesis and FO production. The addition of metal ions and surface active agents suppressed the yield of FOs. The optimum medium composition for FO preparation was 10 g/L xylan and 1 g/L peptone added to 60 g/L WB solution. Under these conditions, an FO yield of 774 nmol/L was achieved. According to observations by scanning electron microscopy, the internal structure of WB was obviously disrupted after fermentation. This process featured one-step fermentation of WB without further hydrolyzing, which greatly decreased the raw material cost and thus facilitated its practical application.

Keywords: A. pullulans; Culture medium; Ferulic oligosaccharides (FOs); Wheat bran; Xylanase

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INTRODUCTION

In recent years, the extraction of natural antioxidants from plants has attracted increasing attention as an alternative to chemically synthesizing antioxidants due to the negative effects of high toxicity, poorer heat stability, lower antioxidation effects, and narrower application ranges. The synthetic antioxidants can be harmful to the organs such as liver, spleen, and lung of humans. Therefore, there has been growing interest in natural antioxidants from plant origin due to their promising biological capacities to protect the human body against free radicals, retard the progression of many chronic diseases, and retard lipid oxidative rancidity in foods. Although substantial breakthroughs have been achieved, the development of natural antioxidants has been restricted as a result of two major problems (Villaño *et al.* 2012). First, some natural antioxidants, such as carotene, phenolic compounds, and vitamin E, are water insoluble. A second problem is the unsuitability of thermal processing because of the heat-sensitive characteristics of antioxidants such as vitamin C. The ferulic oligosaccharides (FOs), which mainly are present in gramineous plants, are functional oligosaccharides formed through the carboxyl esterification of ferulic acid (FA) and sugar hydroxyl groups. The ferulic acyl and hydrophilic oligosaccharide groups in their molecular structures make FOs water soluble and heat resistant (Faulds *et al.* 1995; Yuan *et al.* 2006). Hence, FOs are natural

antioxidants of high research and application value that have solved the aforementioned two problems successfully (Wang *et al.* 2008, 2009a; 2010). The antioxidant activity of FOs is higher than that of FA and vitamin C, exhibiting a strong inhibiting effect on the hemolysis of mice red blood cells, as well as eliminating Fe^{2+}, H_2O_2, and hydroxyl radicals (Wang *et al.* 2010, 2011). They have also been reported to present significant antioxidant capacity in DPPH and lipid peroxidation systems (Wang *et al.* 2008, 2009a). FOs have been found to stimulate the growth of *Lactobacillus* and *Bifidobacteria* while inhibiting that of gram-positive bacteria in the human body (Yuan *et al.* 2005a). In addition, FOs can significantly inhibit the growth of lymphocytes in leukemia patients and accelerate gangrene in leukemia T cells (Akhtar *et al.* 2012; Wang *et al.* 2009b), which means that they have broad application prospects as a health-promoting food. However, further applications of FOs have been limited due to a lack of efficient means of production.

At present, the methods of producing FOs include physical methods, chemical methods, biological enzymes, and biological fermentation. Rose and Inglett (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 were gradually degraded to FA, xylose, and arabinose. Physical methods are used less frequently due to their complicated and strict processing conditions. It has also been reported that 3-fluoroacetic acid could be used to hydrolyze corn bran to obtain several kinds of FOs with different structures (Allerdings *et al.* 2005; Schooneveld-Bergmans *et al.* 1998). 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 enzymatic method of FO production is commonly used due to its mild reaction conditions. Yuan *et al* (2005b, 2006) optimized the conditions for the hydrolysis of WB by xylanase (EC 3.2.1.8) and obtained 1.5 mM FOs. Katapodis and Christakopoulos (2008) used xylanase produced by *Thermoascus aurantiacus* to hydrolyze corn gluten to produce FOs. The FA in WB mostly exists in insoluble dietary fiber, which is covalently cross-linked with xylan by an ester bond (Yuan *et al.* 2005b; Zhang *et al.* 2011). Therefore, using the biological enzymes 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 waste water.

Based on the aforementioned issues, a method of directly using xylanase-producing microorganisms to produce FOs seems attractive and worth investigating. In this approach, the enzyme production process during microorganism fermentation would be combined with the process of obtaining FOs by enzymatic hydrolysis of xylan. Hence, the separation and purification process involved in enzyme and insoluble dietary fiber preparation is avoided, reducing the production cost as well as improving the production rate. This method has been confirmed by Ferreira *et al*. (2007), who used *Streptomyces* to ferment beet pulp, during which FOs were detected. However, excess activity of ferulic acid esterase (EC 3.1.1.73, FAE) was produced during *Streptomycete*, *Aspergillus*, and *Penicillium* fermentations, which hydrolyzes FOs (Topakas *et al.* 2007). Hence, selecting appropriate microorganisms or controlling the fermentation process (so that the endo-xylanase activity is enhanced and the FAE activity is diminished) is the key to solving the above problems.

