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A metabolic-based approach to improve xylose utilization for fumaric acid production from acid pretreated wheat bran by *Rhizopus oryzae*

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HIGHLIGHTS

- Wheat bran treated by acid at 100 °C for 30 min enhanced fumaric acid production.
- A suitable morphology (0.55 mm) was obtained by optimizing the seed medium.
- Metabolic profiling analysis uncovered the limitations of xylose metabolism.
- Regulation strategies significantly improved fumaric acid production.
- Highest production of 20.2 g/L was achieved by combinatorial strategies from WBH.

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ABSTRACT

In this work, wheat bran (WB) was utilized as feedstock to synthesize fumaric acid by *Rhizopus oryzae*. Firstly, the pretreatment process of WB by dilute sulfuric acid hydrolysis undertaken at 100 °C for 30 min offered the best performance for fumaric acid production. Subsequently, through optimizing the seed culture medium, a suitable morphology (0.55 mm pellets diameter) of *R. oryzae* was obtained. Furthermore, a metabolic-based approach was developed to profile the differences of intracellular metabolites concentration of *R. oryzae* between xylose (the abundant sugar in wheat bran hydrolysate (WBH)) and glucose metabolism. The xylitol, sedoheptulose 7-phosphate, ribulose 5-phosphate, glucose 6-phosphate, proline and serine were responsible for fumaric acid biosynthesis limitation in xylose fermentation. Consequently, regulation strategies were proposed, leading to a 149% increase in titer (up to 15.4 g/L). Finally, by combinatorial regulation strategies the highest production was 20.2 g/L from WBH, 477% higher than that of initial medium.

1. Introduction

Fumaric acid, a four-carbon dicarboxylic acid with a double bond and two carboxylic groups, is considered as an important feedstock for polymerization, esterification reactions and for medicines (Zhou et al., 2011). It also serves as a food acidulant and additive. Nowadays, fumaric acid is mainly produced chemically from maleic anhydride through petrochemical process. However, due to the environmental pollution and the depletion of oil, people have generated a renewed interest in the microbial production of biobased fumaric acid. *Rhizopus oryzae* as filamentous fungi has shown the high-efficient production performance of fumaric acid from glucose (Roa Engel et al., 2008). Nevertheless, the glucose which is derived from starch materials, not only contributes to the proportion of fumaric acid production cost but also competes with humans for food and breaks the equilibrium of supply and demand of grain (Aho, 2007; Tollefson, 2008). Therefore, the cheaper and abundant renewable lignocellulose biomass as potential carbon source has attracted much attention.

Wheat bran (WB), rich in cellulose, protein, starch, and nutrients, is a major by-product of white wheat flour production. It has become an inexpensive and extensive substrate for biochemical industry. Generally, in order to increase the accessibility of the substrate for microbial hydrolytic enzymes, a pretreatment becomes indispensable. The dilute acid pretreated WB can be hydrolyzed into the glucose and xylose as major constituents

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(Sun et al., 2004; Zhang et al., 2007). It has been reported that many microorganisms can utilize WB as the sole carbon source, including *Clostridium beijerinckii*, *Lactobacillus amylophilus*, *Aspergillus niger*, and *Microsphaeropsis* sp. However, to our knowledge, there has been no investigation on cell growth and fumaric acid production using wheat bran hydrolysates (WBH) as carbon source by *R. oryzae*.

The morphology of fungus is crucial during industrial fermentation process because cell may suffer from oxygen limitation when large spherical cell pellets generate. The pellets of *R. oryzae* cultured in shake flasks are strongly determined by the preculture conditions, such as carbon source concentrations, nitrogen source concentrations and metal ions, even pH and culture temperature (Liao et al., 2007; Zhou et al., 2000). Thus, it is necessary to screen the optimal seed culture condition to achieve the appropriate morphology for improved fumaric acid production.

Culture condition optimization has proved to be an effective approach in enhancing carbon flux towards the desired products. However, this method is always time-consuming and subjected to laborious experiments. In addition, owing to the strict control of precursor concentration inside cells or unknown metabolic interactions, a simple medium optimization may not benefit final product levels. Therefore, it has become increasingly important to fully understand the mechanism at systematic level, and further identify the bottleneck limiting the production. Metabolomics, focusing on comprehensive metabolites analysis in a biological system, can provide insights into the metabolism (Ding et al., 2012; Selvarasu et al., 2012). Additionally, metabolomics analysis can directly identify key metabolites and pathways associated with the target product by appropriate statistical and visualization tools such as principal component analysis (PCA), and partial least squares (PLS). Many researchers have employed it to identify metabolites which were depleted or accumulated during cultivation, thus developed strategies to optimize process and increase product titers (Korneli et al., 2012; Lu et al., 2012). However, the effects of different carbon sources on fumaric acid production has not been studied by metabolomics technology, which could give clues about how to increase the xylose metabolic efficiency of WBH by *R. oryzae*. In this study, the potential of using WBH as carbon source to produce fumaric acid by *R. oryzae* was investigated. To achieve this goal, the pretreatment conditions of WB were optimized. Besides, a suitable morphology of *R. oryzae* was developed by optimizing the seed culture medium. Moreover, metabolic profiling approach was employed to characterize a global profile of the intracellular metabolite changes in response to the xylose substrate. The potential key metabolites mainly responsible for fumaric acid overproduction on xylose substrate were identified for the first time. Furthermore, regulation strategies of medium conditions were established based on in-depth analysis of these key metabolites for efficient improvement of xylose utilization. Finally, WBH with combinatorial modification strategy was used for improved fumaric acid production.

