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Deletion of Alcohol Dehydrogenase 2 Gene in *Pachysolen tannophilus* Improves Ethanol Production from Corn Stover Hydrolysates

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Research Article

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ABSTRACT

Although ethanol derived from lignocellulosic biomass is a promising alternative biofuel, the conversion rate of xylose to ethanol by fermentation is not ideal due to the low efficiency of many common yeasts in utilizing xylose. *Pachysolen tannophilus* can convert hexose and pentose such as L-arabinose, xylose and glucose in lignocellulosic hydrolysates to ethanol simultaneously. To increase the conversion of corn stover hydrolysates to bioethanol, the effect of alcohol dehydrogenase 2 gene (*adh2*) deletion in *P. tannophilus* on bioethanol production from corn stover hydrolysates was investigated. Two *adh2* deletants (heterozygote ND and homozygote MC) were constructed by using the short flanking homology PCR (SFH-PCR). The ND and MC strains showed lower Alcohol dehydrogenase 2 (ADH2) activity than the initial strain P-01. In the fermented pentose and hexose sugars of MC and ND, the ethanol concentrations (g/L) reached 15.8 and 18.9 versus 14.6 of the initial P-01, while in the corn stover hydrolysate medium, the ethanol concentrations (g/L) were 9.1 for MC and 9.8 for ND versus 7.5 for the initial strain P-01. This research provides useful information for improving the conversion efficiency of hexose and pentose to bioethanol by *Pachysolen tannophilus*.

INTRODUCTION

The increasing industrialization and motorization of the world has led to a steep rise in the consumption of fossil fuels. However, fossil fuels are not only becoming exhausted but also have a major contribution in greenhouse gas emissions and global warming by combustion to fulfill the energy demand, which leads to many negative effects including climate change, receding of glaciers, rise in sea level, loss of biodiversity, etc. [1,2,3]. Therefore, it is an urgent issue to find alternative and sustainable sources with lesser emissions.

Bioethanol is probably one of the most promising alternative biofuels [4]. Firstly, it is environment-friendly because of reducing the emission of carbon monoxide, unburned hydrocarbons and carcinogens when mixed with petrol for combustion in vehicles [5]. Secondly, bioethanol has a higher octane level than petrol. It can not only be blended with petrol or burned in its pure form within modified spark-ignition engines, but can also improve the performance of the petrol, and it is simple to apply [4]. Finally, bioethanol is mainly produced from natural bioresources which are renewable and sustainable, such as corn, potatoes, cassava and lignocellulosic plant biomass [6]. However, the use of such food and feed crops for producing the first-generation bioethanol is contentious because of the conflict with food supply [7]. The second-generation bioethanol is generally produced from agricultural lignocellulosic biomass, either non-edible residues of food crop production or non-edible whole plant biomass (e.g., grasses or straws) [4]. Lignocellulosic biomass derived from plants is abundant, and approximately 800 million tons of various crop residues are produced annually in China, of which corn and wheat straw account for 216 and 135 million tons [8]. Bioethanol not only reduces the reliance on oil imports, but also secures reductions in environmental pollution problems due to its high oxygen content [9].

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Potentially, bioethanol can be produced by fermentation of sugars from hydrolysis of the lignocellulosic biomass consisting mainly of cellulose, hemicellulose and lignin. Only cellulose and hemicellulose can be converted into fermentable sugars, including hexose and pentose. The efficient conversion of lignocellulosic biomass to bioethanol requires optimum utilization of both cellulosic and hemicellulosic sugars. Currently, the fermentation of both types of sugars can be carried out either by co-fermentation or by separate fermentation of pentose and hexose sugars because most naturally occurring ethanol-fermenting microorganisms do not utilize pentose. To date, many xylose-fermenting yeasts, including *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus*, have been widely studied regarding their ability to ferment xylose [10,11].