A. pullulans is one kind of fungus that can produce endo-xylanases (xylanase or 1-4-β-D-xylanxylanohydrolase, EC 3.2.1.8) of high activity and specificity. Endo-xylanases can selectively hydrolyze hemicellulose while not affecting cellulose (Christov *et al.* 1997), which may help to improve the purity of FOs. It is well documented that *A. pullulans* will synthesize different xylanase isomers from different carbon sources, and it is thermally stable (Manitchotpisit *et al.* 2009; Singh *et al.* 2008). 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. Furthermore, the effects of WB concentration and the culture medium, including the carbon source, nitrogen source, metal ions, and surfactants added to WB, on xylanase activity and FO production were also investigated.

EXPERIMENTAL

Microorganism and Materials

A. pullulans 2012 was isolated from the soil surrounding a flour factory. Stock cultures were maintained on potato dextrose agar at 4 °C and subcultured every 2 weeks. WB was supplied by the flour mill of Qinda Co., Ltd. of Jiangsu, China. Lactose, sucrose, glucose, xylose, xylan, $ZnSO_4$, $Fe_2(SO_4)_3$, and $Ca(CO_3)_2$ were all analytical grade reagents. Sorbitan monooleate ethoxylate plant oil, and saccharose ester were all of food grade and purchased from the Jiehua Chemical Co., Ltd., Shanghai, China.

Preparation of the Inoculum Medium

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 °C for 20 min.

Preparation of the Fermentation Medium

Dried WB was crushed into flour and passed through a 40-mesh sieve to remove some starch. Then, the WB obtained was dissolved in distilled water. The pH of the mixture was adjusted to 5.5 with a 2% (v/v) sulfuric acid solution to concentrations of 10, 20, 30, 40, 50, 60, 70, or 80 g/L WB and incubated at 50 °C for 2 h. The resulting WB solutions were prepared as fermentation substrates.

Preparation of Compound Fermentation Medium

The compound carbon source culture medium was prepared by adding 10 g/L glucose, 10 g/L xylose, 10 g/L lactose, 10 g/L xylan, and 10 g/L sucrose to a mixture of 60 g/L WB.

The compound nitrogen source culture medium was prepared by adding 2 g/L yeast extract, 2 g/L peptone, 2 g/L beef extract, 2 g/L bean pulp, and 2 g/L steepwater to a mixture of 60 g/L WB.

The compound metal ion culture medium was prepared by adding 1 mmol/L Zn^{2+}, 1 mmol/L Mg^{2+}, 1 mmol/L Fe^{3+}, 1 mmol/L Ca^{2+}, and 1 mmol/L K^{+} to a mixture of 60 g/L WB.

The compound surfactants culture medium was prepared by adding 1 g/L sorbitan monooleate ethoxylate, 1 g/L plant oil, and 1 g/L saccharose ester to a mixture of 60 g/L WB. The WB solution (60 g/L) alone was used as a control.

Fermentation

Seed culture was prepared by inoculating cells grown on a potato dextrose agar slant into a 250-mL flask that contained 50 mL of the inoculum medium and subsequently incubating at 28 °C for 3 days with shaking at 180 rpm. A 5-L stirred tank fermenter (5M-2002, Shanghai Baoxing Bio-engineering Equipment Co., China) with a working volume of 3 L, was used for the production of FOs in batch culture. The fermenter consisted of a glass vessel with stainless-steel endplates and three equally spaced vertical baffles. Agitation was provided by an impeller with six flat blades (diameter 4 cm) located 3 cm above the bottom of the vessel. The fermenter was sterilized at 121 °C for 20 min. After cooling, 3 L of production medium was added to the fermenter. Then, a 200-mL inoculum was introduced. The fermenter was incubated at 28 °C in a thermostated chamber. The impeller speed was 600 rpm, and the sterile air flow was 3 L/min. The pH was controlled at 6.0 by adding either 2 M NaOH or 2 M HCl.

Xylanase Assays

The xylanase activity was assayed with 10 mg/mL oat xylan (w/v) as substrate, and the liberation of reducing sugars was estimated by the dinitrosalicylic acid (DNS) procedure. One unit of xylanase activity (IU) was defined as the amount of enzyme necessary to produce 1 μmol reducing sugars hydrolyzed from the WB xylan substrate per min at 50 °C.