2. Methods

2.1. Chemicals, microorganism, medium and culture conditions

WB was obtained from Shandong Rongfeng Biotechnology Development Ltd., (Shandong, China). Corn steep liquor (CSL) was purchased from Tianjin Chemical Reagents Company (Tianjin, China). Activated carbon was purchased from the Aladdin chemistry Co., Ltd. (Shanghai, China). All the authentic standards were purchased from Sigma–Aldrich (USA). Other chemicals were of analytical grade and commercially available from Tianjin Guangfu Institute of Fine Chemicals (Tianjin, China). *R. oryzae* wild1.22 derived from *R. oryzae* ATCC20344 (Yu et al., 2012) was used in this work. Potato-dextrose agar (PDA) was used to produce spores. Seed culture medium contained (g/L): WBH 48, CSL 15, $MgSO_4{\cdot}7H_2O$ 0.7. The pH was adjusted to 3.0 with 1 M HCl. Two types of media were used for fermentations. The glucose medium (M1) contained (g/L): glucose 50, urea 0.07, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.6, $ZnSO_4{\cdot}7H_2O$ 0.018, $FeSO_4{\cdot}7H_2O$ 0.001, $CaCO_3$ 40. The xylose medium (M2) contained (g/L): xylose 50, urea 0.07, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.6, $ZnSO_4{\cdot}7H_2O$ 0.018, $FeSO_4{\cdot}7H_2O$ 0.001, $CaCO_3$ 40. Nutrients and sugar solutions were autoclaved at 121 °C for 20 min and 115 °C for 30 min, respectively. The WBH medium contained (g/L): WBH 50, CSL 10, $CaCO_3$ 40.

For the cultivation of *R. oryzae*, spores maintained on PDA slants at 35 °C for 7 days were suspended in sterilized deionized water and filtered through sterile absorbent cotton to remove mycelium. The final concentration of spores was 10^7/mL. One milliliter of spore suspension was transferred to a 250-mL flask containing 50 mL of seed medium and incubated at 35 °C and 200 rpm for 24 h. Fermentation was carried out in a 250-mL flask containing 50 mL of fermentation medium with 10% inoculation for 72 h at 35 °C, 200 rpm. Sterile excess $CaCO_3$ (40 g/L) was added after 8 h fermentation in order to neutralize the accumulated fumaric acid in the medium.

Through controlling the moisture in the shaker, the evaporation during fermentation was reduced to a minimum extent. Besides, a controlled group was performed and the evaporation of experimental group was subtracted from controlled group, generating the true concentrations of product (by-products) and substrates.

2.2. The pretreatment of WB

WB was filtered through mesh screen and the solid between 40 and 80 mesh screens was then hydrolyzed using two different pretreatments as follows.

The mixture of WB and water with a ratio of 1:5 (w/w) was hydrolyzed without any acid at different temperature (100 °C, 1#; 120 °C, 2#; 140 °C, 3#) for 30 min, as indicated in Table 1. Then the suspension was centrifuged at 5000 rpm for 15 min, and the supernatant was collected for next step.

WB was mixed with water by the ratio of 1:5 (w/w), and dilute sulfuric acid (6 M) was added to adjust the pH to 1.0. The mixture was hydrolyzed at different temperature (100 °C, 4#; 120 °C, 5#; 140 °C, 6#) for 30 min, as indicated in Table 1. Then the suspension was centrifuged at 5000 rpm for 10 min, and the supernatant was collected for next step.

The raw hydrolysate was adjusted to pH 4.0 with solid $Ca(OH)_2$ at 50 °C, followed by 2% (w/v) activated carbon to remove the inhibitors that affect microorganism culture growth. Then the supernatant was used as carbon source for fumaric acid production.

2.3. Analytical methods

The biomass yield was determined by dry cell weight (DCW). The collected samples were centrifuged at 4000 rpm for 10 min and the precipitate was washed twice using 4 M HCl to remove residual calcium carbonate and then the biomass was determined by weighing the mass after drying at 80 °C overnight. The composition of fermentation broth (glucose, xylose, fumaric acid and by-products including lactic acid, malic acid) were determined by HPLC (1200, Agilent Technologies, USA) equipped with an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, USA) using a differential refractive index detector (Agilent, HP1047A) and an ultraviolet absorbance detector (Agilent, G1315D). The column was maintained at 65 °C, and the mobile phase was 5 mM sulfuric acid with flow rate of 0.6 mL/min. The pH value of fermentation broth was

Table 1
Effects of different pretreatments of WB on sugars and fumaric acid production.[a]

Sugar/product	Method 1#	Method 2#	Method 3#	Method 4#	Method 5#	Method 6#
Glucose (g/L)	1.5 ± 0.01	1.4 ± 0.01	1.4 ± 0.01	4.8 ± 0.04	4.2 ± 0.06	4.1 ± 0.03
Xylose (g/L)	14.3 ± 0.04	14.8 ± 0.02	13.8 ± 0.01	38 ± 0.52	35.4 ± 1.41	34.8 ± 0.84
SY[b] (g/g)	0.158 ± 0.001	0.162 ± 0.006	0.152 ± 0.004	0.428 ± 0.01	0.396 ± 0.26	0.389 ± 0.017
FAC[c] (g/L)	0.8 ± 0.01	0.6 ± 0.11	0.6 ± 0.08	3.5 ± 0.22	2.9 ± 0.31	2.7 ± 0.32
FAY[d] (g/g)	0.297 ± 0.001	0.335 ± 0.004	0.304 ± 0.002	0.482 ± 0.002	0.373 ± 0.022	0.359 ± 0.005

[a] Fermentation was carried out in WBH pretreated with different methods and WBH were employed as carbon source as carbon source in seed medium with initial pH 3.0.
[b] SY represent sugar yield (total sugar/wheat bran).
[c] FAC represent fumaric acid concentration.
[d] FAY represent fumaric acid yield (fumaric acid/consumed sugars).

detected using pH meter (PB-10, Sartorius, Germany). To study the effect of culture medium and pH on pellet formation in WBH seed medium, the cell diameter was quantified using a microphotograph together with a CCD camera, a PC with a frame-grabber and image analysis software.