P. tannophilus was the first naturally occurring yeast discovered to produce ethanol from both pentose and hexose sugars [12]. *P. tannophilus* was also reported to be able to ferment all the common sugars such as glucose, mannose and galactose except L-arabinose in the hemicellulose hydrolysate [13]. However, most previous studies on *P. tannophilus* were about xylose utilization pathway [14,15]. In bioethanol production, the ultimate ethanol concentration is determined by cellular resistance to ethanol toxicity and ethanol oxidation rate. Generally, alcohol dehydrogenase 2 (ADH2) will catalyze ethanol oxidation when the sugars in the medium are exhausted during yeast fermentation. Therefore, there is an available method to increase the ethanol concentration accumulation by *adh2* deletion. The ethanol concentration in the medium was proved stable during fermentation stage by using *Saccharomyces cerevisiae* with *adh2* deletion because the strain lacking ADH2 cannot utilize ethanol as a carbon source [16]. In our previous study, the ethanol accumulation was reduced obviously after 84 h fermentation of medium containing hexose and pentose sugars by *P. tannophilus* P-01 under optimum conditions, and ADH2 activity was also detected during fermentation [17]. Therefore, we hypothesized that the effect of *adh2* deletion on ethanol production by *P. tannophilus* might be similar to that by *S. cerevisiae*.

To test this hypothesis, we designed the homology primers to clone a fragment of *adh2* from *P. tannophilus* P-01, and then obtained the full-length *adh2* gene by rapid amplification of cDNA ends (RACE). Short flanking homology polymerase chain reaction (SFH-PCR) was used to delete *adh2* of *P. tannophilus* P-01 and the recombinants were validated by PCR. The effects of *adh2* deletion on ethanol production were evaluated by fermenting the initial strain and recombinants in the corn stover hydrolysates.

MATERIALS AND METHODS

Strains and culture conditions

P. tannophilus P-01 was used for *adh2* deletion and fermentation of corn stover hydrolysates to produce ethanol, which was screened by Henan Agricultural University [18]. The strain was grown at 28°C, 150 rpm in a liquid medium (pH 5.0) containing (g/L) xylose, 20; yeast extract, 3; peptone, 5.

Cloning *adh2* from *P. tannophilus*

Figure 1A shows the strategies for cloning full-length *adh2* cDNA by using RT-PCR and RACE methods. Total RNA was isolated from the mycelia using Trizol reagent (Invitrogen, Code: DP405-01) according to the manufacturer's instructions, and cDNA was synthesized based on Takara RNA PCR Kit 3.0 instruction. Next, the homology primers SF and SR **(Table 1)** were designed for cloning partial fragment of *adh2* by blasting the *adh2* of *Saccharomyces kluyveri*, *Pichia stipitis*, *Candida albicans* and *Saccharomyces cerevisiae* from NCBI (http://blast.st-va.ncbi.nlm. nih.gov/Blast.cgi). Based on the cDNA fragment sequences obtained, nested PCR primers **(Table 1)** were designed to perform 3' and 5' RACE, respectively, by using the 5'-and 3'-RACE Kit (Roche Applied Science, Code: PR6931, PR6921). Finally, based on the sequencing result of 5' and 3' RACE, primers JF and JR were designed to obtain the whole length of *adh2* cDNA PCR product. The experiment was carried out according to the manufacturer's recommendations. All the PCR products were cloned into pMD18-T (TaKaRa, Code: D101A) easy vector and sequenced on an ABI PRISM 377 DNA Sequencer.

Table 1. Primers used for full-length cDNA of *adh2* cloning from *P. tannophilus*, *adh2* deletion and identification.

