Conversion of Corn Stalk to Ethanol by One-Step Process using an Alcohol Dehydrogenase Mutant of *Phanerochaete chrysosporium*

Lei Liu,[a] Ping Li,[a] Ge Qin,[a] Yue Yan,[a] Yi Li,[a] JianMing Yao,[b] and Hailei Wang [a,c,*]

The potential of *Phanerochaete chrysosporium* in ethanol fermentation was evaluated. During the initial submerged cultivation, 1.76 g/L ethanol was obtained using glucose as substrate. After mutation, the ethanol concentration of an alcohol dehydrogenase (ADH) mutant reached 5.02 g/L. Both base transition and nine-base frame shift mutation occurred in the ADH gene of the mutant, changing the secondary and tertiary structures of ADH, as well as increasing the ADH activity during cultivation. Moreover, *P. chrysosporium* converted corn stalk to ethanol by a one-step process. After statistical optimizations, 0.26 g/g•substrate of ethanol yield was obtained on day 10. During the fermentation, the maximum lignin peroxidase, Mn-dependent peroxidase, and cellulase activities were 29.0 U/L, 256.5 U/L, and 40 U/mL, respectively, thus explaining why the fungus directly ferments corn stalk to ethanol. This study is the first report of the conversion of corn stalk without pretreatment to ethanol using a white-rot fungus.

Key words: Ethanol; Phanerochaete chrysosporium; Alcohol dehydrogenase; Mn-dependent peroxidase; Cellulase

Contact information: a: College of Life Sciences, Henan Normal University, Xinxiang 453007, China; b: Institute of Plasma Physics, Chinese Academy of Sciences, Hefei 230031, China; c: Key Laboratory for Microorganisms and Functional Molecules, University of Henan Province, Xinxiang 453007, China;
** Corresponding author: whl@htu.cn*

INTRODUCTION

With the inevitable depletion of the world's energy supply, increasing interest has been focused on alternative energy sources (Kerr 1998; Zaldivar *et al*. 2001). Over the last two decades, natural energy resources, such as petroleum and coal, have been consumed at extremely high rates. Therefore, the heavy reliance of modern economy on these resources is unsustainable. Moreover, alternative resources, such as ethanol, have aroused increasing interest because of the negative environmental effects generated by the utilization of fossil fuels (Cheng and Zhu 2009).

Ethanol is one of the cleanest renewable fuels. To date, the varied raw materials used in the manufacture of ethanol *via* fermentation are conveniently classified into three main types (Lin and Tanaka 2006), namely, sugars, starches, and cellulose materials. Sugars from fruits, sugarcane, molasses, and sugar beets can be directly converted into ethanol. Starches must be initially hydrolyzed to fermentable sugars by the action of enzymes or aqueous acids, and starchy materials used for industrial ethanol production include potatoes, cassava, corn, and root crops. Cellulose (from agricultural residues, wood, and waste sulfite liquor from papermaking mills) can likewise be converted into fermentable sugars. Once simple sugars are formed, microorganisms can readily ferment

the sugars to ethanol. Among the three main types of materials, cellulose materials are the most abundant source and have been largely unutilized. In recent years, increasing attention has been focused on the conversion of lignocellulosic materials to ethanol (Sanchez and Cardona 2008; Bellido *et al*. 2011).

Lignocellulose accounts for more than 90% the global production of plant biomass. However, the effective utilization of lignocellulosic feedstock is not always practical because of its seasonal availability, scattered stations, and the high costs of transportation and storage (Polman 1994). Furthermore, lignocellulose, which is composed of a mixture of cellulose, hemicellulose, and lignin, is a complex substrate. Carbohydrate polymers are tightly bound to lignin mainly through hydrogen bonds but also covalent bonds. The conventional biological process for converting lignocellulose to ethanol requires a minimum of three steps: (i) delignification to liberate cellulose and hemicellulose from their complex with lignin, (ii) depolymerization of carbohydrate polymers to produce free sugars, and (iii) fermentation of mixed hexose and pentose sugars to produce ethanol. Among the key processes, delignification is the rate-limiting and most difficult task to be solved. Another problem is that the aqueous acid used to hydrolyze the cellulose to glucose and other simple sugars destroys much of the sugars in the process (Yu and Zhang 2004). In this field, although bioethanol production by biomass has been considerably improved by new technologies, several challenges still need further investigation (Jönsson *et al*. 2013).

Numerous bacteria and fungi have been used for ethanol production, and yeast is the preferred microbe for most ethanol fermentation processes (Ghasem *et al*. 2004; Bellido *et al*. 2011; Saha *et al*. 2011). Information on ethanol fermentation by white-rot fungus is rare. *Phanerochaete chrysosporium* is a strain of white-rot fungus that is studied extensively as a model organism (Tien and Kirk 1983). The fungus degrades lignin by producing ligninolytic enzymes that mainly consist of lignin peroxidase (LiP; EC1.11.1.14) and Mn-dependent peroxidase (MnP; EC1.11.1.13), and it also secretes cellulase and hemicellulase. Interestingly, the fungus is also an ethanol producer under O_2 limitation conditions. This work evaluated the potential of a *P. chrysosporium* mutant in ethanol fermentation using corn stalk, a common and cheap lignocellulosic material. The advantage of ethanol fermentation by *P. chrysosporium* is the direct conversion of corn stalk to ethanol, thereby reducing the pretreatment procedures, including the specific delignification and saccharification in conventional biological process.

EXPERIMENTAL

Chemicals and Microorganism

All chemicals used were of analytical grade unless otherwise stated. *P. chrysosporium* (ATCC24725) was provided by the Laboratory of Microbiology and Functional Molecules, University of Henan Province, China. The fungus was stored on a potato dextrose agar (PDA) plate at 4 °C before use.