2.4. Sample preparation for metabolic profiling

Samples for comparative metabolic profiling were withdrawn from the fermentation vessel. Sampling time points (24 h, 36 h, 48 h) were determined by the fermentation profiles. Sample quenching, extraction were carried out according to the methods described before (Yu et al., 2012) with slight modifications. Firstly, the collected samples were mixed immediately with 25 mL 60% methanol solution in a 50 mL tube pre-chilled on an ethanol bath at −40 °C. After centrifugation at 4000×*g* for 5 min, the precipitates were then neutralized by 4 N HCl at −40 °C to remove residual calcium carbonate. Subsequently, the mixture was centrifuged at 4000×*g* for 5 min and the cells were immediately transferred to a 50 mL tube containing 25 mL 60% methanol solution pre-chilled at −40 °C. After centrifugation under the same condition, the cell pellet was suspended with 2.5 mL of cold methanol–water solution (50% (v/v), −30 °C) and thawed in an ice bath for 4 min. Then the mixture was vigorously mixed with a vortex mixer for 1 min and frozen at −80 °C for 30 min. The above freeze–thaw cycle was repeated for three times. After centrifugation at 5000×*g* for 3 min, the supernatant was collected. Another 2.5 mL of cold methanol–water solution (60% (v/v), −40 °C) was added into the precipitate for further extraction. After mixed and centrifuged at 10,000×*g* for 3 min, the extract was pooled with the former one and centrifuged at 10,000×*g* for 5 min again. Ten microliter of 0.1 mg/mL succinic d_4 (St. Louis, MO, USA) and D-sorbitol-$^{13}C_6$ (St. Louis, MO,USA) were added to 100 μL sample extract before lyophilization (ALPHA 1-2LD PLUS, Christ, Germany) as internal standard, respectively. The former was used as internal standard for gas chromatograph–mass spectrometry (GC–MS) analysis and the latter was for liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) measurements of sugar phosphate intermediates of pentose phosphate pathway (PPP).

2.5. Determination of intracellular metabolites by GC–MS and LC–MS/MS

After lyophilization (ALPHA 1-2LD PLUS, Christ, Germany), the samples were treated with two-stage chemical derivatization (Ding et al., 2010). Parameter settings of GC–MS system were consistent with Wang et al. (2013a). Masslynx software (Version 4.1, Waters Corp, USA) was used for peak recognition, identification, and deconvolution. Metabolites were identified by comparing the mass fragmentation patterns with NIST mass spectral library. The peak areas of all metabolites were normalized with the internal standards and biomass concentrations in the same chromatograms. Metabolite peaks with probability match factors less than 50% for mass spectra comparisons to the NIST MS-library were classified as unknowns.

The extracted metabolites from PPP were measured by LC–MS/MS. Parameter settings of LC–MS/MS system were consistent with Xia et al. (2013). Data were acquired and processed using Analyst™ for Window NT software (Ver. 1.3.1). Quantification of the peaks was achieved by comparing peak area with the standards.

2.6. Data analysis

The datasets were normalized by centering and pareto scaling, then analyzed by PCA and PLS using SPSS 19.0 (IBM, USA) and Matlab (Version R2008b, MathWorks, USA) for subsequent statistical analysis. In PCA, function of princomp was used to judge the differences among samples and process data into a two-dimensional map, which reserved most of the characteristics of raw data. PLS was carried out to investigate the correlations between metabolism and fumaric acid accumulation, and identify the key metabolites that mainly resulted in metabolic discrimination. Mean values and relative standard deviations of metabolites were calculated from four replicates of each sample. Significance level of metabolites abundance in xylose medium relative to the data in glucose medium was identified by a two-tailed Student's *t*-test performed with SPSS 19.0 (IBM, USA).

3. Results and discussion

3.1. Effects of different pretreatments of WB on fumaric acid production

The WBH obtained from WB after the pretreatments, are mainly composed of glucose and xylose, which can be used as the carbon source for fumaric acid production. The effects of different pretreatments on fumaric acid production are shown in Table 1. The result exhibited that the glucose concentration of method 4# was 3.2-fold of method 1#, and the fumaric acid concentration and yield of method 4# was 4.4-fold and 1.6-fold, respectively. Besides, this result reflected that both the total sugars and sugar yield of acid-pretreated WB (4#) were 2.7-fold of the heat-pretreated WB (1#). The corresponding fumaric acid concentration was enhanced to 3.5 g/L from 0.8 g/L. On the other hand, severe pretreatment conditions (temperature) could promote hydrolysis of WB into sugars (Table 1). However, over temperature would cause more serious degradation of hemicellulose, thus leading to a loss of available sugar in solid residue. Indeed, in order to get satisfactory final total sugar, the temperature of acid pretreatment for WB should be no higher than 140 °C, as the polysaccharides may randomly hydrolyze when the temperature was over 140 °C under the acetic conditions (Sixta, 2006). In addition to the temperature, acid played an important role in the pretreatment of WB (Huang et al., 2009). It can be seen that acid ratio contributed more to

the final total sugar concentration, compared to pretreatment temperature (Table 1). According to the fermentation characteristics, dilute sulfuric acid hydrolysis is a suitable pretreatment method. Therefore, this study employed the acid-pretreated WBH at 100 °C for fumaric acid fermentation.

3.2. Optimization of *R. oryzae* morphology in WBH seed medium

Controlling morphology of *R. oryzae* is a major technical challenge for the fumaric acid production on a stable and scalable level (Papagianni and Mattey, 2006). Pellets are often the preferred morphology in industrial fermentation processes, which can avoid many operational difficulties, such as wrapping around the impellers and fouling agitation blades. The factors influencing fungal morphology contained the properties of the individual hyphal elements and environment conditions (Zhou et al., 2011). Therefore, a comprehensive investigation of these factors affecting the pellet formation of *R. oryzae* was carried out in the WBH seed medium.

3.2.1. Effects of culture medium on pellet formation

Both carbon source and nitrogen source may directly influence the pellet diameter and DCW. Different sized pellets were obtained during the cultivation of *R. oryzae* under different WBH and CSL concentrations. As shown in Fig. 1, WBH (measured by total sugar) and CSL could influence pellet diameter and DCW. The biomass of the pre-culture increased from 2.3 to 3.7 g/L with the WBH concentration increased from 20.0 to 48.0 g/L (Fig. 1A). As the WBH concentration increased from 20.0 to 48.0 g/L, the pellet diameter decreased from 5.2 to 1.1 mm. When the concentration of WBH increased from 48.0 to 75.0 g/L, the pellet diameter increased from 1.1 to 2.6 mm. In addition, the pellet diameter decreased from 3.2 to 0.8 mm when CSL concentration increased from 5.0 to 15.0 g/L (Fig. 1B). It has been reported that smaller pellet diameter led to higher fumaric acid production in submerged fermentation by *R. oryzae* (Zhou et al., 2011). Therefore, the more suitable pellet diameter and greater biomass were achieved when the initial WBH and CSL concentrations were 40.0 and 15.0 g/L, respectively.