Primers	Sequence	Purpose
adh2 cloning		
SF	5′-GATATCCCAGTTCCAAAGCCAA-3′	First partial fragment
SR	5′-GAAGTGTCAACAACGTATCTACC-3′	First partial fragment
O-47	5′-GCTGTCATCAATGTTTCTGTCTC-3′	3′-Race primer
I-48	5′-CTGTCATCAATGTTTCTGTCTCTGAAAGAGCC-3′	3′-Race primer
O-276	5′-ATCAATGGCTTCAGCAGTATCACATC-3′	5′-Race primer
I-164	5′-GCTGGTAAACCAACCAAGACAACTGTACCTGTAGG-3′	5′-Race primer
JF	5′-CATATTGATTTACATGC-3′	Full Length cDNA Cloning
JR	5′-AGAGTAAGATTACCTTG-3′	Full Length cDNA Cloning
adh2 deletion		
NKF2	5′-AGATATCGTCAAGGATGTTATTGCTGCAACTGATGGTGGTCGTACGCTGCAGGTCGAC-3′	SFH-PCR
NKR2	5′-CAAAATCTCACCCTTTTCCATTAAATCGTAAACCATTGGTATCGATGAATTCGAGCTCG-3′	SFH-PCR
PCR verification		

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F14	5′-GATGTTATTGCTGCAACTGAT-3′	Identification of recombinants
R1442	5′-TCTCACCCTTTTCCATTAAATCG-3′	Identification of recombinants
YF	5′-TCCACATGCTGTCATCAATG-3′	Identification of homozygote
YR	5′-GATTTAACTAAACCTCTGACG-3′	Identification of homozygote

Gene deletion by SFH-PCR in *P. tannophilus* P-01 strain

The SFH-PCR method was used to produce the deletant in *P. tannophilus* P-01 [19]. **Figure 1B** shows the specific strategies for *adh2* gene disruption. The selectable marker KanMX was routinely obtained from the vector pFA6a-KanMX4, which contains a gene derived from *E. coli* transposon Tn903 that is able to confer resistance to kanamycin in *E. coli* and to geneticin in yeast [20]. With pFA6a-KanMX4 plasmid as template, we amplified a deletion cassette using the pair of primers NKF2 and NKR2 contained at the 5' end 40 bases matching to the *adh2* and at 3' end 19 bases matching to pFA6a-KanMX4 **(Table 1).** The PCR was performed in a reaction volume of 50 µl consisting of 0.5 µl of each primer, NKF2 and NKR2 (20 µM each), 4 µl dNTPs (2.5 mM each), 5 µl 10 × PCR buffer, 10 ng pFA6a-KanMX4 and 0.5 µl EXTaq (20 µM), using a PTC-200 Thermal Cycler (Bio-rad, code: 39855716) with the program of 5 min at 94°C, 30 cycles (of 30 s at 94°C, 45 s at 65°C and 90 s at 72°C), and a final step of 10 min at 72°C. After purification with a Kit for DNA recovery from PCR reaction (TaKaRa, code: DV807A), the SFH-PCR products were used to transform yeast diploid cells by the lithium acetate method [21].