Mutation Procedure of *P. chrysosporium* by ARTP

P. chrysosporium was incubated on a PDA plate and sub-cultured for 3 days at 35 °C before the conidia were harvested using a camel hair brush. Conidia were prepared and used as samples for mutation treatment. The atmospheric pressure glow discharge plasma (ARTP) mutation system consisted of a power supply subsystem (13 to 56 MHz), a gas

supply control subsystem, a co-axial type plasma generator, and a simple plate made of stainless steel (Wang *et al*. 2010). For the mutation, 10 µL of the conidium suspension was dipped onto stainless steel, and helium was used as plasma working gas. Operating parameters were as follows: power input, 100 W; distance between the plasma torch nozzle exit and the sample plate, 3 mm; temperature of the plasma jet, 25 °C to 35 °C; and treatment period, 40 s. After ARTP treatment, conidium samples were all washed with 10 to 20 µL of sterile water. The conidium suspension was diluted gradually and then coated on a PDA plate supplemented with 2,3,5-three phenyl tetrazolium chloride, which can react with alcohol dehydrogenase (ADH) to produce red triphenyl formazan. The red colony was selected and inoculated on a PDA plate at 35 °C.

Initial Ethanol Fermentation using Glucose

The mutant and original strains of *P. chrysosporium* were tested for their ability to produce ethanol. Two groups of 500 mL glass bottles (6 bottles) contained 200 mL of liquid medium (LM1) each; the medium contained 15 g/L glucose, 3.0 g/L ammonium tartrate, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L $CaCl_2 \cdot 2H_2O$. These glass bottles were inoculated with conidium suspensions (5.0 mL) of the two strains. Fermentation was conducted on a rotary shaker at 35 °C and 150 rpm. The bottles were first sealed with gauze, and cultivation proceeded under aerobic conditions. After 36 h, the bottles were tightly sealed using rubber plugs and Parafilm and cultivated continuously. Both ethanol and ADH activity were measured during fermentation.

In addition, the effect of glucose on ethanol production was investigated. The components of the liquid medium were the same as LM1, except glucose was adjusted to different levels (4, 6, 8, 10, 12, 14, and 16 g/L).

PCR Amplification of ADH Genes

The mutant and original strains of *P. chrysporium* were inoculated in LM1 at 35 °C for 3 days, and mycelial pellets were extracted as the total DNA of samples by using the cetyltrimethylammonium bromide method (Griffiths *et al*. 2000). The yield and the fragmentation of DNA were determined by agarose gel electrophoresis and UV visualization after ethidium bromide (EB) staining. The primer pairs of FP (5′-CGGGATCCATGCTCGCCTACCGCTTC-3′) and RP (5′-CCGCTCGAGTTACGCAG-ACGCAGACGC-3′) for the ADH gene were designed according to the information from the *P. chrysosporium* genome database (http://genome,jgi.doe.gov/Phchr2/Phchr2.home.html). The thermo-cycling procedure consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 61 °C for 30 s (annealing), and 72 °C for 1 min (extension), and a final extension at 72 °C for 6 min. Each reaction was conducted in a 25 µL reaction mixture containing 10 ng of template DNA, 2.5 mM of the deoxynucleoside triphosphate mix, 1 µM of each of the primers (20 µM), and 12 µL of FastPfu polymerase (5 U/µL). PCR cycling was performed in a GeneAmp®9700 DNA thermocycler (ABI, Maryland, USA), and the amplified products were visualized on agarose gels containing EB and purified with a DNA gel extraction kit (Axygen Inc., California, USA). Sequencing was carried out at Majorbio Company (Shanghai, China), and ADH gene sequences of the mutant and original strains were deposited in the NCBI database (accession number: KR108328; KU500042).

SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to detect the signal peptide of ADH genes. The secondary and tertiary structures of ADH were

predicted by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Lambert *et al*. 2002), and the obtained 3D structure images were processed by Swiss-Pdb Viewer software (Arnold *et al*. 2006).

Ethanol Fermentation using Corn Stalk

Corn stalks were chopped into small pieces using a fodder grinder, the particles that were passed through 0.90 mm mesh sieve were used for conversion. Two liquid media (LM2 and LM3) were used to produce ethanol. LM2 contained the following components: 20.0 g/L corn stalk, 3.0 g/L ammonium tartrate, 2.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L $CaCl_2 \cdot 2H_2O$. The formula of LM3 was the same as that of LM2, except 20.0 g/L corn stalk was changed to 2.0 g/L glucose and 18.0 g/L corn stalk. Submerged cultivation was conducted in a thermostat shaker at 35 °C and 150 rpm.

Statistical Optimizations

The important factors that influence ethanol production were identified by fractional factorial design (FFD). Six factors were selected as independent variables, and their levels are listed in Table 1. The effects of the important factors screened by FFD on ethanol production were studied by a central composite experimental design (CCD). Two independent variables, namely, ammonium tartrate and pH value, were designated as X_1 and X_2 and coded according to the following equations,

$$X_1 = (Z_1 - 4.5) / 1.5; \quad (1)$$

$$X_2 = (Z_2 - 5) / 1 \quad (2)$$

where Z_i (i = 1 and 2) is the actual value for X_i (i = 1 and 2).

Ethanol concentration, the dependent response, is designated by Y. A second-order polynomial function was fitted to predict the mathematical model between the independent variables and dependent response,

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{12}X_1X_2 + b_{22}X_2^2 \quad (3)$$

where Y is the predicted response, X_1 and X_2 are the code forms of input variables, b_0 is a constant, b_1 and b_2 are linear coefficients, b_{11} and b_{22} are quadratic coefficients, and b_{12} is the cross-product coefficient.

Analytical Methods

The reducing sugar was determined by using the 3,5-dinitrosalicylic acid method. Cellulase, which is expressed as filter paper activity (FPA), was measured according to Reczey *et al*. (1996), with one unit defined as formation of 1 µmol glucose per hour. LiP activity was measured as described by Roy and Archibald (1993), with 1 U defined as 1/µmol of veratryl alcohol oxidized to vertraldehyde per min. MnP activity was measured as described by Michel *et al*. (1991), with 1 U defined as 1 µmol of Mn^{2+} oxidized to Mn^{3+} per min. ADH activity was measured according to the method of Sudar *et al*. (2013), and one unit of ADH activity was defined as the amount of enzyme necessary to convert 1 µmol of NAD^+ to NADH per minute at 25 °C and at pH 8.8 in the glycine-pyrophosphate buffer. Ethanol was measured by high-performance liquid chromatography. Prior to analysis, the clear supernatant after centrifugation of fermentation broth was diluted with double-distilled water and filtered through nylon syringe filter (0.25 µm). Analysis was performed by Prominence Chromatograph (Shimadzu, Tokyo, Japan) equipped with a Rezex ROA-Organic Acid H^+ column (300 × 7.8 mm) and a refractive index detector (RID-10A,

Shimadzu). The following operational parameters were applied: injection volume, 20 µL; elution temperature, 60 °C; mobile phase, 0.005 mol/L H_2SO_4; and flow rate, 0.6 mL/min.

