Effects of Two-stage Controlled pH and Temperature *vs.* One-step Process for Hemicellulase Biosynthesis and Feruloyl Oligosaccharide Fermentation using *Aureobasidium pullulans*

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A two-stage, pH- and temperature-controlled wheat bran fermentation method using *Aureobasidium pullulans* was investigated for feruloyl oligosaccharides (FOs) production and the activities of xylanase, xylosidase, and ferulic acid esterase (FAE). *A. pullulans* secreted xylanase, xylosidase, and FAE at high levels in the initial pH of 4.0 to 5.0 and a fermentation liquid temperature of 31 °C to 33 °C. FOs production *via* two-stage fermentation (FOs 2) reached 1123 nmol/L after fermentation for 96 h, by controlling the initial pH at 4.0 and the initial temperature at 33 °C, and then changing the pH to 6.0 and the temperature to 29 °C at the same time at 36 h. This process was 12 h shorter and 219 nmol/L higher than a one-stage fermentation for producing FOs 1. Xylanase, xylosidase, and FAE activities were highly correlated with controlled pH and temperature and FOs biosynthesis rate. Thus, the combination of two-stage controlled pH and temperature could support mass production of FOs.

Keywords: Aureobasidium pullulans; Feruloyl oligosaccharides; Wheat bran; Two-stage controlled pH and temperature; Hemicellulase

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INTRODUCTION

Feruloyl oligosaccharides (FOs), which are found in gramineous plants, are formed by the esterification of the carboxyl group of feruloyl acid (FA) and the hydroxyl groups of sugars (Yu *et al*. 2013, 2014a,b). As natural antioxidants, FOs are very valuable. The antioxidant activity of FOs is higher than FA and vitamin C, and they strongly inhibit hemolysis in mouse red blood cells and eliminate Fe^{2+}, H_2O_2, and hydroxyl radicals (Wang *et al*. 2010, 2011). FOs also display significant antioxidant capacity in 1,1-diphenyl-2-picrylhydrazyl (DPPH) and lipid peroxidation systems (Wang *et al*. 2008, 2009). However, further application of FOs has been limited because their production is inefficient (Yu *et al*. 2014a).

FOs are produced by physical methods (Rose and Inglett 2010), chemical methods (Schooneveld-Bergmans *et al*. 1998; Allerdings *et al*. 2005), biological enzymes (Yuan *et al*. 2005; Yuan *et al*. 2006; Katapodis *et al*. 2003), and biological fermentation (Xie 2010; Yu and Gu 2013; Yu and Gu 2014a). However, physical methods are used less frequently due to their complicated and strict processing conditions. Chemical methods cannot be applied in the food and pharmaceutical industries because of the detection of chemical residues and the environmental pollution caused by the by-products. Using biological

enzymes to produce FOs requires extraction of the insoluble dietary fiber from the raw materials, which not only increases production costs but produces a large quantity of wastewater. Thus, a single-step biological fermentation method using microorganisms to produce hemicellulases, which subsequently generate FOs, is worth investigating.

The fermentation method has been carried out with *Streptomyces* spp., *Aureobasidium pullulans* (*A. pullulans*), or *Agrocybe chaxingu* to ferment beet pulp or wheat bran (WB) and produce FOs (Ferreira *et al*. 2007; Xie 2013; Yu and Gu 2013, 2014a). In this approach, enzymes produced during microbial fermentation initiate xylan hydrolysis, which releases FOs. However, excess ferulic acid esterase (FAE; EC 3.1.1.73) produced during *Streptomyces*, *Aspergillus*, and *Penicillium* fermentation hydrolyzes FOs (Topakas *et al*. 2007). Hence, the activity of hemicellulases needs to be regulated so that fermentation produces a high yield of FOs with a single-batch operation; this result requires higher xylanase activity and appropriate xylosidase and FAE activities (Yu and Gu 2013, 2014a). Hence, selecting the appropriate microorganisms and controlling the fermentation process is crucial.