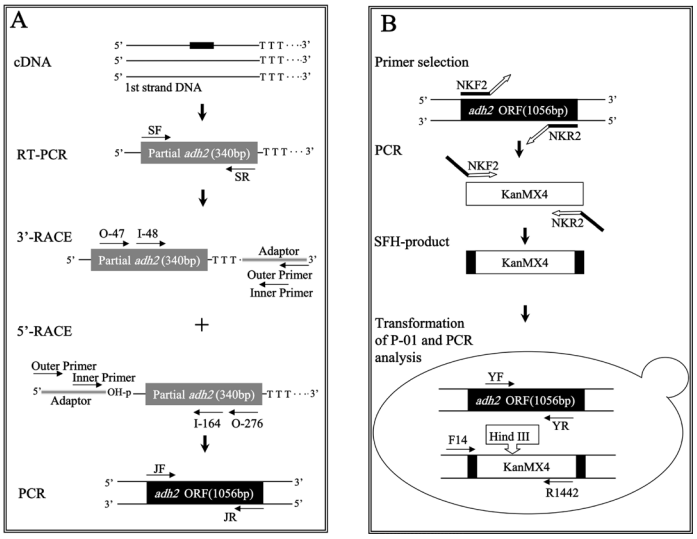

Figure 1. (A) Strategies for cloning full-length adh2 cDNA by using RT-PCR and RACE methods. Homology primers SF and SR were used to obtain the partial fragment of adh2. Four pairs of primers were used for nested PCR (3' RACE Outer primer and O-47, 3' RACE Inner Primer and I-48, 5' RACE Outer primer and O-276, and 5' RACE Inner primer and I-164). Primers JF and JR were used for obtaining the full-length adh2 cDNA. (B) Strategies for gene deletion using the SFH-PCR method. The 5' region of the oligos NKF2 and NKR2 (shown darker) contained a stretch of about 40 bases matching the target gene, whereas the 3' region (shown lighter) matched the KanMX4 cassette. These chimerical oligos were used to transform the p-01 strain. The genotype was verified by PCR, using the following two pairs of primers: YF and YR in adh2, and F14 and R1559 in the KanMX4 cassette.

Verification of transformants

To verify whether the SFH-PCR products replaced the *adh2* correctly, the chromosomal DNA of the yeast transformants and initial strain *P. tannophilus* P-01 was purified and used as the PCR template. A pair of primers, F14 combined with 5' end of *adh2* and R1442 combined with 3' end of KanMX4 **(Figure 1B and Table 1)** was designed. The PCR reactions were incubated for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 65°C and 120 s at 72°C and then 10 min at 72°C, using Taq polymerase (NEB,5 U/ml) in the Thermal Cycler.

Primers YF and YR combined with *adh2* were designed to verify the homozygote by analytical PCR **(Figure 1B and Table 1).** The PCR reactions were incubated for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 65°C and 120 s at 72°C and a final step of 10 min at 72°C, using Taq polymerase (NEB,5 U/ml) in the Thermal Cycler.

Genetic stability of *adh2* deletants was also tested. As the deletants had the geneticin (G418) resistance for carrying the selectable marker KanMX, the positive deletants, but not the initial *P. tannophilus* P-01, could grow on the YPD plate containing 10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Dextrose (glucose), 20 g/L agar powder, and 80 mg/L G418.

Pentose and hexose medium fermentation

To compare ethanol productivity between the recombinants of MC, NM, MK and ND with the initial *P. tannophilus* strain P-01, fermentation was carried out in a 300 ml glass flask with 100 ml of pentose and hexose medium containing (g/L) glucose 4, xylose 2, yeast decoction 0.3, peptone 0.5, urea 0.02, and $(NH_4)_2HPO_4$ 0.01, which was adjusted to pH5.5 with 2 M H_2SO_4, and sterilized at 120°C for 20 min. After cooling to room temperature, the flasks were inoculated with 1 ml of the recombinant culture (three replicates for each recombinant) on a shaker at 28°C and 120 rpm for 96 h, and then the ethanol content was determined.

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Corn stover hydrolysates fermentation

Corn was cultivated at Hebi of Henan, China. The corn stover was dried and milled with a mini-shredder to pass through a 100 mesh, and was pretreated under the following conditions: 100 g corn stover powder, 600 ml distilled water, 4.5 ml concentrated hydrochloric acid, 121°C, and 1h. The pretreatment solution was adjusted to pH 4.8 with 2M NaOH for saccharification process in the saccharification tank at 45°C, and 120 rpm for 48 h with an enzyme dosage of 2.4 × 104 U/g cellulose (SUKAHAN, Code: 9042-54-8) and 5.3 × 104 U/g xylanase (SUKAHAN, Code: 9025-57-4) dry matter. Saccharification solution was filtrated by filter paper, and supplemented with the following nitrogen sources (g/L): yeast extract, 3; peptone, 5; and urea 0.2 before adjustment to pH 5.5 with 2M NaOH. Then, the as-treated saccharification solution was aliquoted into 90 ml in 300 ml glass flasks, and sterilized at 121°C for 20 min. After cooling to room temperature (24°C), *P. tannophilus* strain P-01, and recombinants of MC and ND were inculcated with 9 ml of culture (three replicates for each strain) on a shaker at 28°C and 120 rpm for 96 h before the determination of the ethanol content.