Minitab 16 (Minitab Inc., Pennsylvania, USA) was used for regression analysis of the obtained data and coefficient estimation of regression equations. The quality of the fit of the polynomial model was expressed by the determination coefficient R^2. Statistical significance was validated by an *F*-test, and the significance of regression coefficients was tested by *t*-test.

RESULTS AND DOSCUSSION

Mutant Screening

After ARTP mutagenesis, six strains of *P. chrysosporium* with higher ADH activity were obtained on PDA plates (Fig. 1).

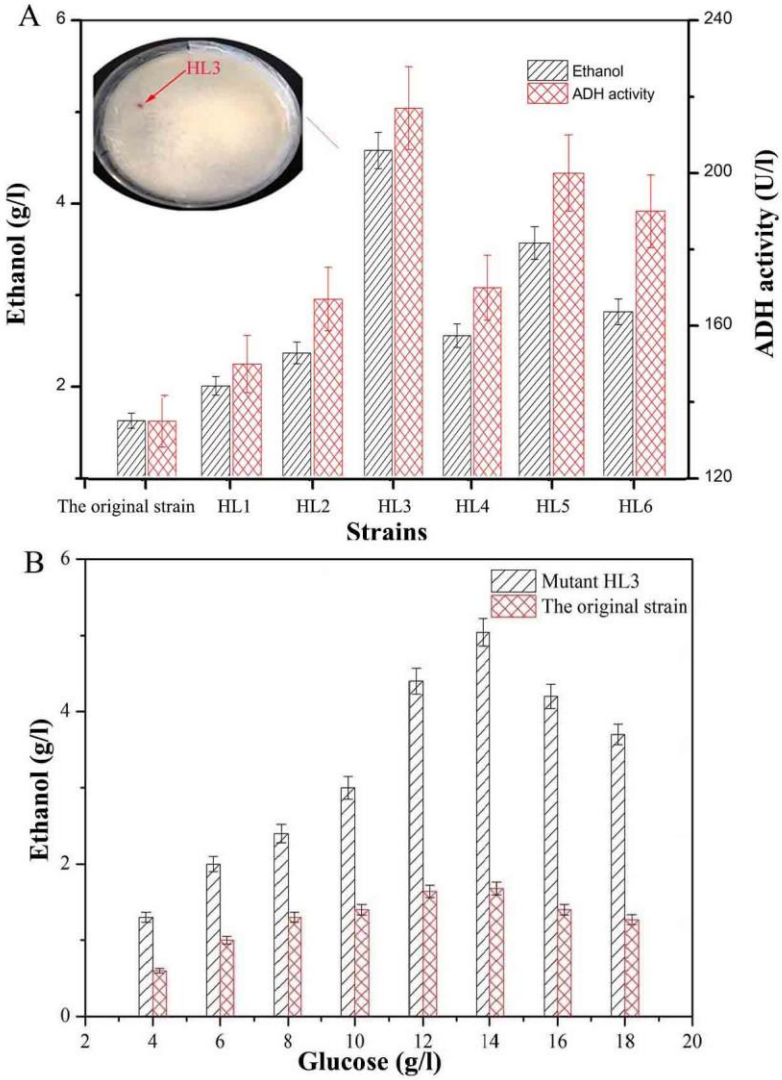

Fig. 1. Ethanol and ADH activity of different strains, as well as the effect of glucose on ethanol production during submerged fermentation. (A) Ethanol and ADH activity of different strains; (B) effect of glucose on ethanol production of mutant HL3 and the original strain

The results of submerged cultivation showed that mutant HL3 is a better ethanol producer (Fig. 1A), and its ethanol concentration reached 3.58 g/L on day 9, which is 2.2 times higher than that of the original strain (1.63 g/L). Glucose exerted an important influence on ethanol production (Fig. 1B), and 14 g/L was considered as the ideal level for both mutant HL3 and the original strain to produce ethanol. A maximum ethanol concentration of mutant HL3 reached 5.02 g/L, and it was 5.2 times higher than that (about 0.96 g/L on day 9) in the previous report (William and Diane 2004). The corresponding ethanol yield reached 0.29 g/g·glucose. Higher glucose concentration (> 14 g/L) inhibited ethanol production.

Moreover, the correlation between ethanol concentration and ADH activity was analyzed. Ethanol was positively correlative with ADH activity, and the two-tailed test indicated that the correlation coefficient was 0.958 and correlation was significant at the 0.01 leve1. In sum, the high ADH activity of mutants resulted in higher ethanol yield. ADH catalyzed the reduction of acetaldehyde to ethanol in the last step of the ethanol production pathway (Ida *et al*. 2012). Thus, the activity is expected to significantly influence ethanol production.

Comparison of ADH Genes

Figure 1 also shows that the ADH activity of mutant HL3 (217 U/l) was significantly higher than that of the original strain. The ADH genes of the original and mutant strains are shown in Fig. 2.