The dimorphic fungus *A. pullulans* is an excellent production microbe for the xylanase system; it simultaneously produces intracellular xylanase and xylosidase and extracellular FAE (Ohta *et al*. 2010; Yu and Gu 2014a). Yu and Gu (2013, 2014a) reported that FO yield and xylanase activity are positively correlated (r = 0.992), and FO yield and FAE activity are positively correlated (r = 0.802). The induction of hemicellulases by *A. pullulans* 2012 can be regulated and controlled by varying the carbon source, fermentation temperature, and pH (Yu and Gu 2013, 2014a,b). Because culture pH, incubation temperature, and hemicellulases are pivotal to FOs production (Yu and Gu 2014b), there is motivation to study this topic. To date, the effects of two-stage controlled pH and temperature on hemicellulose activity and FOs production have not been reported.

In this study, *A. pullulans* 2012 fermentation was optimized for FOs production with WB (60 g/L liquid), 10 g/L xylan, and 1 g/L peptone. The effects of two-stage control of pH and temperature in a single-batch operation with respect to the production of FOs (FOs 2) and hemicellulose activity (such as xylanase, xylosidase and FAE) were investigated, whether the optimal pH and temperature for hemicellulases and FOs production are the same or not, and the relevant conditions were optimized. The optimal technology for FOs production *via* fermentation will support the application of FOs as functional biological materials.

EXPERIMENTAL

Materials and Microbes

WB was supplied by the Qinda Co. Ltd. flour mill, Yancheng, China. Ferulic acid, xylose, xylan, and methyl ferulate standards were purchased from Nanjing Scigene Technology Co Ltd, Nanjing, China. *A. pullulans* (2012 strain) was isolated from the soil surrounding a flour factory; the organism and its inoculum and fermentation media were described previously (Yu and Gu 2014a). The seed culture was inoculated with a full loop of *A. pullulans* from a fresh slant tube in an Erlenmeyer flask (500 mL) containing 100 mL of fresh medium. The culture was cultivated by agitation using a reciprocal shaker (180 rpm) at 28 °C for 72 h.

Optimization of the Two-stage Fermentation Process

Effects of the initial pH and temperature on xylanase, FAE, xylosidase, and FOs

The initial conditions for two-stage fermentation were based on the pH and temperature used in one-stage fermentation of FOs (Yu and Gu 2013; Yu and Gu 2014b).When the fermentation temperature was maintained at 29 °C, the initial pH was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, or 7.0. When the initial pH was adjusted to 6.0, the fermentation temperature was controlled at 25 °C, 27 °C, 29 °C, 31 °C, 33 °C, or 35 °C. The activity of xylanase, FAE, and xylosidase and FOs yield in the supernatant were detected after fermentation of *A. pullulans* 2012 in a 5-L automatic fermenter for 96 h.

Temperature and pH in the second stage of fermentation

Fermentation conditions were designed as follows: 1) constant temperature (29 °C) and pH (6.0); 2) constant temperature (29 °C) and variable pH (4.0 for the first stage and changed to 6.0 at 24 h, 36 h, or 48 h by feeding with 2 M NaOH); 3) constant pH (6.0) and variable temperature (33 °C for the first stage and changed to 29 °C at 24 h, 36 h, or 48 h); and 4) variable pH and temperature (4.0 and 33 °C, respectively, for the first stage and changed to 6.0 and 29 °C, respectively, at 24 h, 36 h, or 48 h by feeding with 2 M NaOH). WB was fermented by *A. pullulans* 2012 in a 5-L automatic fermenter for 108 h. Samples were taken every 12 h to determine FOs yield and the activity of xylanase, FAE, and xylosidase.

Analysis of Enzyme Activity and FO Yield

Crude enzyme was prepared from the extracellular matrix and the hyphal filaments as described by Yu and Gu (2014a). Xylanase and xylosidase activity were determined as described by Katapodis *et al.* (2003) and Yu and Gu (2014a). FAE activity was determined by the method reported by Xie *et al.* (2010). One unit of enzyme activity (IU) was defined as the amount of enzyme producing 1 μmol of hydrolyzed product from the substrate per min at 50 °C. FOs yield from *A. pullulans* 2012 fermentation of WB was determined as described by Yu and Gu (2014a).

