Enhanced Fumaric Acid Production by Fermentation of Xylose Using a Modified Strain of *Rhizopus arrhizus*

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An enhanced, xylose-utilizing strain of *Rhizopus arrhizus* was successfully developed via modification using media with increasing concentrations of xylose. Xylose, a relatively cheap monosugar that can be easily obtained from corncobs, rice straw, and vinasse, was the sole carbon source used for both the seed culture phase and the fermentation process. After modification, this newly improved strain showed tremendous industrial potential for fumaric acid production using xylose. The fumaric acid production increased to 28.48 g/L (parental strain was 13.23 g/L) at 8% initial xylose, a carbon:nitrogen ratio of 200, and a residence time of 7 days. The volumetric productivity was 169.52 mg/L (78.75 mg/L) per hour.

Keywords: Xylose; Fumaric acid; Fed-batch; Modification; Rhizopus arrhizus

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INTRODUCTION

Fumaric acid (FA), a commercially important chemical, has a number of industrial applications including its use in foods, beverages, animal feeds, paper sizing, pharmaceuticals, and detergents (Altmeyer *et al.* 1994; Lee *et al.* 2004; McGinn *et al.* 2004; Tsao *et al.* 1999). In the present industrial practice, FA is produced chemically from petroleum-based materials (Moresi *et al.* 1992), but as the world's crude oil resources diminish, more interest has been focused on fermentation processes for its production.

Previous research on FA production predominantly used petrochemical maleic anhydride or maleic acid as a substrate (Cao *et al.* 1997; Fu *et al.* 2010; Roa Engel *et al.* 2011). However, because such a large quantity of hemicellulosic materials in the form of lignocellulosic biomass and agricultural residues, such as corncobs, rice straw, vinasse, and sugarcane bagasse, is available in the world, hemicellulosic materials have become potential feedstocks for chemical production. Using a broad range of physical, chemical, and enzymatic treatments, these hemicellulosic materials can be hydrolyzed to fermentable monosaccharaides (including xylose) as the ultimate product (Melzoch *et al.* 1997; Mosier *et al.* 2005). Therefore, xylose could then be an alternative source for FA production. Much research has been conducted on the fermentation of hemicellulose hydrolysate to produce ethanol and L-lactic acid, as well as the negative influence of inhibitory compounds in lignocellulose hydrolysates on the fermentation process (Garde *et al.* 2002; Larsson *et al.* 2000; Martin *et al.* 2007; Wheals *et al.* 1999). However, few studies have focused on fermentation that uses xylose as the sole carbon source to produce FA. A previous report showed that the maximum FA product concentration was 16.4 g/L, while the highest volumetric productivity was 87 mg/L per hour when xylose

was used for fermentation (Kautola and Linko 1989). Because the production of FA from xylose was relatively poor compared with the results of glucose fermentation, which is capable of reaching a production rate of 4.25 g/L per hour through immobilization with a rotary biofilm contactor (Cao *et al.* 1996), greater effort should be made toward higher production of FA using xylose in fermentation.

Due to its capability to form cellulolytic and xylanolytic enzymes and its simple nutritional requirements (Bakir *et al.* 2001; Murashima *et al.* 2002), *Rhizopus* species were identified as the best fumarate-producing strains among a variety of microorganisms tested (Kenealy *et al.* 1986; Zhou *et al.* 2002). In earlier work, the *Rhizopus arrhizus* mutant RH-7-13 was selected, which significantly improved the efficiency of substrate utilization and enhanced production of FA from non-grain biomass. As a result, there was a maximum FA production of 46.36 g/L in flasks with a productivity of 0.32 g/L/h (Han 2009).

The purpose of this work was to offer more support for FA production from hemicellulose hydrolysate, therefore a better xylose-utilizing strain of *Rhizopus arrhizus* was developed using a directed-evolution strategy. Furthermore, factors in a preculture medium affecting fungal growth morphology were investigated, and the production of FA from xylose was evaluated at different C/N ratios, inoculum sizes, and rotation speeds in the flask culture.

EXPERIMENTAL

Materials and Methods

Microorganism

R. arrhizus RH-7-13, a mutant of *R. arrhizus*, was used in this study. The fungus was first cultured on a potato dextrose agar (Difco) slant at 30 °C for 7 days to form spores. To prepare spore inoculum, agar plates containing fungus spores were washed with sterile water and filtered through sterile cotton wool to obtain a spore suspension. In order to rupture the sporangium wall, and liberate spores, the suspension was transferred into a 50-mL flask containing glass pellets to disperse the spores, and then the sporangium was filtered out through a disc filter. The spore suspension was finally diluted in sterile water at a concentration of 10^7/mL, which was counted with a hemacytometer under a microscope and used for inoculation.