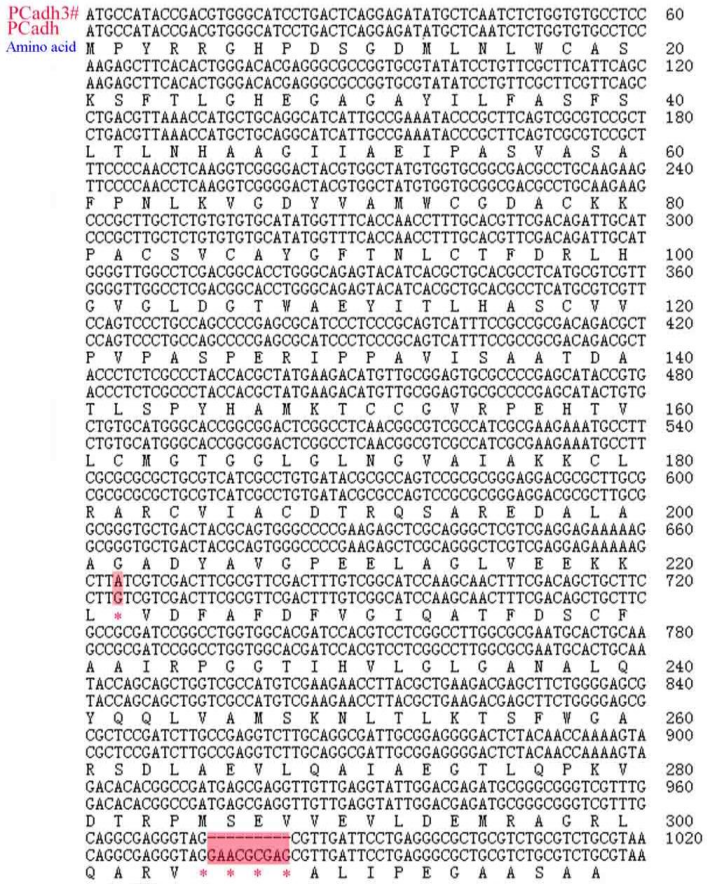

Fig. 2. Comparison of ADH genes of mutant HL3 and the original strain

The ADH genes of mutant HL3 (Pcadh3#) and original strain (PCadh) were amplified and compared. PCadh possesses a length of 1020 bp and encodes 340 amino acids. However, Pcadh3# only contains 1011 bases, and 9 bases disappear because of frame shift mutation from ARTP treatment (Fig. 2). In addition, base transition (G→A) also occurred in the codon coding the 222th amino acid (V222→I222). Evidently, these mutations will change the secondary and tertiary structures of ADH.

Structures of ADH of mutant HL3 and original strain were predicted on the basis of the amino acid sequences, and neither strain possessed the signal peptide analyzed by the SignalP 4.1 server. The secondary structure shows that the ADH of the original strain contains 9 α-helices and 13 β-folds. In contrast to the original strain, the ADH of mutant HL3 loses one α-helices, but two β-folds were added (Fig. 3). According to the homology comparison of protein template, Swiss-PDB Viewer was used to predict the 3D structure of ADHs. The number and binding regions of several key genes in two enzymes were different (see Appendix). For example, both enzymes possessed 21 zinc ion-binding sites, but the location and structure of these sites were not consistent. In addition, after mutagenesis, an active site named 1pl6_C was added to the coenzyme nicotinamide adenine dinucleotide (NAD) site. NAD is an important coenzyme in all living cells, and the NAD+ and NADH levels exert a significant effect on microbial growth and ethanol production (Bhatt and Srivastava 2008; Li *et al*. 2011). Thus, mutagenesis by ARTP results in the frame shift mutation and base transition in Pcadh3#, leading to changes in the secondary and tertiary structures of the enzyme. These changes in gene and protein structure can explain the higher ADH activity of mutant HL3.

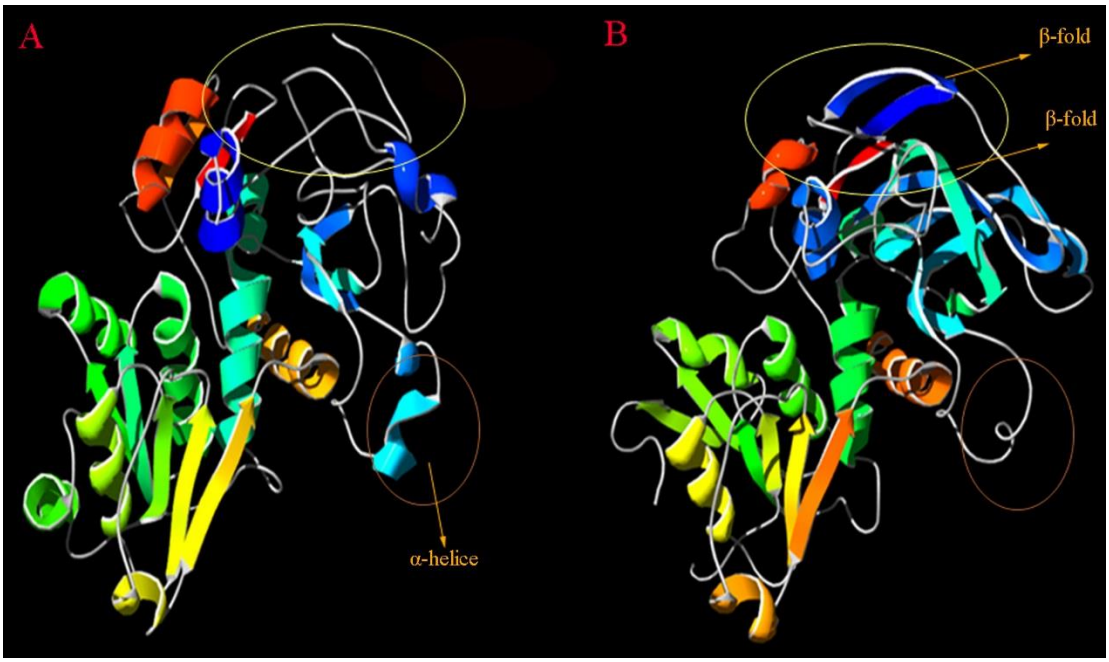

Fig. 3. 3D structure prediction of ADHs. (A) ADH of mutant HL3; (B) ADH of the original strain

Ethanol Fermentation using Corn Stalk

Corn stalks, which mainly consist of cellulose, hemicellulose, and lignin (Supplementary material 2), are abundant renewable resources (Guo *et al*. 2011), and the feasibility of utilizing a low-cost material to produce ethanol was evaluated. During cultivation, ethanol appeared in LM2 on day 5, and then its concentration increased with

time and reached 2.2 g/L (ethanol yield, 0.16 g/g·corn stalk) on day 11 (Fig. 4). After day 11, the curve of ethanol concentration presented a decreasing trend. The appearance of 2.0 g/L glucose significantly improved ethanol yield. Ethanol appeared in LM3 on day 3, and the maximum ethanol concentration of 2.9 g/L (ethanol yield, 0.21 g/g·substrate) was obtained on day 10. This finding is explained by the greater biomass requirement to produce higher yield of ethanol, and glucose is helpful for the growth of *P. chrysporium* at the early phase of cultivation as a rapid metabolizable carbon source (Brückner and Titgemeyer 2002). The facts mentioned above show that corn stalk can be used to produce ethanol as carbon supplement.