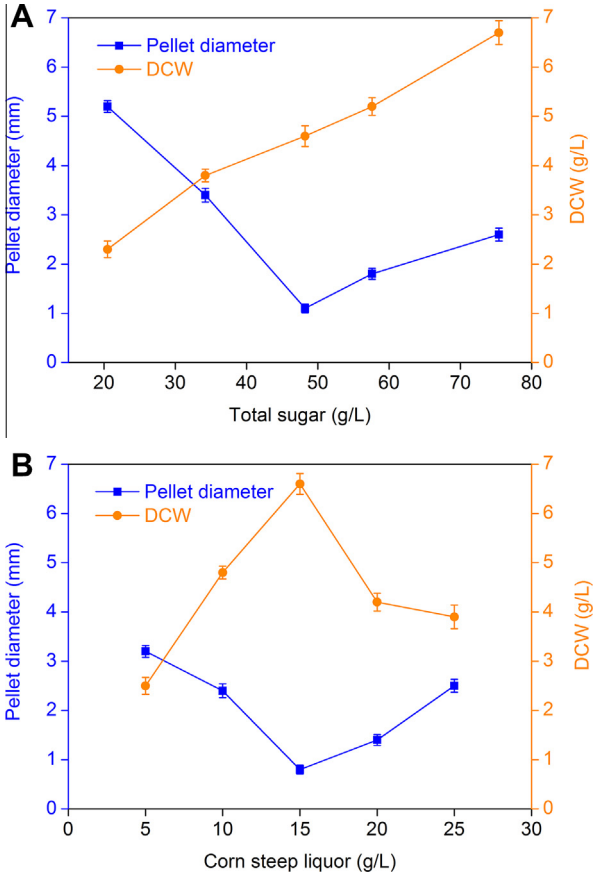

Fig. 1. Effects of WBH (total sugar) (A) and corn steep liquor (B) concentrations in seed medium with initial pH 3.0 on pellet diameter and DCW.

3.2.2. Effects of pH on pellet formation

The morphologies during the seed cultivation of *R. oryzae* at different initial pH are shown in Table 2. It can be seen that the biomass increased from 2.3 to 3.7 g/L with pH from 1.8 to 5.0. Besides, it should be noted that slow mycelial growth was observed at pH 1.8 in the seed culture. At higher pH values, extensive formation of swollen cells (diameter was over 0.8 mm but still maintained to be pellet) generated. At pH 5.0, coagulation clumps prevailed, while at pH 2.0, pellets with the diameter between 0.8 and 1.0 mm predominated. The diameter of pellets at initial pH 2.5 ranged between 1.5 and 3.8 mm. Besides, bigger swollen pellets and suspended mycelia accumulated at pH 3.0. These results indicated that low pH inhibited the cell growth but was beneficial for pellets formation (Zhou et al., 2000). Therefore, it is important to choose appropriate initial pH for smaller pellets formation and higher fumaric acid production. It can be seen that the initial pH 2.0 resulted in small radial pellets. At the same time, a drop in final pH was observed when the initial pH was equal to or higher than 2.5, perhaps on account of the accumulation of fumaric acid. Moreover, though greater biomass was obtained when pH increased, most of the mycelia stick together, which was unfavorable for fumaric acid production. Therefore, the initial pH 2.0 was chosen to be the optimal condition in the seed culture.

3.3. Improvement of xylose utilization for enhanced fumaric acid production

The dilute acid pretreated WB generated the hydrolysates with the major constituents of glucose and xylose (Sun et al., 2004; Zhang et al., 2007). However, the utilization rate of xylose is rather low compared to glucose (Fig. 2). *R. oryzae* did not uptake xylose until the glucose was almost exhausted. Besides, the fumaric acid was only 5.8 g/L after a series of medium optimization (Fig. 2). Consequently, it is difficult to uncover the bottlenecks limiting the conversion of xylose into fumaric acid. Therefore, we developed a metabolic-based approach to get deep insights into the xylose metabolism by *R. oryzae* and proposed the corresponding regulation strategies.

3.3.1. Comparisons of fermentation characteristics with glucose and xylose medium

To identify the limitations of the utilization of xylose in *R. oryzae*, it is necessary to figure out the extracellular metabolites differences between xylose and glucose metabolism. The relevant

Table 2
Effects of pH on the morphology of *R. oryzae*.[a]

Initial pH	Final pH	Biomass	Morphology
1.8	1.8 ± 0.1	1.0 ± 0.03	Little mycelial
2.0	2.2 ± 0.1	2.5 ± 0.03	Pellets (0.8–1.0 mm)
2.5	2.4 ± 0.1	2.7 ± 0.04	Pellets (1.5–3.8 mm)
3.0	2.8 ± 0.1	2.9 ± 0.04	Pellets and suspended mycelia
3.5	2.9 ± 0.1	3.0 ± 0.04	Suspended mycelia
4.0	2.9 ± 0.1	3.3 ± 0.1	Suspended mycelia and clumps
4.5	2.7 ± 0.1	3.6 ± 0.1	Clumps
5.0	2.6 ± 0.1	3.7 ± 0.1	Clumps

[a] WBH was used as carbon source in seed medium with different initial pH.