Cultural conditions

Several media were applied in this study. The medium used for the preculture consisted of 35 g xylose (J&K, Shanghai, China), 1.5 g urea, 0.6 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.76×10^{-3} g $ZnSO_4 \cdot 7H_2O$, and 4.98×10^{-4} g $FeSO_4 \cdot 7H_2O$ per liter (Sinopharm Chemical Reagent, Beijing, Beijing, China). The pH of the preculture medium was adjusted to 2.5–2.8. For pellet formation, 4 mL of spore suspension (1×10^6 spores/ml) were inoculated into a 500-mL flask containing 200 mL of medium and were cultivated at 32 °C with shaking at 180 rpm for 24 h. The fermentation medium consisted of 80 g xylose, 0.4 g urea, and the other ingredients were in similar amounts to the preculture medium (per liter). For each experiment, the fungal pellets were transferred (10% v/v) into a 250-mL flask containing 50 mL of fermentation medium and allowed to grow at 32 °C with shaking at 220 rpm for 7 days. 2.25g sterilized $CaCO_3$ powder was

added to the fermentation medium to maintain a pH of 5.5. All media were sterilized by autoclaving at 115 °C for 30 min before being used.

Strain modification and selection

For the modification procedure, four different medium with increasing concentrations of xylose (30g/L, 50 g/L, 80 g/L, and 100 g/L) were prepared, and the other contents in this medium were similar to the fermentation medium. The pH of the media was not adjusted. Strains were modified by a sequential transfer of culture samples to modification media containing increasing concentrations of xylose. *R. arrhizus* pellets were first grown in 30 mL of modification medium containing 30 g/L xylose with 220 rmp agitation at 32 °C in a 250-mL Erlenmeyer flask. After 24-h incubation, a large number of fungi pellets formed. Cells were collected by centrifugation and transferred to a fresh modification medium for 24-h continued cultivation, and then collected and transferred again. This collection-transformation-cultivation procedure was repeated at least 10 times until a stable culture was established. Afterward, a sample of 1-mL culture broth was taken, diluted in sterile water, and spread on an isolation medium plate. The transferred culture plates were maintained at 32 °C for 24 h. A single colony was selected and named as mod30, then transferred into a preculture medium. Part of these pellets were used for fermentation experiments to evaluate its FA productivity, while part of these newly formed pellets were set into a high-level modification procedure with a higher concentration of xylose that gradually increased in concentration from 50 g/L to 80 g/L and 100 g/L, and the improvement strain was named mod50, mod80, and mod100, respectively.

Analytical methods

The culture broth was centrifuged, and the resulting supernatant was used for measurement of fumarate by high-performance liquid chromatography (HPLC) using a BonChrom-C18 column (416 mm × 250 mm, 5 μm, 100 A, Agela Technologies) at a column temperature of 30 °C. The mobile phase was 0.5%（v/v）CH_3COOH at a flow rate of 1.0 mL/min. The retention time of FA was 7.6 min. The residual xylose was determined by DNS colorimetry (Miller 1959). The dry cell weight was determined by harvesting the culture samples, filtering through a Buchner filter, washing the mycelia twice with distilled water, and drying at 65 °C until a constant weight was achieved.

RESULTS AND DISCUSSION

Pellet Formation of the Initial Strain

The pellet formation is an important topic in fermentation research. Many factors influence the formation of fungal pellets, such as pH, medium nutrients, agitation, *etc*. Due to this fact, carbon/nitrogen ratio, inoculum size, and rotation speed were chosen as the research subjects. A reported ideal pellet morphology was described as small (diameter <1 mm), spherical, and uniformly distributed. As the examples show in Fig. 1, after cultivation for 24 h, the morphology of the fungus tended to be pellet-matrix when the C/N ratio was lower, while when the C/N ratio was higher, floc pellets formed. The pellet size was 0.92 ± 0.12 mm when the C/N ratio was 23.33. Therefore, the chosen C/N

ratio was 23.33 with 35 g/L xylose and 1.5 g/L urea for pellet formation in xylose preculture medium, and the cultural conditions were the same as with its parental strain.