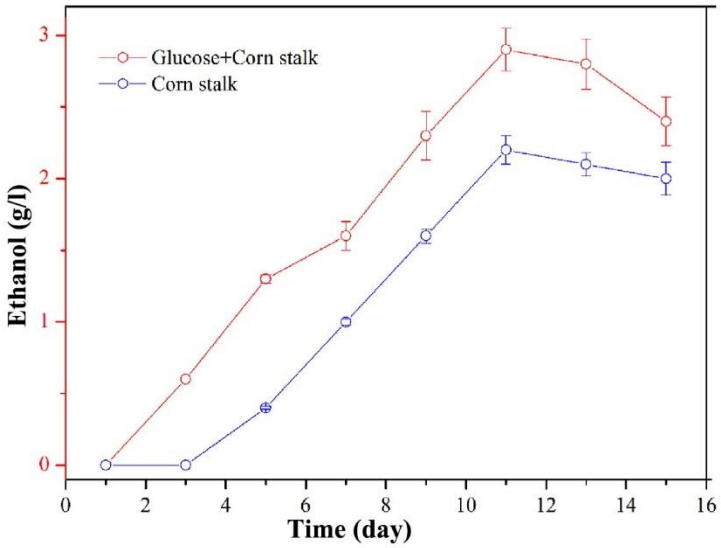

Fig. 4 Ethanol concentration variation with time during submerged fermentation using corn stalk and corn stalk plus glucose as substrates

Optimization of Culture Conditions

The design and results of the FFD experiment are shown in Table 2, and statistical analysis indicated that ammonium tartrate and pH are important factors that affect ethanol production. The t-test showed that p-values of ammonium tartrate and pH values were both 0.000, indicating that they were significant at the 0.01 level (Table 1).

Table 1. Levels and Significance of Factors in Fractional Factorial Experiment

Factor	Level		Coefficient	t-value	p-value
	-1	1			
A Corn straw (g/L)	15.0	20.0	0.0750	1.35	0.209
B Ammonium tartrate (g/L)	3.0	6.0	0.6750	12.18	0.000**
C Rotary speed (rpm)	50	150	0.0500	0.90	0.391
D $MnSO_4$ (g/L)	1.0	2.0	-0.0625	-1.13	0.289
E pH	4	6	-0.3750	-6.76	0.000**
F Temperature(℃)	28	32	-0.0125	-0.23	0.827

** Represents significance at the 0.01 level

The possible explanation is that *P. chrysosporium* needs to hydrolyze cellulose to sugar before ethanol production, and nitrogen resource (ammonium tartrate) and pH are crucial factors that influence the secretion of lignin-degrading enzymes and cellulase (Chen *et al*. 1991; Wang *et al*. 2012).

Table 2. Experiment Design and Results of the Fractional Factorial Experiment

Trial	A	B	C	D	E	F	Ethanol (g/L)
1	1	1	-1	1	-1	-1	3.5
2	1	-1	-1	-1	1	-1	1.7
3	-1	-1	1	1	1	-1	1.4
4	1	-1	1	-1	-1	1	2.0
5	1	1	1	1	1	1	2.9
6	-1	-1	-1	1	-1	1	2.1
7	1	-1	1	1	-1	-1	2.3
8	1	1	-1	-1	-1	1	3.6
9	-1	1	1	-1	-1	-1	3.4
10	-1	1	1	1	-1	1	3.5
11	-1	-1	-1	-1	-1	-1	2.1
12	1	-1	-1	1	1	1	1.1
13	-1	1	-1	1	1	-1	2.2
14	1	1	1	-1	1	-1	3.0
15	-1	1	-1	-1	1	1	2.8
16	-1	-1	1	-1	1	1	1.4

Table 3. Variables and Experimental Results of CCD

Trial No.	Code Value		Real Value		Ethanol (g/L)
	X_1	X_2	X_1	X_2	Y
1	1.414	0	6.62	5	3.4
2	1	1	6	6	2.3
3	0	0	4.5	5	3.6
4	0	-1.414	4.5	3.59	2.3
5	-1.414	0	2.38	5	2.0
6	0	0	4.5	5	3.6
7	-1	1	3	6	2.1
8	0	0	4.5	5	3.5
9	1	-1	6	4	3.3
10	0	0	4.5	5	3.8
11	-1	-1	3	4	2.4
12	0	1.414	4.5	6.41	1.9
13	0	0	4.5	5	3.6

CCD is one of the most commonly used response surface designs that is used extensively in optimizing the performance and design of products (Wang *et al*. 2011; Alberti *et al*. 2014). In this work, CCD was used for further optimization of ammonium tartrate and pH. The variables and experimental results are shown in Table 3. After data

processing by using Minitab 16 software, the second order polynomial equation of Y was expressed as:

$$Y = 3.62 + 0.385X1 - 0.2332X2 - 0.4288\,X12 - 0.175X1X2 - 0.7288X22 \quad (4)$$

Table 4 shows the significance analysis of regression terms for the quadratic response surface model. Regression terms of the quadratic model were significant (p-values < 0.01) except for the interaction term (p-value = 0.103), indicating that the selected factors are critical to ethanol production but independent of each other. The ANOVA of the quadratic regression model demonstrates that the model presented high correlation with the experimental data (p = 0.001). The goodness of fit of the model was checked by determination coefficient (R^2). In this case, R^2 of the response surface model, 0.9624, established that the model fitted the experimental results well. The fitted quadratic polynomial equation was expressed as 3D response surface diagram and contour plot (Figs. 5A and 5B) to visualize the relationship among ethanol yield, ammonium tartrate, and pH value. Optimum parameters were obtained by solving Eq. 4, and the optimal ammonium tartrate and pH were 5.24 g/L and 4.78, respectively.