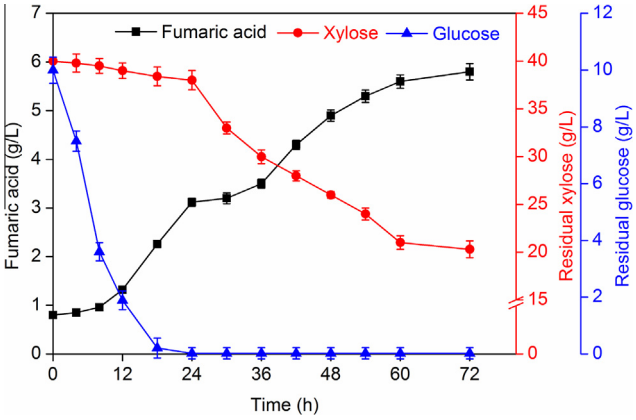

Fig. 2. Time profiles of sugar consumption and fumaric acid production in the pretreated WBH (6 M sulfuric acid and 100 °C).

fermentation parameters, such as fumaric acid, dry cell weight and productivity, were detected with glucose-substrate (M1) and xylose-substrate (M2), respectively. As shown in Fig. 3A, strain grew faster in M2 than M1 during the fermentation. After 72 h, the biomass in M2 was 9.45 g/L, which was about 3.0-fold of that in M1, indicating that xylose was better for biomass accumulation. It is likely that the specific xylose metabolic pathways result in the conversion of more substrate into biomacromolecules of cell (Maas et al., 2008). However, fumaric acid production was higher in M1 and the highest productivity of 2.075 g/ (L h) was achieved at 36 h (Fig. 3B), which was about 12.3-fold of that in M2, indicating that M2 was better in supporting cell growth but weaker in fumaric acid production. During the exponential acid secretion phase

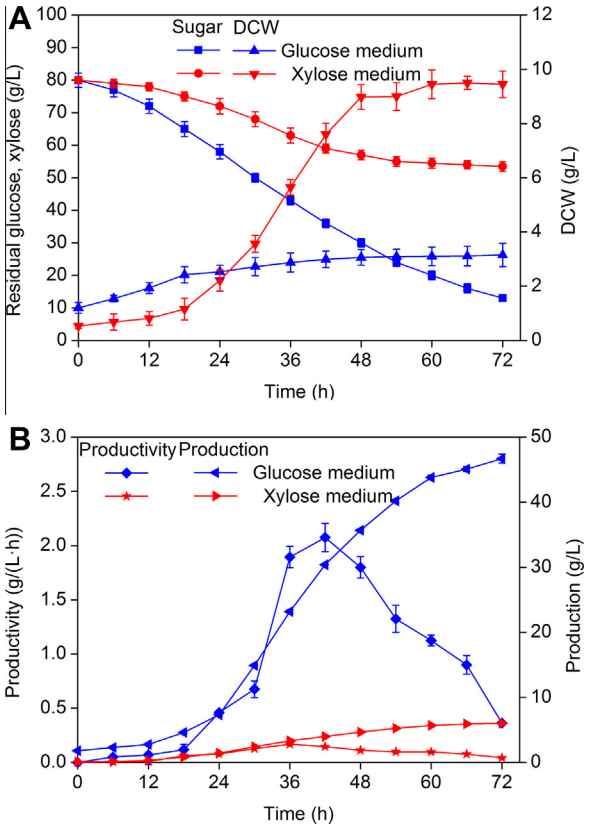

Fig. 3. Time profiles of residual sugar and DCW (A), fumaric acid concentration and productivity (B) using glucose and xylose as carbon source, respectively.

(24-48 h), the increment of the fumaric acid in M2 (from 1.42 g/L to 4.67 g/L) was only 11% of that in M1 (from 7.32 g/L to 35.7 g/L) (Fig. 3B). It was almost at the same time (24–48 h) that production rate reached the highest in both medium, indicating that the exponential acid secretion phase was the key period for fumaric acid production.

According to the above results, strain exhibited different fermentation characteristics between M2 and M1. To identify the obstacles of xylose metabolism, multivariate statistical analysis using PCA and PLS on the intracellular metabolites were conducted to unravel the limitations of xylose-fermenting in *R. oryzae*.

3.3.2. Analysis of key metabolites and pathways for potential fumaric acid improvement from xylose

The intracellular metabolites of *R. oryzae* with different carbon sources for fumaric acid production capabilities were analyzed by a combination of GC–MS and LC–MS/MS. Generally, a total of 136 putative intracellular metabolites were detected and 102 of them were identified in database, including amino acids, organic acids, sugars, alcohols, lipids and others. Subsequently, PCA was performed to elucidate the nature of multivariate data and further validate the different metabolism in xylose-substrate M2 and glucose-substrate M1, as shown in Fig. S1. In the score plot (Fig. S1), the tight clustering of four parallels for individual time point and a clear separation between the different media revealed a quite clear discrimination among samples under various culture conditions and fermentation times. Examination of PCA score plot also indicated that the xylose-substrate metabolism exhibited different metabolic characteristics compared with those in glucose. In the loading plot, the contribution of each metabolite to a specific component was used to identify the significant factors responsible for the discrimination. To further evaluate the metabolites contributing to fumaric acid production in xylose metabolism, PLS (using fumaric acid titer as the *Y* matrix) was carried out. The significant discrimination on the PLS profile reflected the metabolic difference caused by medium and cultivation time (Fig. S1). This was consistent with the results of PCA, ensuring the quality of sampling and the reliability of analytical technique. Moreover, the PLS analysis verified the strong correlation between metabolism and fumaric acid biosynthesis in *R. oryzae*. Subsequently, the variable importance of the projection plots (VIP) score for each mass peak was generated by PLS analysis (Fig. S1). A higher VIP score revealed that the peak contributed more significantly to the differentiation of xylose and glucose metabolism in the model. In this work, six metabolites separated clearly from others were identified to be the most significant metabolites responsible for the discrimination between M1 and M2, including xylitol, sedoheptulose 7-phosphate (Sed7P), ribulose 5-phosphate (Rib5P), glucose 6-phosphate (Glu6P), proline and serine. These six metabolites mainly distributed in central carbon pathways and amino acids metabolism (Fig. 4).