FO Assays

FOs produced during WB fermentation by *A. pullulans* were determined as described by Xie *et al*. (2010) with slight modifications. Briefly, the fermentation broth was centrifuged at 4000 rpm for 15 min, and the supernatant containing FA and FOs were carefully collected. One milliliter of the collected supernatant was filtered using a 0.45 μm membrane and analyzed by HPLC for measuring the content of free form FA. One milliliter of the supernatant was mixed with 1 mL of 1 mol/L NaOH and allowed to react at 100 °C in the dark for 90 min to completely hydrolyze the FOs. Next, the mixture was cooled and neutralized with 1 M HCl, and analyzed by HPLC. FO determination was carried out by measuring the added content of FA hydrolyzed from the FOs using NaOH.

HPLC analysis for the FA assay was conducted on an Agilent 1200 (Agilent, USA) system using a UV detector (325 nm) and an Agilent TC-C18 column (4.6×250 mm, 5 μm). The solvents consisted of A (0.5% acetic acid in water, v/v) and B (acetonitrile), and the linear gradient was run at 25 °C for 15 min from 5% to 20% and for 15 to 40 min from 20% to 40% of B at a flow rate of 0.6 mL/min. Portions of 20 μL were injected for HPLC analysis.

Analytical Methods

Moisture, total protein, crude fat, soluble dietary fiber, mineral elements, and starch content were estimated by standard AOAC Methods (AOAC, 1990). The nitrogen conversion factor for protein was 5.95. All contents are expressed on a dry basis. WB before and after fermentation by *A. pullulans* was sampled, fixed in 3% glutaraldehyde

solution for 12 h at 4 °C, dehydrated, and dried, and the surface of the WB was coated with metal and observed with an Hitachi S-3000 N scanning electron microscope.

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

Analysis of the WB Composition and the Effect of the Concentration of the WB Solution on FO Preparation

The contents of major fractions of WB were tested. IDF content was the largest component in WB (45.62%), followed by starch (12.96%) and protein (12.56%). These findings are similar to the results of Chen *et al*. (2006).

A. pullulans was cultivated in medium with WB as the only carbon source (Fig. 1). As its concentration increased, the xylanase activity and FO yields increased. The optimum concentration of WB solution was 50 to 60 g/L for enhancing xylanase synthesis and the preparation of FOs. This suggests that the nutrients in WB could meet the needs of microbial growth as carbon, nitrogen, and mineral sources (Gessesse and Mamo 1999).

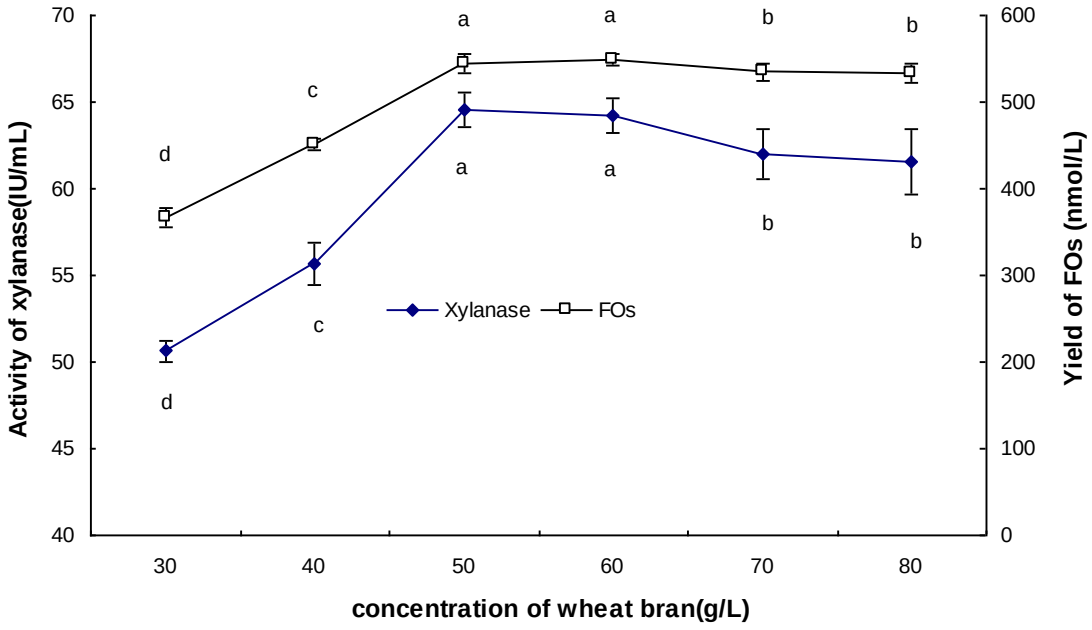

Fig. 1. Effects of the concentration of WB on the biosynthesis of xylanase by *A. pullulans* 2012 and the yield of FOs

Effects of WB Combined with Carbon Sources on Xylanase Activity and FO Production

Xylanase activity and FO production followed a trend of increasing up to the fourth day and decreasing afterwards during the monitored fermentation period (Fig. 2). The decrease in xylanase activity might be attributed to the degradation of xylanase caused by the proteinases formed by *A. pullulans* metabolism after the fourth day, whereas FO was consumed by *A. pullulans* to satisfy its growth needs. Hence, a 4-day fermentation time was judged to be the most suitable to obtain the highest FO content and xylanase activity.