Table 4. Estimated Regression Coefficients for Eq. 4

Model	b0	b1	b2	b11	b12	b22
Coefficient	3.6200	0.3850	-0.2332	-0.4288	-0.1750	-0.7288
SE-Coe.	0.0834	0.0659	0.0659	0.0707	0.0932	0.0707
t-value	43.407	5.839	-3.537	-6.064	-10.307	-1.877
p-value	0.000**	0.001**	0.010**	0.001**	0.1030	0.000**

** Represents significance at 0.01 level

Confirmatory Ethanol Fermentation Experiment

Under optimal conditions, the confirmatory experiments of ethanol fermentation were conducted, and the lignin-degrading enzymes and cellulase were also measured during fermentation. The *P. chrysosporium* can secrete a series of enzymes, including LiP, MnP, and cellulase (Szabó *et al*. 1996; Jin *et al*. 2009; Zeng *et al*. 2014). In previous reports, the fungus was used extensively in the pretreatment of lignocellulose because the secreted LiP and MnP catalyze the oxidization of lignin complex (Jin *et al*. 2009; Zhi and Wang 2014).

During ethanol fermentation, the maximum LiP and MnP activities reached 29.0 and 256.5 U/L, respectively, on day 5 (Fig. 6), suggesting that the used *P. chrysosporium* is a good MnP producer. The deconstruction of lignin is helpful for the improved accessibility of cellulose for cellulase, and in this test, the maximum FPA was 40 U/mL. To date, no evidence has shown that *P. chrysosporium* can convert hydrolytes of hemicellulose to ethanol. Therefore, the appearance of hemicellulase was not discussed in this study.

The reducing sugar increased with FPA and reached the maximum value of 3.9 g/L on day 5. The experimental value of ethanol concentration, 3.6 ± 0.2 g/L (n = 6), was obtained on day 10. The corresponding ethanol yield reached 0.26 g/g·substrate. In view of 0.9 g/L reducing sugar on day 2, a minimum of 2.7 g/L ethanol resulted from the conversion of corn stalk, indicating that ethanol from corn stalk reached 0.23 g/g·corn stalk.

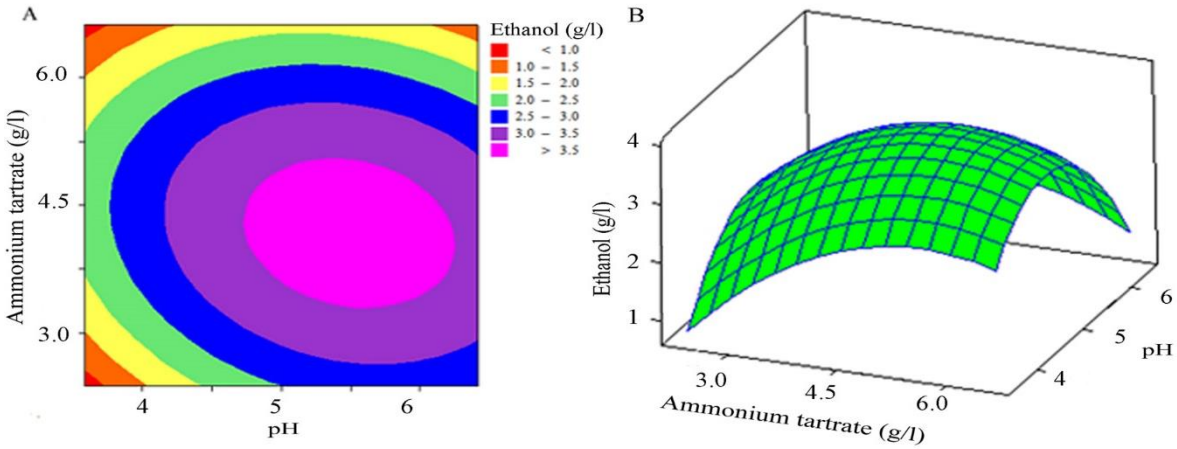

Fig. 5. Contour plot and 3D response surface diagram to visualize the relationship among ethanol, ammonium tartrate, and pH value. (A) Contour plot between ethanol, ammonium tartrate, and pH value; (B) 3D response surface among ethanol, ammonium tartrate, and pH value

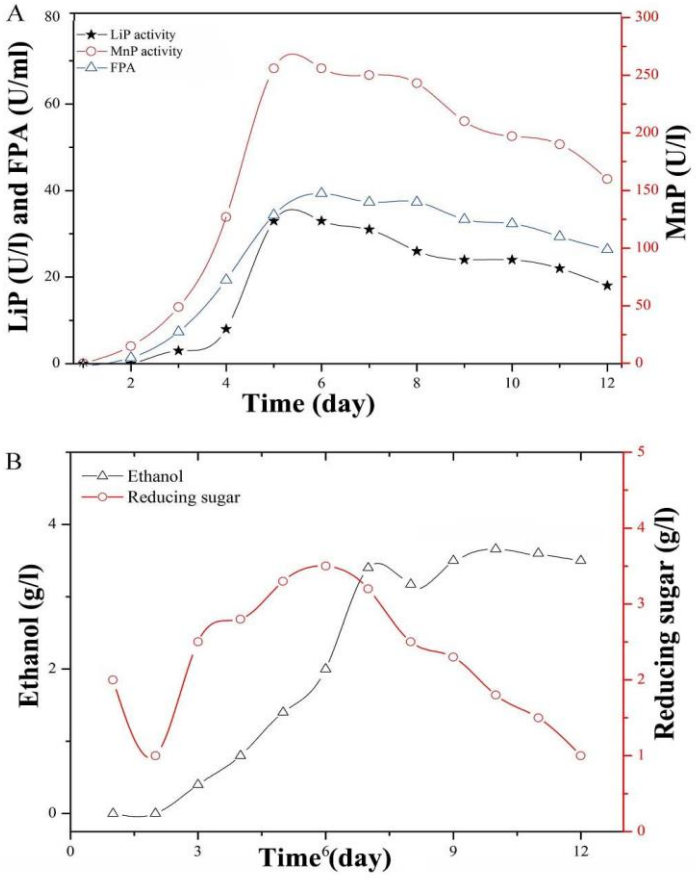

Fig. 6. Variations in reducing sugar, ethanol, FPA, LiP, and MnP activity with time during confirmatory ethanol fermentation. (A) FPA, LiP, and MnP activity; (B) reducing sugar and ethanol concentration.