Statistical Analysis

All data was expressed as means ± SD of triplicates. Statistical analyses were performed in SPSS 11.0 software (SPSS Inc., Chicago, USA). Differences were considered to be statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Effect of pH and Temperature on Xylanase, Xylosidase, and FAE Activity and FOs Yield

Fermentation parameters influence the activity of *A. pullulans* xylanase, xylosidase, and FAE (Cately 1979; Yu and Gu 2014a), which subsequently affects FOs production. The effects of different initial medium pH values ranging from 2.0 to 7.0 and fermentation temperature ranging from 25 to 35 °C on hemicellulases and FOs production by *A. pullulans* 2012 were investigated (Figs. 1 and 2). As the initial pH and fermentation temperature increased, hemicellulase activity and FOs yield increased at first and then decreased. *A. pullulans* 2012 secretes high levels of xylanase, xylosidase, and FAE at an initial pH of 4.0 to 5.0 and a fermentation liquid temperature of 31 to 33 °C.

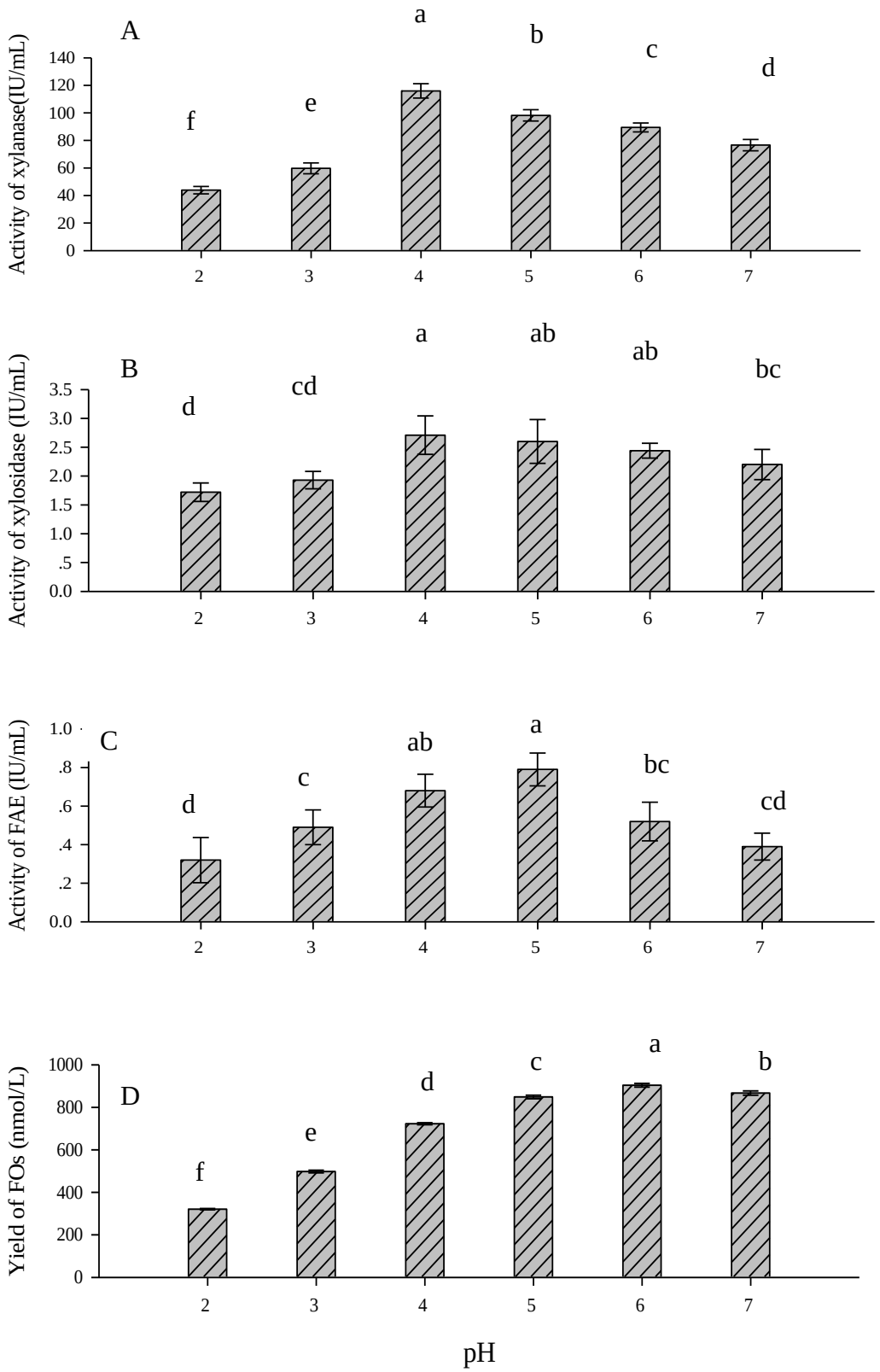

Fig. 1. The effects of initial pH on the biosynthesis of xylanase (A), xylosidase (B), and FAE (C) and the yield of FOs 1 (D) in *Aureobasidium pullulans.* Fermentation conditions: controlled temperature 29 °C and time 96 h. Within each graph, means with different lowercase letters are significantly different ($P < 0.05$).