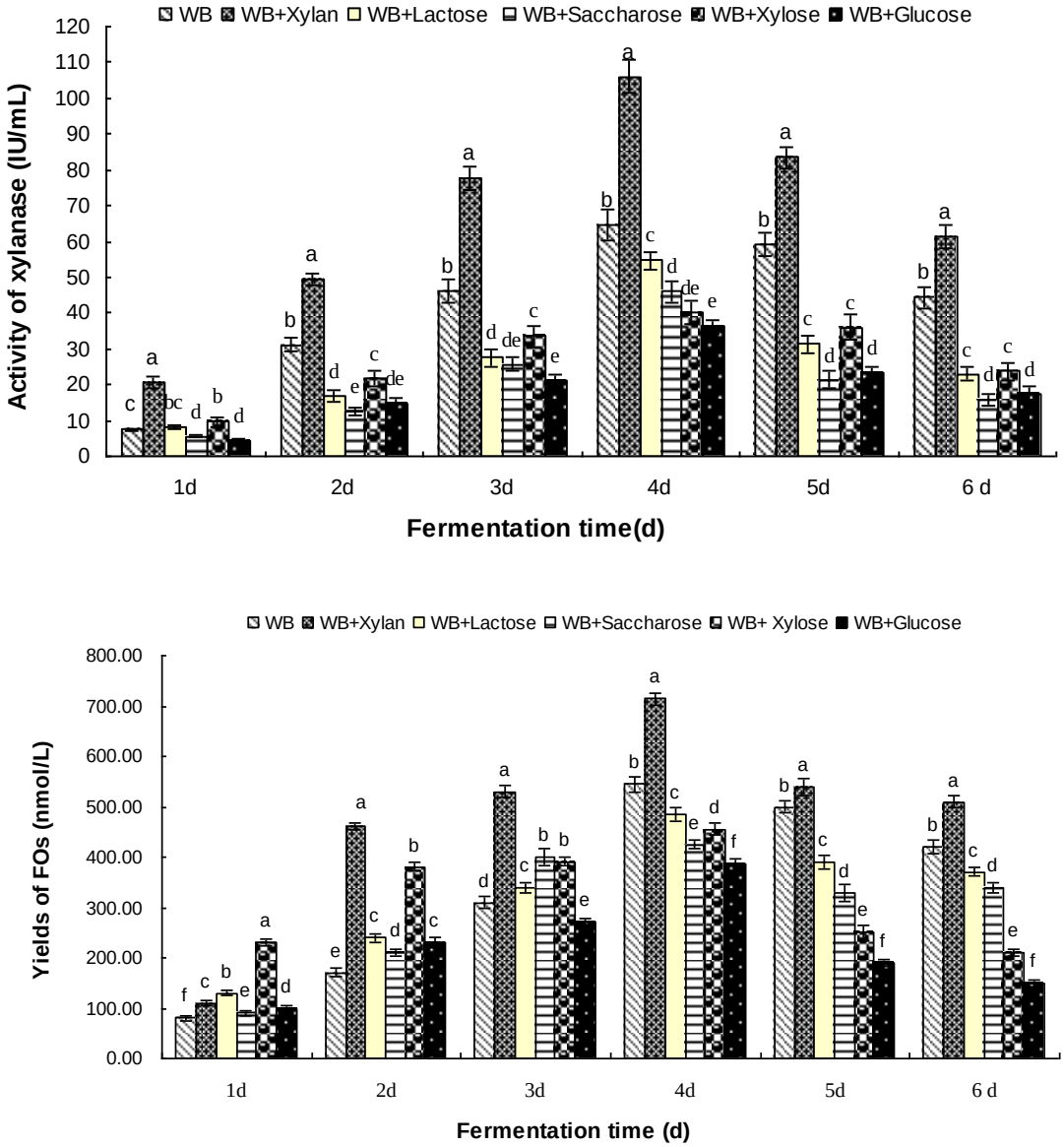

Fig. 2. Effects of WB combined with different carbon sources on the biosynthesis of xylanase by *A. pullulans* 2012 and the yields of FOs