Analysis

The amino acid sequence of *P. tannophilus* p-01 ADH2 was obtained as described in our previous study [22]. Multiple sequence alignments and an unrooted phylogenetic tree of the amino acid sequences of ADH2 in yeasts were generated using MEGA version 6.0, and the statistical significance of the tree nodes was evaluated using the bootstrap procedure with 1,000 replications [23]. The ADH2 protein sequences in *Candida tropicalis* (LOCUS: EER31030), *Pichia anomala* (LOCUS: CAH56496), *Candida boidinii* (LOCUS: BAD12483), *Pichia stipitis* (LOCUS: CAA73827), *Saccharomyces cerevisiae* (LOCUS: AAA34411) and *Saccharomyces kluyveri* (LOCUS: AAP51047) were identified by searching public databases available at NCBI (http://www.ncbi.nlm.nih.gov). The amount of reducing sugar in the corn stover saccharification solution was determined by the DNS method [24]. The ethanol content was determined with an Agilent 7890A gas chromatograph (Agilent, USA) equipped with a HP-FFAP (30 m × 0.32 mm × 1 μm) column and a flame ionization detector (GC-FID), with the column temperature set at 45°C for 1 min to 55°C at 10°C/min and held for 1 min, then heated to 80°C at 15°C/min and kept for 0.5 min, and finally heated to 120°C at 20°C/min and held for 1 min. The temperatures of the injection port and detector were 200°C and 250°C, respectively. The injection volume was 0.2 μl, and the gas flow rates were 1.5 ml/min for N_2, 30 ml/min for H_2 and 350 ml/min for air.

The yeast crude extracts were prepared in the cold by grinding the washed cells with twice their weight of alumina and by subsequent extraction with twice their weight of 50 mM phosphate buffer (pH 7.5), containing 10 μM EDTA. After centrifugation for 20 min at 20000 g, the supernatant was collected. ADH activity was measured at 25°C in cuvettes with 20 μl of crude extract and 2.98 ml of reaction mixture containing 100 mM sodium pyrophosphate buffer (pH 8.5), 2 mM β-mercaptoethanol, 70 mM semicarbazide, and 1 mM NAD^+. The reaction was started by addition to the experimental cuvette of 100 mM ethanol. Enzyme units are expressed as 1 μmol of NADH formed/min, under the conditions of the assay. Differentiation between the isoenzymes ADH1 and 2 was accomplished by thermal denaturation of the former from the crude extracts according to the reported methods [25]. The activity of each of the two isoenzymes was determined in the extracts by assaying alcohol dehydrogenase activity before (total ADH activity) and after (ADH2 activity) incubation at 61°C for 15 min. The activity of ADH1 was obtained by subtracting the activity of isoenzyme2 from the total activity [26].

RESULTS

Cloning adh2 from *P. tannophilus*

Figure 2A shows the gel electrophoresis of the *adh2* complete open reading frame (ORF) PCR products from P-01 using primers JF and JR. The sequencing result indicates that the ORF of *P. tannophilus adh2* encoded 351 amino acid residues. The molecular weight of the protein was calculated to be 35.137 kD with an isoelectric point of 6.65 using the software ProtParam (http://au.expasy.org/tools/protparam. html). The *P. tannophilus* ADH2 contained 55.8% hydrophobic amino acids, 21.8% hydrophilic amino acids, 11.9% alkaline amino acids and 10.3% acidic amino acids. The protein structure of ADH2 was predicted by Prosite Server (http://kr.expasy.org /cgi- bin/prosite/ScanView) with a zinc binding site located in the 47-157 region and the NAD^+ binding domain located in the 171-240 region. The conserved region of *P. tannophilus* ADH2 was consistent with that of the other known ADH2 [27,28].