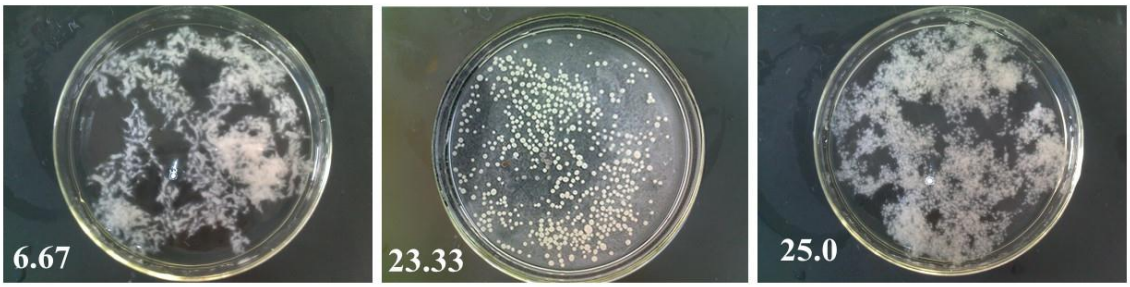

Fig. 1. Effect of C/N ratio in preculture medium on growth morphology of *R. arrhizus RH-7-13*

Enhanced Xylose Utilization and FA Production by Modified Strains

The results of fermentation using the parental and modified strains are summarized in Table 1. Using the modified strains of *R. arrhizus* significantly improved FA production and xylose utilization, compared with its parental strain RH-7-13. FA production of the mod50 strain, which was modified with 50 g/L xylose modification medium, increased more than two-fold, while the consumption of xylose increased from 62% to 83%. The volumetric productivity of FA improved by about 115%, which meant that it reached 169.52 mg/L·h when the concentration of initial xylose was 80 g/L, which is much higher than previously reported (Kautola and Linko 1989). After modification, the biomass of the strain increased slightly with increasing xylose consumption, which could be one of the reasons for overproduction. The production of ethanol occurred in insignificant amounts, for which data is not shown. It is directly reflected in Table 1 that when the xylose concentration in the modification medium increased, the modified strains showed an increase of FA production at first, and the $Y_{p/x}$ amount increased from 3.31 (parental strain) to 4.22 (improved strain mod50). However, after modification in the medium with a xylose concentration of 80 g/L or 100 g/L, the FA production of modified strains no longer increased, and the $Y_{p/x}$ even decreased to 2.86 (modified strain mod100). Early research suggests that during xylose metabolism, relatively more substrate is respired into carbon dioxide, which results in higher energy generation accompanied by higher amounts of biomass (Maas et al. 2006). Under different modified conditions, the improved strains tend to show different mutation trends. Under higher xylose concentration of the modified condition, strains tend to metabolize xylose into cell growth instead of producing output, while mod30 and mod50 with increasing biomass produced more fumaric acid at the same time. Compared with its parental strain, the Yp/s of modified strains showed varying degrees of increase. With 80g/L xylose as the sole carbon source, the Yp/s of the modified strain mod50 arrived at 0.43 g/g, which indicated that the overproduction of fumaric acid is mainly due to the strain-improvement strategy instead of to the accession of biomass.

Results in Fig. 2 show that the newly modified strain (mod50) consumed xylose more effectively than its parental strain during the first 72 h of fermentation and produced more FA during the whole fermentation process. This suggested that the conversion from xylose to FA was greatly enhanced by modification. The modification of fungi, such as *R. oryzae* (Bai *et al.* 2007), *C. tropicalis* (Oberoi *et al.* 2010), and *P. stipitis* (Huang *et al.* 2009), has been carried out in other research to improve strain performance for enhanced substrate utilization, inhibitor tolerance, and product output.

Table. 1. Fermentation Characteristics of Modified Strains and the Parent Strain of *R. arrhizus* with an Initial Xylose Concentration of 80 g/L or 100 g/L as the Sole Carbon Source