The proposed pathways for xylose catabolism are the same as those in most eukaryotic organisms (Fig. 4) (Maas et al., 2008; Zaldivar et al., 2001). The catabolism of xylose occurs via the pentose catabolic pathway (PCP). As exhibited in Table S1, the relative abundance of xylitol were 0.49 in M2 but 0.01 in M1 at 36 h, indicating that the xylitol was accumulated during xylose metabolism in M2. The high VIP from PLS suggested that the accumulation of xylitol contributed to the low rate of metabolism. Therefore, it provides a target to enhance fumaric acid production from xylose by improving the conversion rate of xylitol to xylulose, and further to xylulose5-phosphate (Xyl5P). Then Xyl5P was channeled into the glycolysis via PPP, as depicted in Fig. 4. Recently, cofactors have emerged to be appealing targets to enhance cell metabolism, thus playing an essential role in the microbial products (San et al., 2002; Yu et al., 2011). The level and availability of cofactors can become a

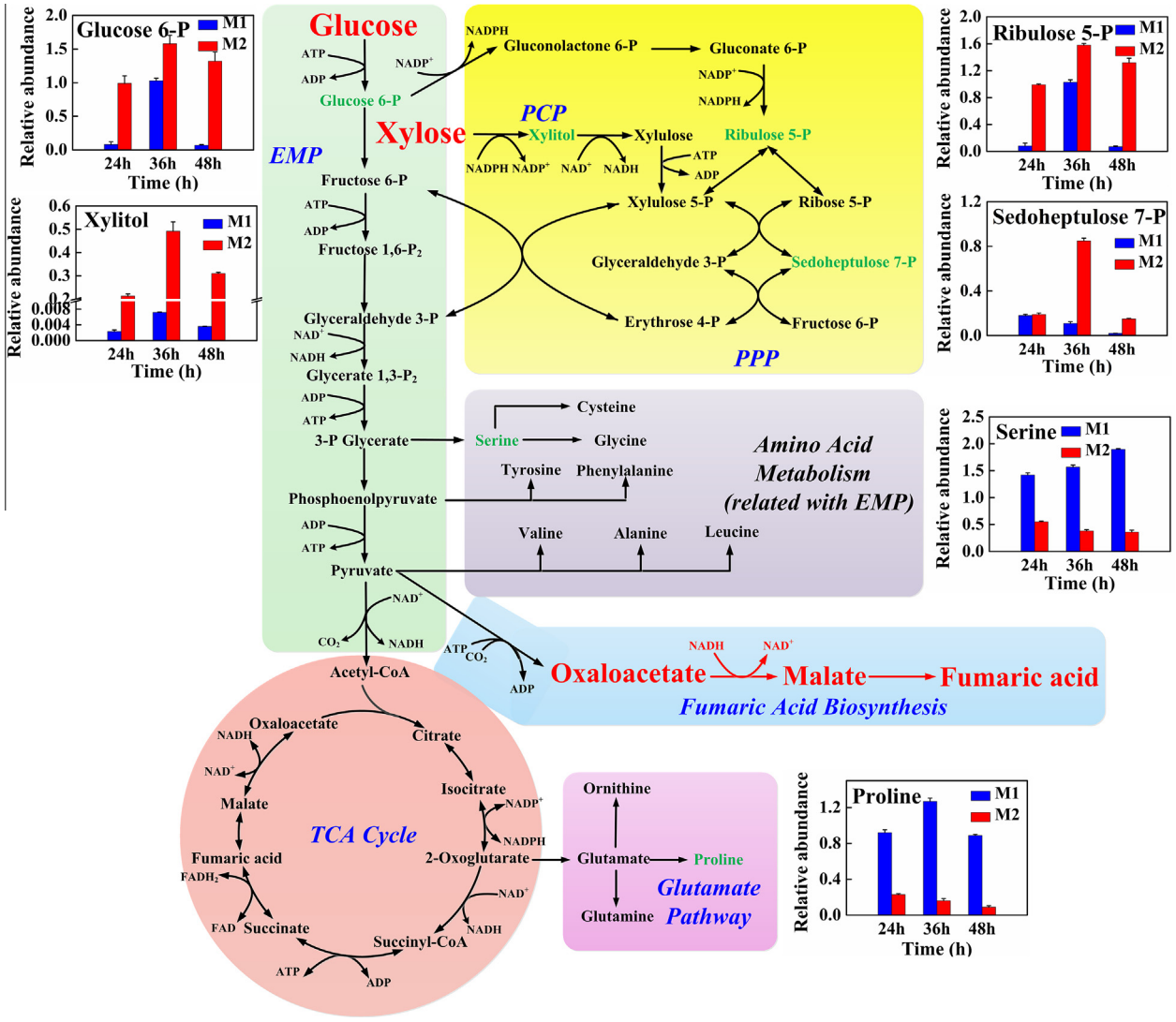

Fig. 4. Schematic diagram of the proposed metabolic pathways of glucose and xylose in *R. oryzae*. Abbreviation: sedoheptulose 7-P, sedoheptulose 7-phosphate; ribulose 5-P, ribulose 5- phosphate; glucose 6-P, glucose 6-phosphate; fructose 6-P, fructose 6-phosphate; gluconolactone 6-P, gluconolactone 6-phosphate; gluconate 6-P, gluconate 6-phosphate; ribose 5-P, ribose 5-phosphate; xylulose 5-P, xylulose 5-phosphate; erythrose 4-P, erythrose 4-phosphate; glyceraldehyde 3-P, glyceraldehyde 3-phosphate; glycerate 1,3-P_2, 1,3-bisphosphoglycerate; 3-P glycerate, 3-phosphoglycerate; EMP, Embden-Meyerhof-Parnas; PPP, pentose phosphate pathway; TCA, tricarboxylic acid; PCP, pentose catabolic pathway.

limiting step in cofactor-dependent production systems (Berríos-Rivera et al., 2002; Knepper et al., 2008). The cofactor imbalance between the NADPH-dependent xylose reductase (XR) and NAD^+-dependent xylitol dehydrogenase (XDH) may lead to the accumulation of xylitol. In addition, the lack of ATP, which is essential for the conversion of xylitol to Xyl5P, might be also considered to be a key factor contributing to the accumulation of xylitol. Although the ATP consumption for Glu6P formation might also result in the ATP shortage in glucose medium, the concentrations of intracellular ATP in xylose and glucose medium showed that the concentration of ATP in xylose was only 40% of that in glucose (data not shown), which was probably due to the ATP imbalance between consumption and generation. The different levels of ATP indicated that higher level of ATP derived from glucose fermentation might promote the rate of glucose utilization, compared to that of xylose utilization. Furthermore, the first step of fumaric acid biosynthesis is the conversion of pyruvate to oxaloacetate, which is an ATP-dependent step (Fig. 4). During aerobic fermentation, the demand for ATP is mainly derived from carbon metabolism. Noted that ATP can be mainly synthesized in glycolysis pathway and tricarboxylic acid (TCA) cycle (Fig. 4). In addition, oxygen supply impacted by agitation speed, was closely linked to energy production as well as the fumaric acid production during the fermentation. The dissolved oxygen has a profound effect on production with the adjustment of the redox balance (Neves et al., 2000). The above result provided a key potential regulation approach to modify the energy status.