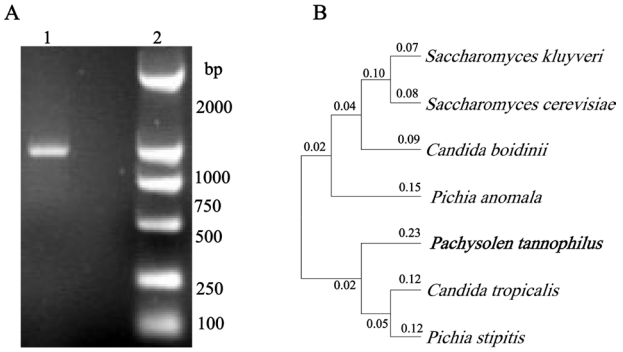

Figure 2. (A) Gel electrophoresis of PCR products obtained from *P. tannophilus* P-01 using primers JF and JR. Lane 1: adh2, and lane 2: DNA ladder. (B) Unrooted phylogenetic tree based on alignment of predicted protein sequences. The tree was bootstrapped 1,000 times.

To investigate the phylogenetic relationship between ADH2 in *P. tannophilus* and their homologues in other yeast strains, an alignment was performed with *Candida tropicalis* ADH2, *Pichia anomala* ADH2, *Candida boidinii* ADH2, *Pichia stipitis* and other predicted or published ADH2 available from GenBank. The unrooted phylogenetic trees are presented in **Figure 2B.** The length of *P. tannophilus* ADH2 protein was the same as that of *Saccharomyces kluyveri*, but relatively shorter than that of *Candida boidinii* or *Pichia anomala* ADH2 proteins. *P. tannophilus* ADH2 was found to have 73% and 71.6% amino acid sequence identities to *Pichia stipitis* and *Candida tropicalis*, respectively. The rest four yeasts were grouped together, but they were in a separate branch from *P. tannophilus* ADH2.

Gene deletion by SFH-PCR

Figure 3 shows the gel electrophoresis of PCR products amplified from partial recombinants (NM, MK, ND, MC) and P-01 DNA with primers F14 and R1442. There were obvious four bands from NM, MK, ND and MC strains but no bands appeared from the P-01 strain in gel electrophoresis of PCR products, indicating that the SFH-PCR products replaced *adh2* in the four recombinants, because primers F14 and R1442 were complementary with KanMX4. However, this result could not distinguish between heterozygote and homozygote. As displayed in **Figure 4A**, no band appeared from the MC strain in gel electrophoresis of PCR products using primers YF and YR, but obvious bands were observed in the other four strains (NM, MK, ND and P-01), indicating that the MC strain was homozygote, and the other three recombinants were heterozygotes. Strains MC and ND could grow on the YPD plate with 80 mg/L G418 until the twentieth generation, indicating that both the heterozygote ND and the homozygote MC obtained the phenotype of G418 resistance.

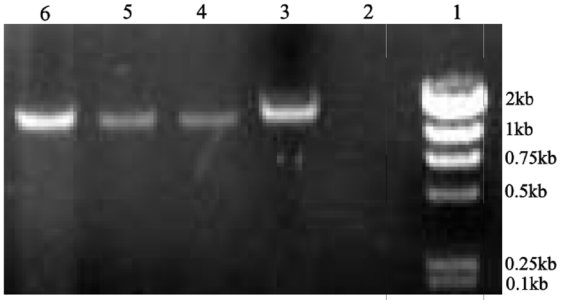

Figure 3. Gel electrophoresis of PCR products obtained from recombinants with primers F14 and R1442. Lane 1: DNA ladder, lane 2: strain P-01, lane 3: recombinant NM, lane 4: recombinant MK, lane 5: recombinant ND, and lane 6: recombinant MC.