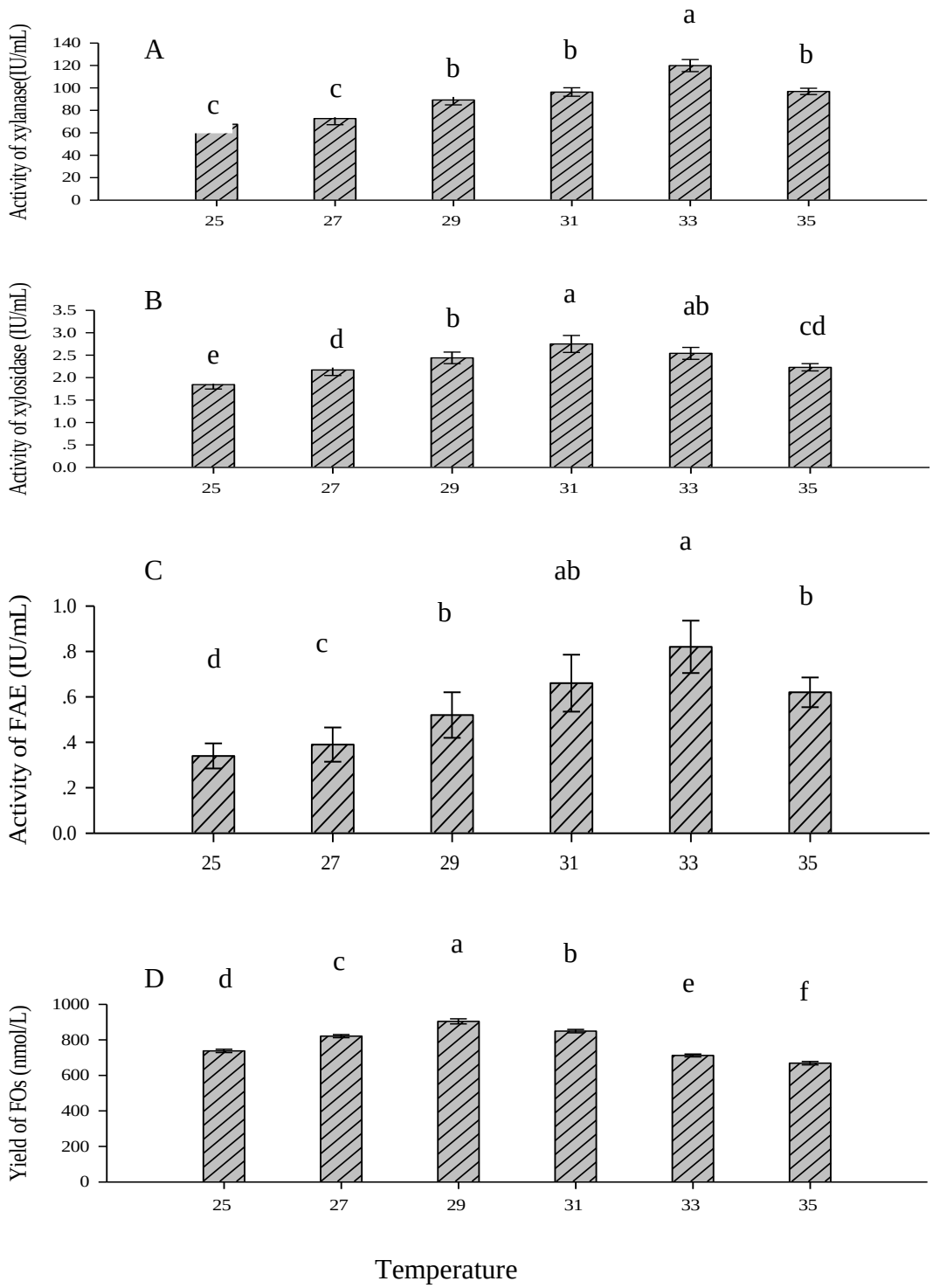

Fig. 2. Effects of fermentation temperature on the biosynthesis of xylanase (A), xylosidase (B), and FAE (C) and the FOs 1 yield (D) in *Aureobasidium pullulans*. Fermentation conditions: controlled pH 6 and time 96 h. Within each group, means with different lowercase letters are significantly different ($P < 0.05$).

However, *A. pullulans* ferments WB and produces FOs at a high level when the initial pH is maintained at 6.0 and the temperature is 29 °C. The differences agreed with the study of Xia *et al.* (2011) who found that optimal pH and temperature of cell growth is not in accordance with that of pullulan production by *A. pullulans*. Sirma *et al.* (2014) found maximum xylanase activity of *A. pullulans* after 96 h of cultivation at 28 °C and initial pH 3.0. Similarly, 0.44 U/ml β-xylosidase activity was present in the culture filtrate grown on medium with initial pH of 5.0 at 30 °C for 5 days by *A. pullulans* strain ATCC 20524 (Ohta *et al*. 2010). In contrast, under the conditions of pH 6 and 26.5 °C, 1542 mU/g FAE activity was produced by the fungus *Penicillium brasilianum* (Panagiotou *et al*. 2006). The optimum pH for *A. pullulans* to produce pullulan is 5.0 by (Seo *et al*. 2004) or 6.0 (Lee and Yoo 1993). Furthermore, Xie (2010) confirmed that pH 5.5 supported *Agrocybe* production of FO. The different optimum fermentation pH and temperature conditions reported in the literature may be due to the differences in the types of strain or microorganism, composition of fermentation medium, and culture conditions. Results indicated that the optimal pH and temperature for hemicellulases production is not in accordance with that of FOs production. Hence, there are differences between hemicellulases and FOs fermentation process conditions.