Most strikingly, proline and serine both declined during the main fumaric acid production stage (24–36 h) in M2 while increased in M1. The low level of proline in M2 reflected the reduced flux of glutamate metabolism which is derived from the intermediate 2-oxoglutarate (AKG) of TCA cycle (Fig. 4). In addition, the flux rearrangement from PPP to glycolysis pathway also changed the profiling of the associated amino acids metabolism. Proline accumulation occurred predominantly through the enhanced glutamate biosynthesis (Hayashi et al., 2000), which played a pivotal role in nitrogen catabolism and anabolism (Wang et al., 2013a). The relative abundance of serine, which is derived from the 3-phosphoglycerate intermediate of central carbon metabolism, exhibited a slightly decrease from 0.38 (24 h) to

0.36 (48 h) during the fermentation. These results revealed that a positive glycolysis and PPP could maintain cell viability and promote utilization of xylose. Hence, cofactors, energy, proline and serine were regarded as the key metabolic factors influencing the production during the utilization of xylose. Medium regulation strategies were proposed based on the comparative metabolic analysis.

3.3.3. Improvement of fumaric acid production based on regulation strategies

Fumaric acid is an intermediate of the TCA cycle, but *R. oryzae* accumulated large amounts of fumaric acid via cytosolic reductive TCA pathways using NADH (Fig. 4) (Ferreira et al., 2013). The intracellular redox state directly affects the activity of many enzymes (Chen et al., 2003). Besides, according to the analysis of metabolites in xylose metabolism, xylitol accumulated due to the imbalanced NADH cofactor. Therefore, nicotinic acid (NA) was added into the medium to strengthen the PCP and cytosolic reductive TCA, thus fumaric acid production was significantly improved (Fig. 5A). Consistent with the above analysis, NA could enhance the availability of NAD(H) (Li et al., 2014), which further boosted the fumaric acid production. Particularly, a final NA concentration of 25 mg/L added at 36 h resulted in an increase of fumaric acid titer from 6.02 to 8.54 g/L (Fig. 5A) but no significant difference in cell growth (data not shown). In addition, vitamin C (Vc) has served as a reductant in numerous pathways (Dai et al., 2014). Therefore, to regulate the cellular redox status, Vc was added to the fermentation culture. The best result was observed when 0.1 g/L was added at 24 h, with a 28% improvement of fumaric acid titer, up to 7.71 g/L (Fig. 5B). As mentioned, conversion of xylose to Xyl5P via the two-step reduction/oxidation route suggested an enhanced negative effect of oxygen limitation on the product formation compared with glucose (Maas et al., 2008). The dissolved oxygen level has a significant effect on the production of fumaric acid (Xu et al., 2012). Moreover, the limitation of oxygen may guide more metabolic flow to produce by-products. Thus, the regulation of dissolved oxygen level during xylose fermentation was considered through optimizing rotating speed between 180 and 240 rpm (Fig. 5C). The highest production titer of 8.01 g/L was achieved when speed was settled at 230 rpm (Fig. 5C). However, the excess of oxygen led to a decreased production (Fig. 5C), which was probably due to the increased biomass accumulation and energy consumption.

Additionally, the previous analysis suggested that both the conversion of xylitol to Xyl5P and fumaric acid biosynthesis required