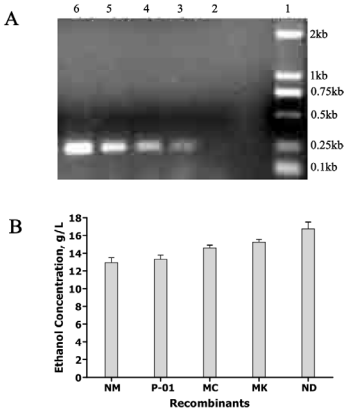

Figure 4. (A) Gel electrophoresis of PCR products obtained from recombinants with primers YF and YR. Lane 1: DNA ladder, lane 2: recombinant MC, lane 3: recombinant NM, lane 4: recombinant MK, lane 5: recombinant ND, and lane 6: strain P-01. (B) Fermentation results of several recombinants. Pentose and hexose medium (g/L): glucose, xylose 2, yeast decoction 0.3, peptone 0.5, urea 0.02, and $(NH_4)_2HPO_4$ 0.01, pH 5.5; 28°C, 120 rpm, 96 h for P-01 and NM, MC, MK, ND strains.

Fermentation

Figure 4B presents the fermentation result with pentose and hexose medium for P-01 and NM, MC, MK, ND strains. The ethanol yield of ND strain (16.78 g/L) was higher than that of P-01 or the other three strains. The ethanol yield of NM strain (12.97 g/L) was nearly equal to that of the initial strain P-01. The ethanol yield of MC, although lower than that of ND, was little higher than that of initial strain P-01.

After the genetic stability test, the twentieth generation P-01, MC and ND strains were used for fermenting the pentose and hexose medium and the corn stover hydrolysate containing 46 g/L total reducing sugars. **Table 2** displays the ethanol yields from the pentose and hexose medium and the corn stover hydrolysate with P-01, ND and MC strains. In the pentose and hexose medium, the ethanol yields of P-01, ND and MC are 65.7%, 85.1% and 70.9%, respectively. The ethanol yields of recombinants ND and MC are obviously higher than the initial strain P-01 by 29.53% and 7.91%, respectively. A similar tendency was observed in the corn stover hydrolysate fermentation, with the ethanol yields of ND, MC and P-01 strains being 57.8%, 53.2% and 43.9%,

respectively. It is worth noting that the utilization of total sugar, glucose and xylose was not significantly different among ND, MC and P-01 strains in the pentose and hexose medium, but very different in the corn stover hydrolysates. The heterozygote ND strain showed a higher utilization of total sugar (62.05%), glucose (69.22%) and xylose (58.33%) than the wild strain P-01, but was very close to the homozygote strain MC in the utilization of corn stover hydrolysates. It indicates that the deletion of *adh2* gene in *Pachysolen tannophilus* can not only improve ethanol production but also increase the utilization of sugars in corn stover hydrolysates.

Table 2. Utilization of sugar and ethanol production of P-01, ND and MC strains from the pentose and hexose medium and the corn straw hydrolysate (Mean ± SE).

Fermentation medium	Strain	Utilization of total sugar (%)	Utilization of glucose (%)	Utilization of xylose (%)	Ethanol concentration(g/L)	Ethanol yield(%)
Pentose and hexose medium	P-01	93.75 ± 0.06	96.47 ± 0.08	77.08 ± 0.66	14.6 ± 0.01	65.7 ± 1.12
	ND	94.31 ± 0.92	96.97 ± 1.03	76.56 ± 1.06	18.9 ± 0.10	85.1 ± 1.38
	MC	93.04 ± 1.05	95.84 ± 1.11	75.95 ± 0.58	15.8 ± 0.03	70.9 ± 2.23
Corn stover hydrolysates	P-01	50.23 ± 0.05	60.35 ± 0.04	47.22 ± 0.10	7.5 ± 0.02	43.9 ± 1.23
	ND	62.05 ± 0.06	69.22 ± 0.09	58.33 ± 0.07	9.8 ± 0.09	57.8 ± 1.01
	MC	61.25 ± 0.08	68.35 ± 0.02	56.09 ± 0.11	9.1 ± 0.08	53.2 ± 2.11

Figure 5A shows the ADH1 activities of strains P-01, ND and MC in the pentose and hexose fermentation medium. The heterozygote ND strain had obviously higher ADH1 activity than either the ND or the MC strain after 24 h fermentation, but the homozygote MC strain had the lowest ADH1 activity from 36 to 72 h. **Figure 5B** shows the ADH2 activities of strains P-01, ND and MC in the pentose and hexose fermentation medium. The initial strain P-01 had obviously higher ADH2 activity than either the ND or the MC strain, and the activity decreased at 96 h. The heterozygote ND was higher than the homozygote MC in terms of ADH2 activity after 36 h fomentation, and the highest ADH2 activity of the three strains was detected at 84 h.