The peak values of xylanase activity and FO production at the fourth day were 64.56 IU/mL and 545 nmol/L in the control group (Fig. 2), respectively. The addition of different carbon sources significantly changed the xylanase activity and FO production,

indicating that carbon sources could influence the biosynthesis of xylanase and FOs. Xylan exhibited the greatest effect. The addition of xylan increased the xylanase activity and FO production (fourth day) by 169.10% and 133.33%, respectively, compared to the control. At the end of the fermentation time, xylanase activity and FO production had increased by 116.04% and 377.78%, respectively. However, simple carbon sources such as glucose, xylose, lactose, and saccharose decreased the xylanase activity and FO production. The biosynthesis of xylanase can be inhibited by xylose in most cases, while there are a few cases that can be induced by xylose (Maximo *et al*. 1998). Numerous studies have been conducted on the induction of microbial enzymes by carbon sources, whereas little information is available on the biosynthesis of FOs by xylanase during *A. pullulans* fermentation.

The results showed that the addition of 10 g/L xylan into the 60 g/L WB fermentation solution enhanced xylanase synthesis and FO production. Thus, it was preliminarily inferred that the production of xylanase by *A. pullulans* was inducible. Kulkarni *et al.* (1999) and Zhang *et al.* (2013) found that sustained release-type inducers were more effective at improving the biosynthesis of xylanase. For *A. pullulans*, xylan was a type of sustained-release inducer, so when xylan was added to the WB culture medium, the biosyntheses of xylanase and FOs were enhanced more than when other carbon sources were added.

Effects of WB Combined with Nitrogen Sources on Xylanase Activity and FO Production

Organic nitrogen sources tend to be more suitable for fungal growth and metabolism compared with inorganic nitrogen sources (Kim *et al.* 2005). Organic nitrogen sources had substantial impacts on the biosyntheses of xylanase and the production of FOs (Fig. 3), whereas different nitrogen sources had various effects.

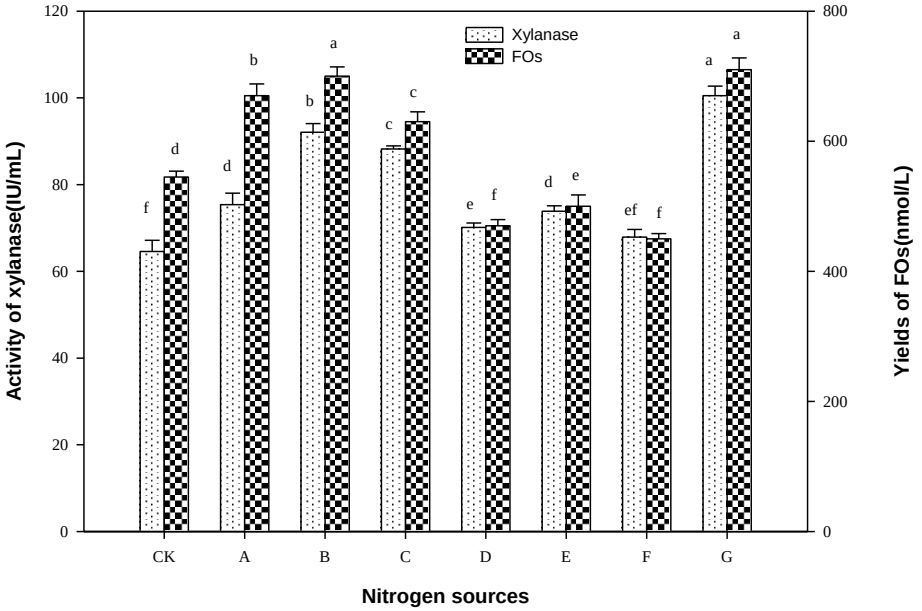

Fig. 3. Effects of WB combined with different nitrogen sources on the biosynthesis of xylanase by *A. pullulans* 2012 and the yield of FOs; CK: WB, A: WB+yeast extract, B: WB+peptone, C: WB+beef extract, D: WB+bean pulp, E: WB+ammonium sulfate, F: WB+ammonium nitrate, and G: WB+steepwater

Xylanase activity and FO production were greatly promoted by steepwater, peptone, beef extract, and yeast extract. These findings are in agreement with Oliveira *et al.* (2006) and Cai *et al.* (2004). It was reported that peptone had the greatest effect in increasing the xylanase activity (Xu 2005). Cai *et al.* (2004) also showed that the xylanase activity of *Pluribus* cultivated in a medium containing 0.8% peptone was the highest compared with other nitrogen sources. Hence, considering their stronger effects on xylanase activity and FO production, steepwater and peptone were selected as nitrogen sources for future research.