Carbon Source	Strain	Produced Fumaric Acid (g/L)	Produced Cell Dry Weight (g/L)	$Y_{p/x}$ (g/g)	$Y_{p/s}$ (g/g)
80g/L Xylose	Parent Strain	13.23±0.13	4.0±0.03	3.31±0.03	0.27
	Mod 30	26.83±0.37	6.39±0.10	4.20±0.01	0.42
	Mod 50	38.48±0.12	6.76±0.09	4.22±0.04	0.43
	Mod 80	18.42±0.24	5.36±0.02	3.44±0.03	0.36
	Mod 100	16.74±0.21	5.86±0.04	2.86±0.02	0.34
100g/L Xylose	Parent Strain	14.66±0.10	3.98±0.04	3.68±0.03	0.27
	Mod 30	24.28±0.40	6.41±0.07	3.79±0.02	0.39
	Mod 50	24.68±0.22	6.93±0.02	3.56±0.02	0.39
	Mod 80	16.73±0.38	5.43±0.04	3.08±0.06	0.34
	Mod 100	15,59±0.10	5.77±0.01	2.70±0.01	0.32

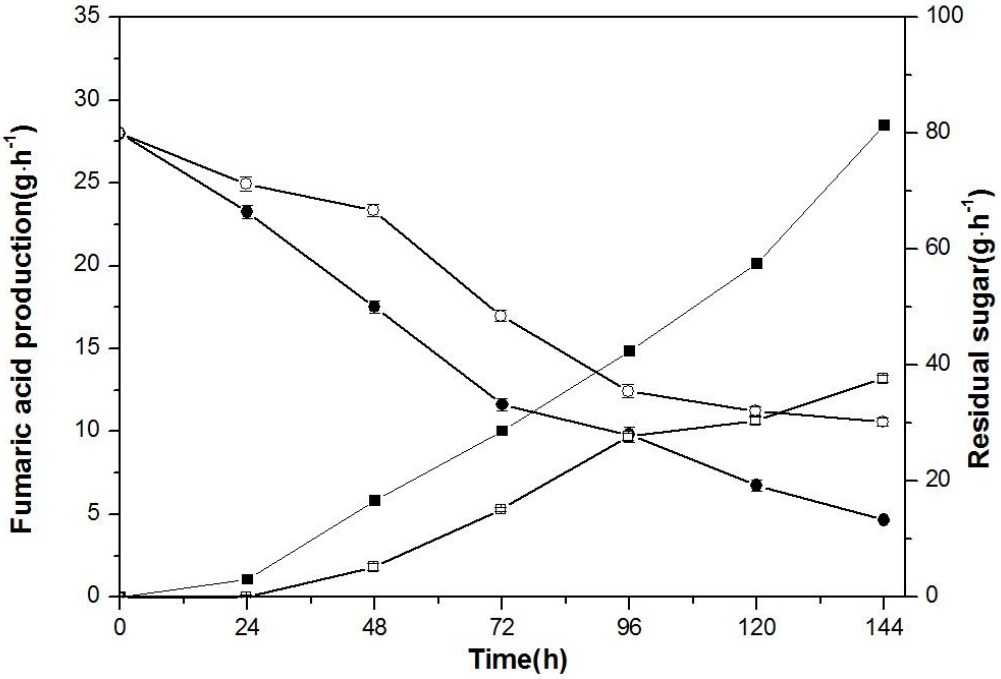

Fig. 2. Comparison of FA production by the mod50 strain of *R. arrhizus* (filled circle) and its parental strain *R. arrhizus* RH-7-13 (open circle), and residual sugar by the modified strain (filled square) and strain RH-7-13 (open square) over time in 80 g/L xylose-based medium

FA production by modified strains using glucose

To investigate the mutation of the newly developed strains, mod30 and mod50 strains were cultured in xylose-based media and glucose-based media. During the preculture phase, neither of these modified strains could form pellets under the same culture conditions using glucose as the carbon source, while pellets were formed optimally in the xylose-based medium. The modified strains produced more FA using

xylose as the sole carbon source rather than glucose, which can be seen in Fig. 3. Both of these fermentation processes produced a small amount of ethanol. It was reported that *R. arrhizus* RH-7-13, which was chosen as the initial strain, could produce almost 39.63 g/L of fumaric acid when cultured in a glucose-based medium. Therefore, the glucose-consumption ability of these modified strains must be weakened during the modification process, and pellet formation influenced the production as well. However, compared to the results of previously reported fermentation of glucose to FA (Cao *et al.* 1996), the production of FA from xylose by our modified strain was relatively poor. Even so, we have seen that xylose, as the ultimate product of hemicellulosic materials, holds a promising prospect and commercial value in the industry of FA production.