Culture pH and incubation temperature are important for FOs production (Yu and Gu 2014b). To ensure a higher xylanase activity, appropriate activities of xylosidase and FAE, and optimal FOs output, it was necessary to adopt a two-stage process to control the initial pH of the fermentation, the pH during the fermentation, the starting temperature, and the temperature during the fermentation when FOs are generated.

Two-step Process Study for Activity of Xylanase, Xylosidase, and FAE Induction and FOs 2 Production from Wheat Bran

Because the optimal pH and temperature for hemicellulases production is not the same as the optimal values for FOs production, the effect of two-stage pH and temperature on xylanase, xylosidase, FAE, and FOs production was investigated (Fig. 3). As the fermentation time increased, xylanase activity increased at first and then decreased. Xylanase production (Fig. 3A) preceded the xylosidase (Fig. 3B) and FAE production (Fig. 3C), reaching the highest level of production at 72 h, whereas the xylosidase and FAE production did not start until a significant build-up of xylanase activity, but continued until 108 h. For xylanase, xylosidase, and FAE production, the highest activity was achieved at one-stage fermentation. When the second stage began at 36 h, xylanase and xylosidase production had the second highest activity and FAE had the third highest. The delay in xylosidase and FAE production in relation to xylanase production suggested that xylose and esterified ferulic acid are required for the induction of xylosidase and FAE biosynthesis, respectively.