Effects of WB Combined with Metal Ions on Xylanase Activity and FO Production

Xylanase activity and FO production induced by different metal ions varied. For example, Ca^{2+}, Mg^{2+}, and K^{+} accelerated the xylanase activity of *A. pullulans* while decreasing FO production (Fig. 4). This interesting phenomenon could be the result of FO consumption during the growth of *A. pullulans*. However, another possibility is that the formation of other enzyme series activated by Ca^{2+}, Mg^{2+}, and K^{+} could hydrolyze FOs (Xu *et al.* 2008).

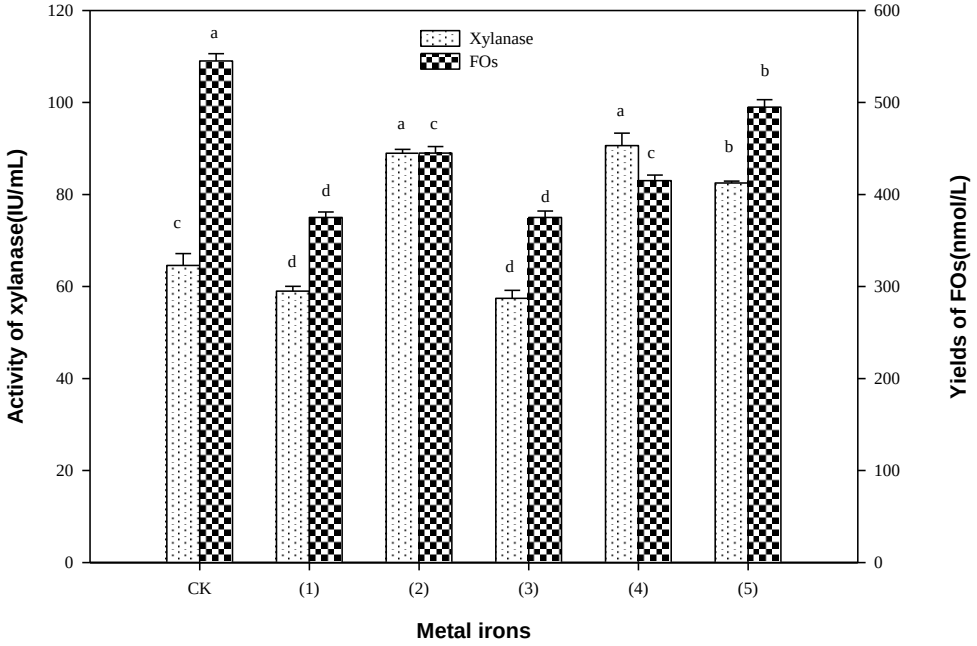

Fig. 4. Effects of WB combined with different metal ions on the biosynthesis of xylanase by *A. pullulans* 2012 and on the yield of FOs; CK: WB, (1): WB+Zn^{2+}, (2): WB+Mg^{2+}, (3): WB+Fe^{3+}, (4): WB+Ca^{2+}, and (5): WB+K^{+}

It had been demonstrated that metal ions can activate a number of enzymes (Irshad *et al.* 2013; Li *et al.* 2008). For example, Mg^{2+} was the activating factor of both proteinase and (1→4)-β-d-glucuronanase of *Rhizobium meliloti* (Michaud *et al.* 1994). Heck *et al.* (2005) found that xylanase released from *Bacillus circulan* could be activated by Co^{2+} and Fe^{3+}. The results of Khandeparkar and Bhosle (2006) also suggested that Ca^{2+} and Mg^{2+} could improve the activity of the xylanase produced by *Arthrobacter* sp. MTCC 5214, which agreed with our results. Hence, the reason for the reduction in FO

production could be further confirmed by the fact that there were many other enzymes hydrolyzing FOs that were activated by Ca^{2+}, Mg^{2+}, and K^{+}. In addition, xylanase is a kind of protein and thus could be hydrolyzed by proteinase (Xu. 2005), so the FO production might also be reduced through this mechanism.

Effects of WB Combined with Surfactants on Xylanase Activity and FO Production

Xylanase activity and FO production were both reduced after surfactants were added to the fermentation system (Fig. 5). A previous study indicated that a low concentration of surfactants could stimulate the biosynthesis of xylanase, whereas a high concentration would achieve the opposite result (Zeng *et al.* 2006). This phenomenon was also found during the addition of plant oil to *Cordyceps militaris* fermentation for polysaccharide production (Park *et al.* 2002). Plant oil is similar to surfactants; they could both change the permeability of the cell membrane and improve the growth of fungi and metabolite production (Hsieh *et al.* 2008). WB was used as the fermentation substrate in this study, and it contained 2.74% crude fat, which means there was a certain concentration of oil in the medium. Consequently, the addition of surfactants such as Sorbitan monooleate ethoxylate and plant oil failed to promote the biosynthesis of xylanase and FOs by *A. pullulans*.