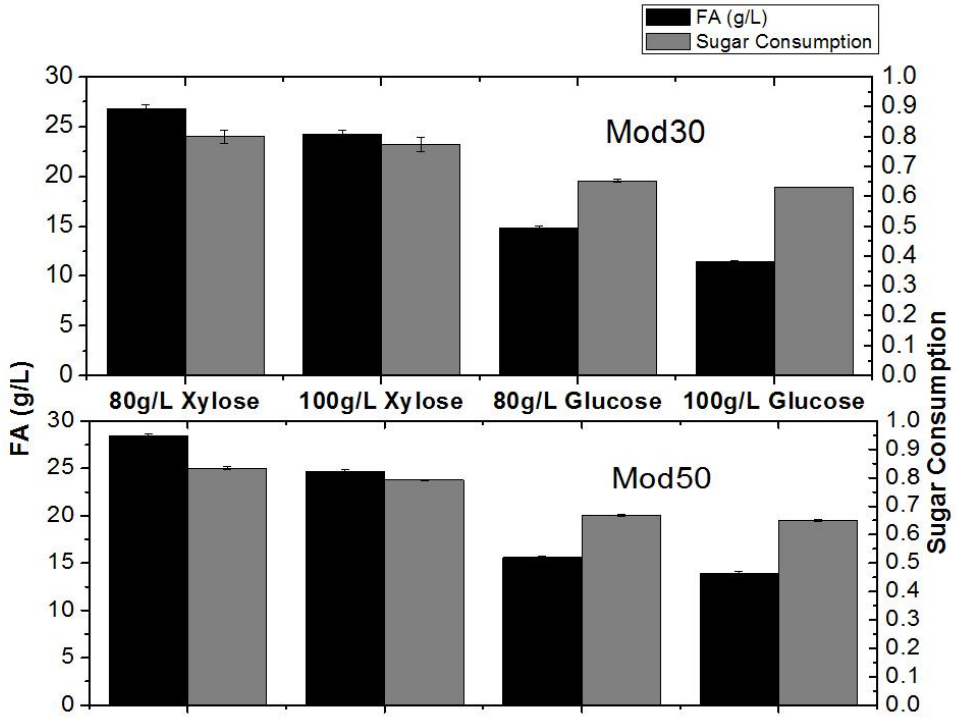

Fig. 3. Comparison of FA production and sugar consumption by the modified strains mod30 and mod50, using xylose and glucose as carbon sources

In recent years, a great deal of attention has been paid to the bioconversion of hemicellulose and accordingly to its practical applications in various industrial and agricultural processes. Dilute sulfuric acid hydrolysis is thought to be a promising, simple, fast, and convenient pretreatment method for producing sugars from cellulosic biomass and has been widely used in the fermentation industry. However, this process is accompanied by a production of weak acids and furan derivatives, which have been characterized as inhibitors of microbial growth and therefore detrimental to fermentation performance. This research attempted to find a new strain-modification process to raise its utilization rate of xylose, and successfully increase the production of FA in fermentation with xylose as the sole carbon source by improved strains, which has made for a considerable basis for further research on biomass fermentation. In this way,

modification of fermentation microorganisms to inhibitory hydrolysates is a possible and promising strategy for dealing with the inhibitor problem, while the production of organic acid will be improved in the hydrolysate fermentation process.

CONCLUSIONS

1. In this work, xylose was used as the sole carbon source for both the seed culture phase and fermentation process, for which there was little previously reported literature. The volumetric productivity of FA increased using xylose concentration modification; FA production increased to 28.48 g/L at 80% initial xylose, a carbon:nitrogen ratio of 200, and a residence time of 7 days. The volumetric productivity was 169.52 mg/L per hour, using the modified mod50 strain.
2. The present study demonstrated that modification of the original strain *R. arrhizus* using media with increasing concentrations of xylose resulted in greater production of FA and faster utilization of xylose when the concentration was below 50 g/L.
3. From the results obtained in this study, it can be concluded that the filamentous fungus *R. arrhizus* is a promising microbial producer of fumaric acid from xylose, which is abundantly present in lignocellulosic hydrolysates.
4. In order to utilize and efficiently develop the potential value of *R. arrhizus*, much more can be done. During acid hydrolysis of plant biomass, compounds that inhibit fermenting microorganisms are formed as well, so that the modification of inhibitory hydrolysates is one of several possible strategies to improve the production of FA in actual fermentation with hydrolysate. Moreover, the appropriate addition of glucose and the optimization of glucose/xylose ratios might also increase production. The results of this work indicated that using *R. arrhizus* to produce fumaric acid from xylose was encouraging and could have great potential in the development of FA production fermentation.

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