Three types of two-stage fermentation were designed. The pH and temperature were changed for the second stage, which started after 24 h, 36 h, or 48 h. The FOs outputs for variable pH and constant temperature fermentation, variable temperature, and constant pH fermentation or variable temperature and pH were higher than the output for a constant pH and temperature fermentation (one-stage fermentation) (Fig. 3D). FOs output was maximized when the conditions were changed after 36 h. The optimum FOs 2 output resulted from the initial conditions of pH 4.0 and at 33 °C, with a shift to pH 6.0 and 29 °C at 36 h (Fig. 3D); the output was 23% higher and occurred 12 h earlier with constant pH and temperature. Thus, it is beneficial to combine pH and temperature control in a two-stage FOs 2 production process.

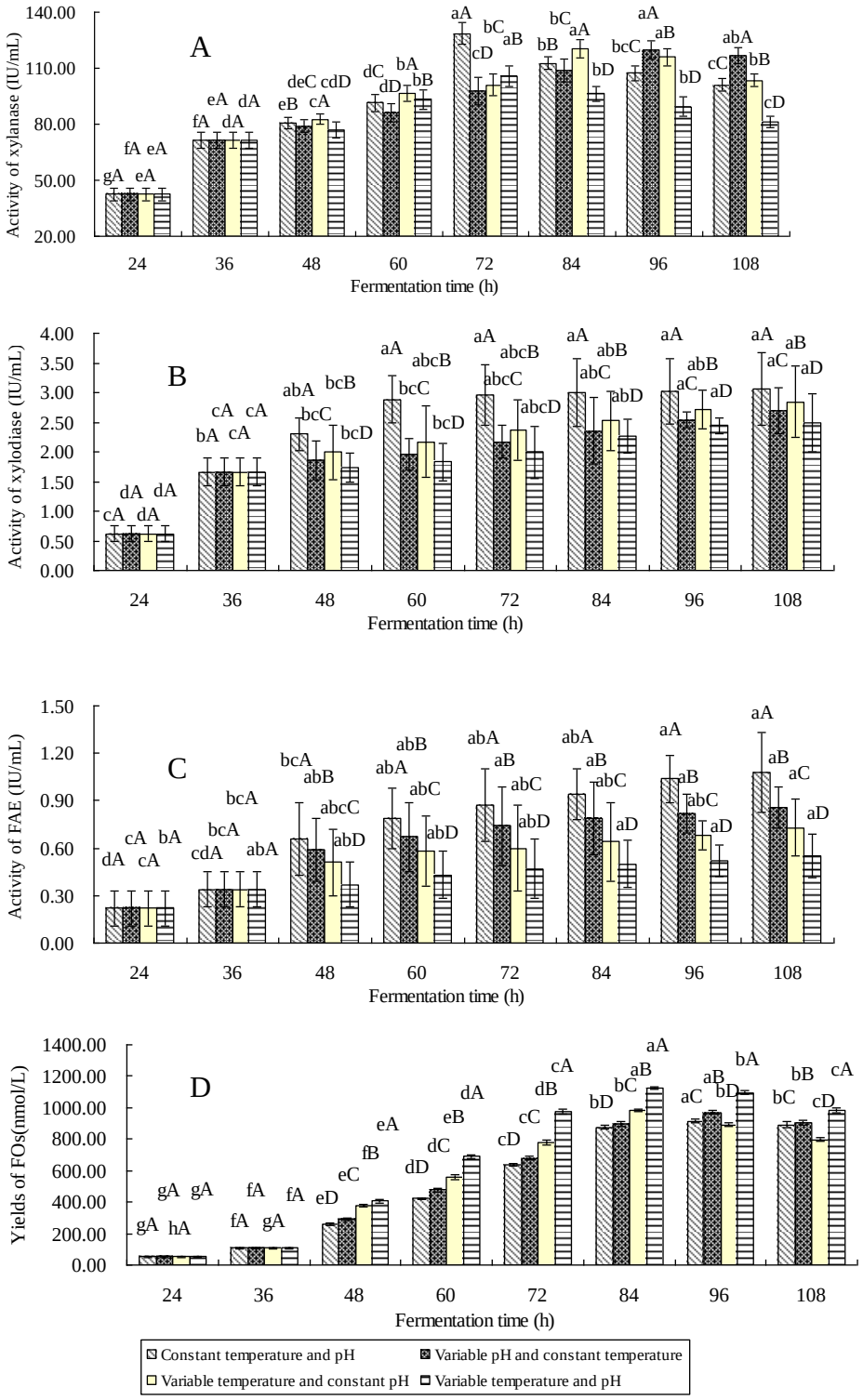

Fig. 3. Effects of two-stage control of pH and temperature on the biosynthesis of xylanase (A), xylosidase (B), and FAE (C) and FOs 2 production (D) by *Aureobasidium pullulans*. Fermentation conditions: The pH and the temperature was adjusted to 4.0 and 33 °C, respectively, for the first stage and then changed to 6.0 and 29 °C, respectively at 36 h for the second stage. Within each dimension, means with different capital letters are significantly different among different groups ($P < 0.05$). Within each group, means with different lowercase letters are significantly different among different dimensions ($P < 0.05$).

FOs 2 production *via* two-stage fermentation reached 1123 nmol/L after fermentation for 96 h with initial pH at 4.0 and 33 °C, with a shift to pH 6.0 and 29 °C at at 36 h; this yield occurred at 12 h less time and was 219 nmol/L higher than one-stage fermentations. The optimal growth conditions for *A. pullulans* are pH 4.0 and 33 °C, with a shortened fermentation time to achieve the highest xylanase activity. However, at the higher temperature, nutrient consumption by cells depletes the media. Lower temperatures slow consumption of nutrients during late fermentation when xylanase is secreted. The prolonged expression of xylanases improves FOs yield.