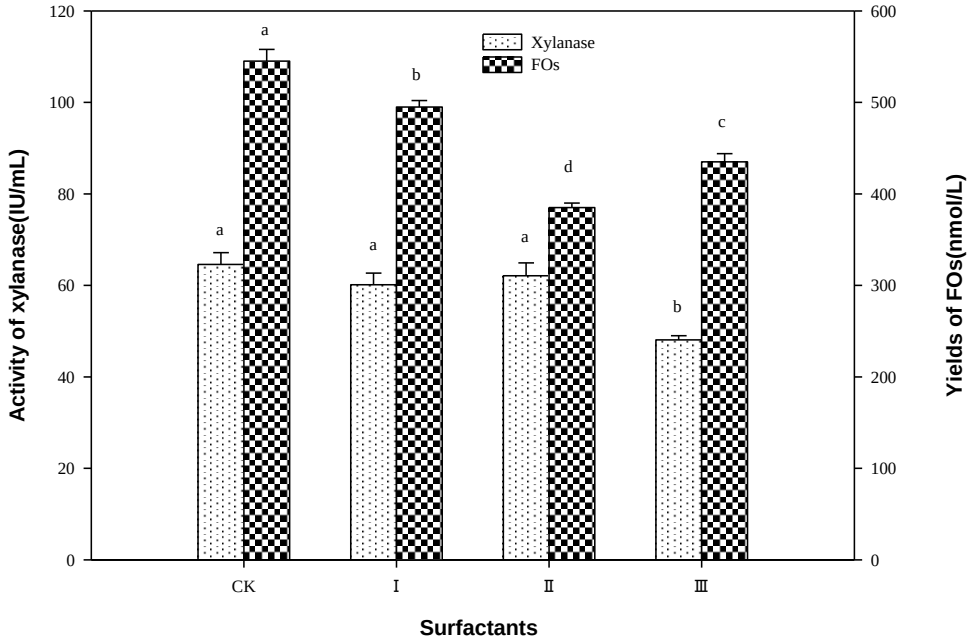

Fig. 5. Effects of WB combined with surfactants on the biosynthesis of xylanase by *A. pullulans* 2012 and on the FO yield; CK: WB, Ⅰ: WB+Sorbitan monooleate ethoxylate, Ⅱ: WB+plant oil, Ⅲ: WB+saccharose ester

To summarize, adding metal ions and surfactants failed to improve FO production. Hence, metal ions and surfactants were no longer added in the subsequent experiments designed to optimize the fermentation conditions.

Optimization of the Compound Culture Media for FO Production

On the basis of single-factor experiments, the culture components for FO production, namely xylan, peptone, and steepwater, were optimized using orthogonal experimental design. The factors and levels investigated are shown in Table 1. The factors had significant effects on FO production, and the sequence of the influence of xylan, peptone, and steepwater on FO production was RA > RB > RD > RC. This indicated that xylan had the greatest impact on FO production, followed by peptone. Steepwater had less impact on FO production than that of the null columns.

Table 1. Design and Results of Orthogonal Experiment

No.	Xylan (g/L) A	Peptone (g/L) B	Steepwater (g/L) C	Null columns D	FO yield (nmol/L)
1	1(0)	1(0)	1(0)	1	353±8
2	1	2(1)	2(1)	2	718±12
3	1	3(2)	3(2)	3	322±4
4	2(5)	1	2	3	407±7
5	2	2	3	1	684±13
6	2	3	1	2	545±14
7	3(10)	1	3	2	693±16
8	3	2	1	3	767±11
9	3	3	2	1	665±9
k_1	464.33	484.33	555.00	567.33	
k_2	545.33	723.00	596.67	652.00	
k_3	708.33	510.67	566.33	498.67	
R	244.00	238.67	41.67	153.33	
RA: range of Xylan; RB: range of Peptone; RC: range of Steepwater; RD: range of Null columns.					

Table 2. Analysis of Variance for Orthogonal Experiment

Factor	Bias squares	Degrees of freedom	F ratio	F critical value	Significant
A	92666.00	2	33.28	19.00	*
B	102740.67	2	36.90	19.00	*
C	2784.67	2	1.00	19.00	
D	35394.67	2	12.71	19.00	
error	2784.67	2			
*mean α=0.05 level, significant difference.					

Changes in WB Configuration Before and After Fermentation by *Aureobasidium pullulans* 2012

The structure of WB was compact and smooth before fermentation. However, this structure broke down, loosened, and formed cavities, and its internal structure was obviously disrupted after fermentation by *A. pullulans* (Fig. 6). This showed that the WB fibers were hydrolyzed by enzymes generated during the *A. pullulans* fermentation process, and the WB fibers can be hydrolyzed for the purpose of producing FOs in a single-step process. The changes in fiber structure were similar to those seen in WB fermented by *Agrocybe chasingu* (Xie *et al.* 2010).