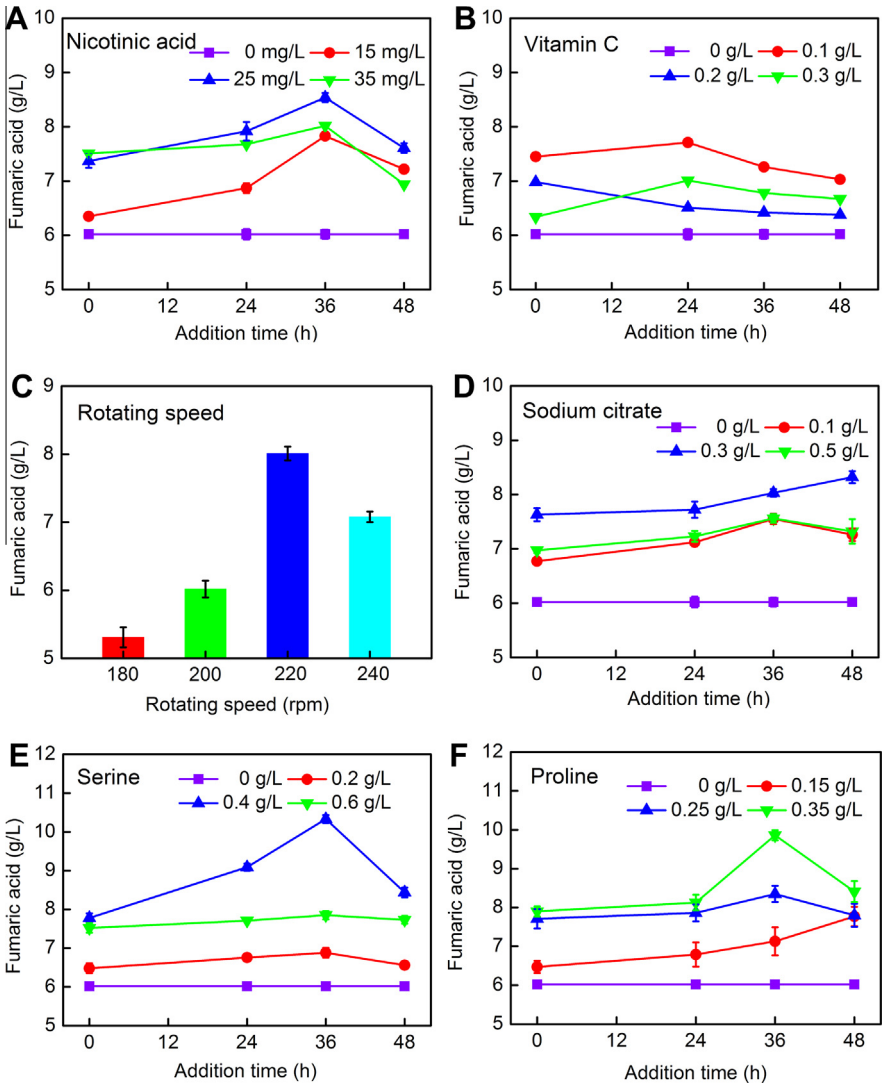

Fig. 5. Effects of regulation strategies on the fumaric acid production with xylose as carbon source. The strategies contain nicotinic acid (A), Vc (B), rotating rate (C), sodium citrate (D), serine (E) and proline (F) at various feeding time.

sufficient ATP (Fig. 4). The generation and consumption of ATP were mainly through the central carbon metabolism and fumaric acid biosynthetic pathways. According to the origin of ATP mentioned above, the addition of sodium citrate could enhance TCA cycle, which increased ATP production and boosted the synthesis of NADPH and NADH cofactors (Wang et al., 2013b). Therefore, sodium citrate were added with various final concentrations at different time. As shown in Fig. 5D, when 0.3 g/L sodium citrate was added at 48 h, fumaric acid titer increased by 38% up to 8.32 g/L, suggesting that exogenous sodium citrate could enhance the flux of TCA cycle, which was beneficial for xylose metabolism in *R. oryzae*.

In the case of amino acid catabolism metabolites as analyzed above, the exogenous addition of serine was to reduce the carbon flux from 3-phosphoglycerate to serine and conversely promote fumaric acid pathway at the same time. The exogenous proline was added in M2, which may redistribute the AKG flux between the glutamate pathway and TCA cycle. Results showed that when 0.4 g/L of serine and 0.25 g/L proline were added at 36 h, fumaric acid production was boosted up to 10.33 g/L and 9.75 g/L, respectively (Fig. 5E and F).

The pH between the additives and the control group was determined and the result showed that there was no difference, indicating that the additives did not play a role in the buffering action. With the combination of the above strategies: 0.1 g/L Vc was added at 24 h; 25 mg/L NA, 0.25 g/L proline and 0.4 g/L serine were added at 36 h; 0.3 g/L sodium citrate was added at 48 h; the rotating speed was settled at 230 rpm, the fumaric acid production was enhanced to 15.4 g/L in xylose medium.

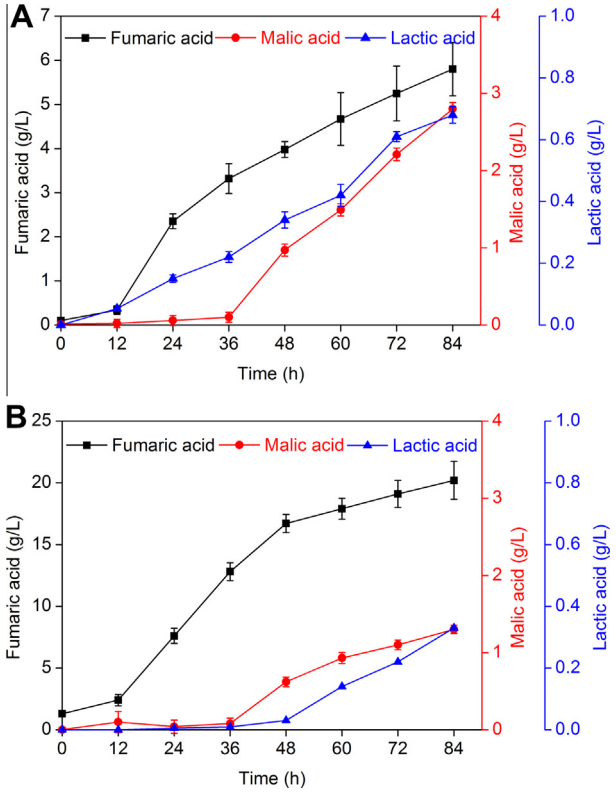

Fig. 6. Cultivation profiles of fumaric acid and main by-products with pretreated WBH (6 M sulfuric acid and 100 °C) as carbon source by combinatorial regulation strategies. (A) pretreated WBH without combinatorial regulation strategies; (B) pretreated WBH with combinatorial regulation strategies.

3.4. Fumaric acid production from WBH by combinatorial regulation strategies

As the bottlenecks for xylose have been confirmed and relieved, a combinatorial regulation strategy based on WBH medium was carried out. The combinatorial regulation strategy was the same as described above. In addition, a suitable morphology of *R. oryzae* was applied by optimizing the seed culture medium. As shown in Fig. 6, the strategy successfully enhanced the fumaric acid titer from 5.8 g/L to 20.2 g/L, with a 477% improvement compared with that in initial WBH medium. Moreover, lactic acid and malic acid as common by-products were compared to examine the effects of the regulations on the fermentation profile. Surprisingly, both by-products displayed a remarkable decrease (Fig. 6), indicating that regulation strategies played a significant role in the redistribution of pathway flux.

4. Conclusions

As xylose and glucose were the main monosaccharides of WBH, a novel strategy was developed for the efficient and economical fumaric acid biosynthesis using WBH as carbon substrate. WB treated by acid at 100 °C for 30 min resulted in the enhanced fumaric acid production. A suitable morphology (0.55 mm) was obtained by optimizing the seed medium. Comparative metabolic profiling analysis uncovered the limitations of xylose metabolism. Fumaric acid production ultimately reached 20.2 g/L using the optimized WBH medium. This study provides an encouraging means of producing fumaric acid from low-cost renewable resource.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.12.091.

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