The facts mentioned above suggest that the fungus possesses versatile abilities, including delignification, cellulose depolymerization, and sugar fermentation to produce

ethanol. Therefore, *P. chrysosporium* can directly convert corn stalk to ethanol. This study is the first report about the conversion of corn stalk by a one-step process and is an interesting commendable attempt because ethanol fermentation by white-rot fungus reduces the higher cost of pretreatment of lignocellulosic materials in the conventional biological process. However, the limitations of this work are also evident. The production of ethanol by *P. chrysosporium* is a slow process, and the fungus cannot accumulate high ethanol concentration. Thus, the strain should be modified further before its application in ethanol production in industrial scale, although the yield of mutant HL3 is increased significantly in contrast to the original strain.

CONCLUSIONS

1. An ADH mutant was screened after AFTP treatment. Both base transition and frame shift mutation occurred in the ADH gene, resulting in changes in secondary and tertiary structures of ADH and improvement of ADH activity.
2. Compared with the original strain, ethanol yield of the mutant was significantly increased by using glucose as substrate. Moreover, the mutant directly converted corn stalk to ethanol, and the maximum ethanol yield was 0.26 g/g·substrate.
3. During ethanol fermentation, FPA, LiP and MnP activities explained why the fungus ferments corn stalks to ethanol by a one-step process.

ACKNOWLEDGMENTS

This work is supported by the National Science Foundation of China (No. 51008119), PhD Research Startup Foundation (5101049170143), and Young Scholar's Foundation (2015QK17) of Henan Normal University. The article and its contents are original. The material was written by the author(s), and it has not been published elsewhere.

REFERENCES CITED

Alberti, A., Zielinski, A. A. F., Zardo, D. M., Demiate, I. M., Nogueira, A., and Mafra, L. I. (2014). "Optimisation of the extraction of phenolic compounds from apples using response surface methodology," *Food Chemistry* 49, 151-158. DOI: 10.1016/j.foodchem.2013.10.086

Arnold, K., Bordoli, L., and Kopp, J. (2006). "The SWISS-MODEL workspace: A web-based environment for protein structure homology modeling," *Bioinformatics* 22, 195-201. DOI: 10.1093/bioinformatics/bti770

Bellido, C., Bolado, S., Coca, M., Lucas, S., González-Benito, G., and García-Cubero, M. T. (2011). "Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*," *Bioresource Technology* 102, 10868-10874. DOI: 10.1016/j.biortech.2011.08.128

Bhatt, S. M., and Srivastava, S. K. (2008). "Mannitol increases lactic acid production by shifting NADH/NAD+ ratio inhibiting ethanol production," *Journal of Biotechnology* 136, S27. DOI: 10.1016/j.jbiotec.2008.07.049

Brückner, R., and Titgemeyer. F. (2002). "Carbon catabolite repression in bacteria: Choice of the carbon source and autoregulatory limitation of sugar utilization," *FEMS Microbiology Letters* 209, 141-148. DOI: 10.1111/j.1574-6968.2002.tb11123.x 141-148

Chen, A. H. C., Dosoretz, C. G., and Grethlein, H. E. (1991). "Ligninase production by immobilized cultures of *Phanerochaete chrysosporium* grown under nitrogen-sufficient conditions," *Enzyme and Microbial Technology* 13, 404-407. DOI: 10.1016/0141-0229(91)90202-L

Cheng, S., and Zhu, S. (2009). "Lignocellulosic feedstock biorefinery - The future of the chemical and energy industry," *BioResources* 4, 456-457. DOI: 10.15376/biores.4.2.456-457

Ghasem, N., Habibollah, Y., Ku, S., and Ku, I. (2004). "Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*," *Bioresource Technology* 92, 251-260. DOI: 10.1016/j.biortech.2003.09.009

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2000). "Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition," *Applied and Environmental Microbiology* 66, 5488-5491. DOI: 10.1128/AEM.66.12.5488-5491.2000

Guo, P., Mochidzuki, K., Cheng, W., Zhou, M., Gao, H., Zheng, D., Wang, X. F., and Cui, Z. J. (2011). "Effects of different pretreatment strategies on corn stalk acidogenic fermentation using a microbial consortium," *Bioresource Technology* 102, 7526-7531. DOI: 10.1016/j.biortech.2011.04.083

Ida, Y., Furusawa, C., Hirasawa, T., and Shimizu, H. (2012). "Stable disruption of ethanol production by deletion of the genes encoding alcohol dehydrogenase isozymes in *Saccharomyces cerevisiae*," *Journal of Bioscience and Bioengineering* 113,192-195. DOI: 10.1016/j.jbiosc.2011.09.019

Jin, S. B., Ko, J. K., Choi, I. G., Park, Y. C., Seo, J. H., and Kim, K. H. (2009). "Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw," *Biotechnology and Bioengineering* 104, 471-482. DOI: 10.1002/bit.22423

Jönsson, L. J., Alriksson, B., and Nilvebrant, N. O. (2013). "Bioconversion of lignocellulose: inhibitors and detoxification," *Biotechnology for Biofuels* 6, 1-10. DOI: 10.1186/1754-6834-6-16

Kerr, R. A. (1998). "The next oil crisis looms large—and possibly close," *Science* 281, 1128-1131. DOI: 10.1126/science.281.5380.1128

Lambert, C., Leonard, N., De Bolle, X., and Depiereux, E. (2002). "ESyPred3D: Prediction of proteins 3D structures," *Bioinformatics* 18, 1250-1256. DOI: 10.1093/bioinformatics/18.9.1250

Li, L. B., Wang, Y. H., Zhuang, Y. P., Chu, Z., and Zhang, S. L. (2011). "Determination of coenzyme NAD+ and NADH of *Saccharomyces cerevisiae* cells in ethanol production," *Journal of Food Science and Biotechnology* 30, 287-294.