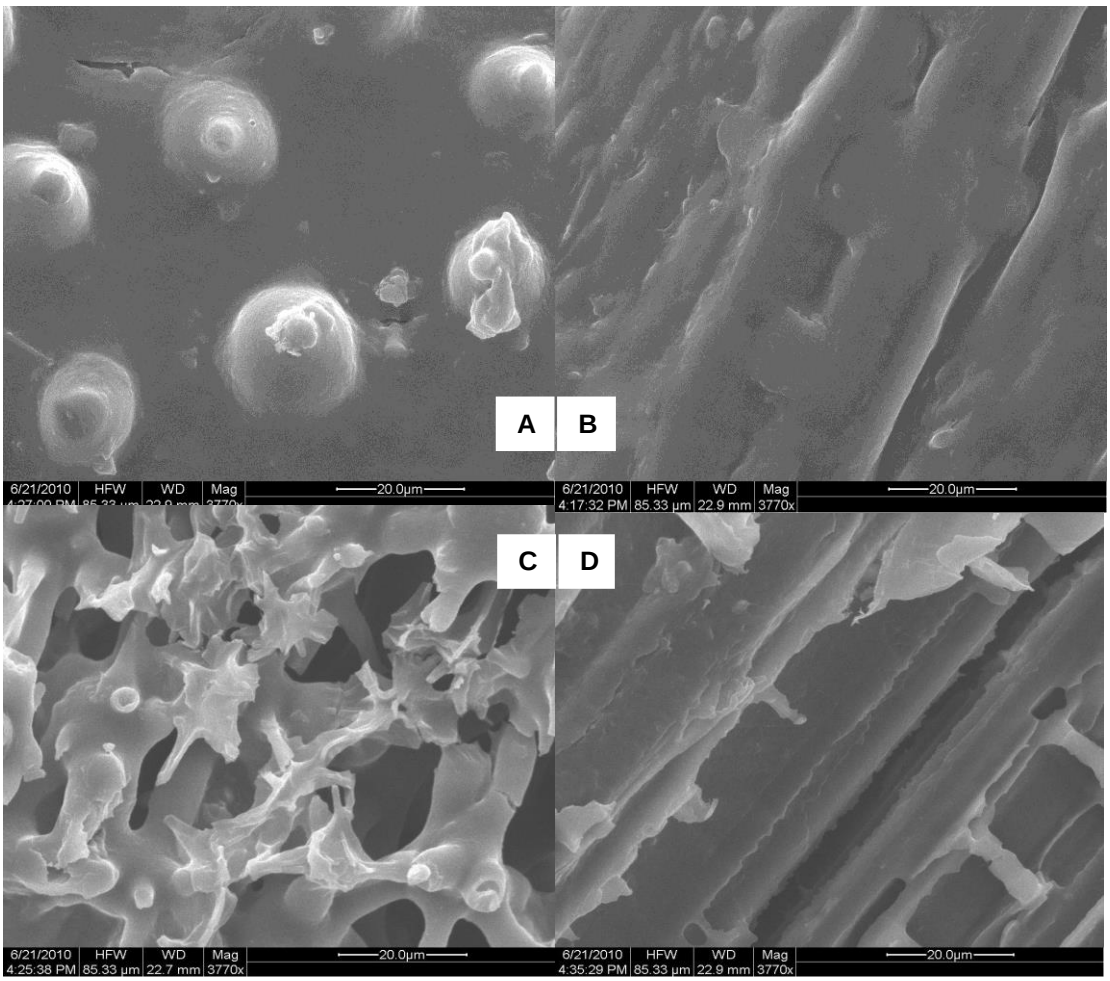

Fig. 6. Changes in the WB configuration before and after fermentation by *A. pullulans*; Fig. A and B depict the structure of the WB fibers before fermentation; C and D depict the structure of the WB fibers after fermentation

CONCLUSIONS

1. One-step fermentation of wheat bran (WB) by *A. pullulans* 2012 to produce ferulic oligosaccharides (FOs) was investigated. The optimum medium composition for FO preparation was 10 g/L xylan and 1 g/L peptone added to a 60 g/L WB solution. Under these conditions, a yield of 774 nmol/L FOs was achieved.

2. A moderate amount of xylan and peptone added to 60 g/L WB solution promoted xylanase synthesis and FO production. The addition of metal ions and surface active agents failed to improve the yield of FOs.
3. The internal structure of WB was obviously disrupted after fermentation.

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