The dimorphic fungus *A. pullulans* is an excellent production microbe for the xylanase system (Xu *et al*. 2005); it simultaneously produces intracellular xylanase and xylosidase and extracellular FAE (Ohta *et al*. 2010; Wu *et al*. 2010; Yu and Gu. 2014a). Xylosidase activity and FAE production are positively correlated (r = 0.966) (Yu and Gu 2014a). Xylanase hydrolyzes xylan in WB to oligomerized xylose (Yuan *et al*. 2006). Xylosidase further hydrolyzes oligomerized xylose into xylose, which reduces the polymerization of xylan and loosens the polyxylose network structure.

The optimum conditions for the xylosidase of *A. pullulans* ATCC 20524 are pH 3.5 and 70 °C (Ohta *et al*. 2010) and pH 4.5 and 70 °C for the CBS 58475 enzyme (Dobberstein and Emeis 1991). Topakas *et al.* (2005) found that the FAE of *Fusarium oxysporum* is more likely to hydrolyze the FA ester bond within the arabinose side chain of short-chain xylan C2 to C4, this reaction exhibits synergism with xylanase. Therefore, two-stage fermentation with an initial pH at 4.0 and temperature of 33 °C, which are changed to pH 6.0 and 29 °C at 36 h, results in the highest xylosidase activity, which reduces xylan polymerization. Concurrently, moderate FAE activity hydrolyzes ester linkages in lignin and polysaccharide molecules in WB cell walls (Topakas *et al*. 2007), releasing xylan from the xylan-lignin polymer; xylan is then hydrolyzed by xylanase. After 36 h, pH 6.0 inhibits xylosidase and FAE, thus blocking the complete hydrolysis of oligomerized xylose. FAE cannot hydrolyze ester linkages between lignin and polysaccharides, and xylan chains of moderate length promote xylanase catalysis, which increases the FOs output. Overall, moderate hemicellulase activity must be maintained during fermentation to produce a high yield of FOs in a single-step process; that is, higher xylanase activity of and moderate xylosidase and FAE activities are required.

CONCLUSIONS

1. Feruloyl oligosaccharides (FOs) were prepared *via* the controlled fermentation of wheat bran (WB) by *A. pullulans* 2012 in a two-stage process. The optimal pH and temperature for hemicellulases and FOs production were not the same. The pH and temperature were optimized for each stage.
2. The maximum FOs yield of 1123 nmol/L was generated by a 96-h fermentation where the initial pH of 4.0 and temperature of 33 °C were shifted to pH 6.0 and 29 °C after 36 h.
3. This process was 12 h shorter than a one-stage fermentation for producing FOs. The shift in pH and temperature promotes higher xylanase activity and moderate xylosidase and FAE activity, resulting in a high FOs yield.

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