Lin, Y., and Tanaka, S. (2006). "Ethanol fermentation from biomass resources and prospects: current state and prospects," *Applied Microbiology and Biotechnology* 69, 627-642. DOI: 10.1007/s00253-005-0229-x

Michel, F. C., Dass, S. B., Grulke, E. A., and Reddy, C. A. (1991). "Role of a manganese peroxidase and lignin peroxidase of *Phanerochate chrysosporium* in the

decolorization of kraft bleach plant effluent," *Applied and Environmental Microbiology* 57, 2368-2375.

Polman, K. (1994). "Review and analysis of renewable feedstocks for the production of commodity chemicals," *Applied Biochemistry and Biotechnology* 45, 709-722. DOI: 10.1007/BF02941842

Reczey, K., Szengyel, Z., Eklund, R., and Zacchi, G. (1996). "Cellulase production by *T. reesei*," *Bioresource Technology* 57, 23-30. DOI: 10.1016/0960-8524(96)00038-7

Roy, B. P., and Archibald, F. (1993). "Effect of kraft pulp and lignin on *Tramestes versicolor* carbon metabolism," *Applied and Environmental Microbiology* 59, 1855-1863.

Saha, B. C., Nichols, N. N., and Cotta, M. A. (2011). "Ethanol production from wheat straw by recombinant *Escherichia coli* strain FBR5 at high solid loading," *Bioresource Technology* 102, 10892-10897. DOI: 10.1016/j.biortech.2011.09.041.

Sanchez, O., and Cardona, C. (2008). "Trends in biotechnological production of fuel ethanol from different feedstocks," *Bioresource Technology* 99, 5270-5295. DOI: 10.1016/j.biortech.2007.11.013

Sudar, M., Valinger, D., Findrik, Z., Vasić-Rački, Đ., and Kurtanjek, Ž. (2013). "Effect of different variables on the efficiency of the Baker's yeast cell disruption process to obtain alcohol dehydrogenase activity," *Applied Biochemistry and Biotechnology* 169, 1039-1055. DOI: 10.1007/s12010-012-0056-3

Szabó, I. J., Johansson, G., and Pettersson, G. (1996). "Optimized cellulase production by *Phanerochaete chrysosporium*: Control of catabolite repression by fed-batch cultivation," *Journal of Biotechnology* 48, 221-230. DOI: 10.1016/0168-1656(96)01512-X

Tien, M., and Kirk, T. K. (1983). "Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds," *Science* 221, 661-663. DOI: 10.1126/science.221.4611.661

Wang, H. L., Ren, Z. F., Li, P., Gu, Y. C., Liu, G. S., and Yao, J. M. (2011). "Improvement of the production of a red pigment in *Penicillium* sp. HSD07B synthesized during co-culture with *Candida tropicalis*," *Bioresource Technology* 102, 6082-6087. DOI: 10.1016/j.biortech.2011.01.040

Wang, L. Y., Huang, Z. L., Li, G., Zhao, H. X., Xing, X. H., Sun, W. T., Li, H. P., Gou, Z. X., and Bao, C. Y. (2010). "Novel mutation breeding method for *Streptomyces avermitilis* using an atmospheric pressure glow discharge plasma," *Journal of Applied Microbiology* 108, 851-858. DOI: 10.1111/j.1365-2672.2009.04483.x

Wang, Y., Chung, J. S., Cao, G. L., Zhao, L., and Wang, A. J. (2012). "Producing cellulase on *Phanerochaete chrysosporium* by fermentation and breeding by induced mutation of cellulose-degrading *Phanerochaete chrysosporium*," *Advanced Materials Research* 2012; 535-537, 2353-2356. DOI: 10.4028/www.scientific.net/AMR.535-537.2353

Yu, Z. S., and Zhang, H. X. (2004). "Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with *Saccharomyces cerevisiae*," *Bioresource Technology* 93, 199-204. DOI: 10.1016/j.biortech.2003.09.016

Zaldivar, J., Nielsen, J., and Olsson, L. (2001). "Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration," *Applied Microbiology and Biotechnology* 56, 17-34. DOI: 10.1007/s002530100624

Zeng, J. J., Singh, D., Gao, D., and Chen, S. L. (2014). "Effects of lignin modification on wheat straw cell wall deconstruction by *Phanerochaete chrysosporium*," *Biotechnology for Biofuels* 7, 161-174. DOI: 10.1186/s13068-014-0161-3

Zhi, Z., and Wang, H. (2014). "White-rot fungal pretreatment of wheat straw with *Phanerochaete chrysosporium* for biohydrogen production: Simultaneous saccharification and fermentation," *Bioprocess and Biosystems Engineering* 37, 1-12. DOI: 10.1007/s00449-013-1117-x

Article submitted: August 16, 2016; Peer review completed: September 18, 2016; Revised version received and accepted: September 25, 2016; Published: October 3, 2016.
DOI: 10.15376/biores.11.4.9940-9955

APPENDIX

Differences of the Number and Binding Regions of the Key Genes between Original Strain and Mutant

Key genes	NAP		Zn		NAD	
	Number	Difference	Number	Difference	Number	Difference
PCadh	5	2nvb_B* 1ykf_D	21	2hcy_C 1h2b_A 1yqd_A 1yqx_B	7	
PCadh3#	3		21	1e3j_A 1ykf_D	8	1pl6_C

* represents an active site.

Composition of Corn Stalk Used in this Work

Composition	Cellulose	Hemicellulose	Lignin	SS	Others
Content (%, dry matter)	38.2	23.7	12.7	15.0	11.4

SS: Soluble substance, includes soluble saccharides, starch and a small amount of protein.