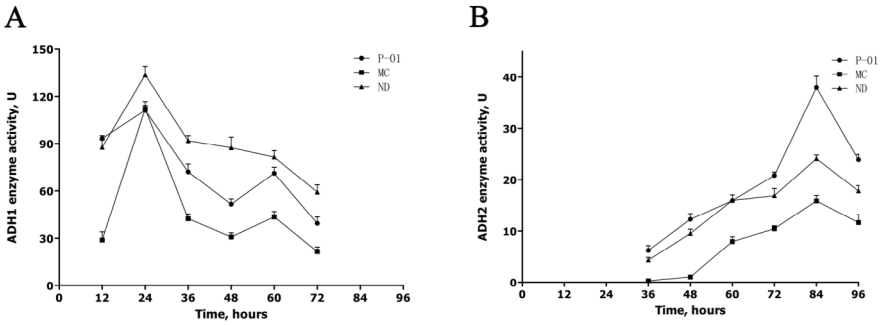

Figure 5. (A) Comparison of ADH1 activities of P-01, ND and MC strains. The activity of ADH1 was obtained by subtracting the activity of ADH2 from the total ADH activity. (B) Comparison of ADH2 activities of P-01, ND and MC strains. ADH2 activity was measured according the Mauricio and Ortega's method [26]. Enzyme units are expressed as 1 μmol of NADH formed/min, and the activity of each of the two isoenzymes was determined in the extracts by assaying alcohol dehydrogenase activity before (total ADH activity) and after (ADH2 activity) incubation at 61°C for 15 min.

DISCUSSION

In this study, to test the effects of *adh2* deletion on ethanol production by *P. tannophilus*, we cloned the full-length ADH2 gene (*adh2*) from *P. tannophilus*. Alcohol dehydrogenases (ADHs: EC 1.1.1.1) occur in a wide variety of organisms, including animals, plants, yeasts, and bacteria [27]. There are five alcohol dehydrogenases (ADH1 to ADH5) in yeast, and ADH2 expression is repressed by growth on glucose, mainly due to the involvement in ethanol consumption, converting ethanol into acetaldehyde [29]. Currently, there are few reports available regarding the *adh2* gene of unconventional yeasts. It is worth noting that the *adh2* genes (DNA sequence) of *P. tannophilus* showed 72% identity to the *adh2* genes of *P. stipitis*. The latter has been cloned for investigating the importance of *P. stipitis* metabolism by means of gene deletion [30]. Actually, gene deletion is an effective method for yeast to improve bioethanol production. SFH-PCR has been proved a standard technique in gene deletion and the modification in *Saccharomyces cerevisiae* [31]. It was reported that *adh2* deletion in *S. cerevisiae* could improve ethanol production by 2.5% compared with the initial strain [32].

Based on the ethanol result in **Table 2**, P-01, ND and MC strains can co-ferment the glucose and xylose for ethanol production in the pentose and hexose medium, but the utilization of xylose was obviously lower than that of glucose. It was obviously found that the deletion of *adh2* had slightly effects on the utilization of total sugars, glucose and xylose when co-fermented with the pentose and hexose medium. However, in the corn stover hydrolysates, the tendency of utilization of sugars was similar among P-01, ND and MC strains, but obviously lower than that in the pentose and hexose medium. The possible reason for this was the influence of some poisonous matters or unsuitable proportion of pentose and hexose on ethanol fermentation. It is noteworthy that the sugar utilization of the mutant strains increased on corn stover hydrolysates with the *adh2* deletion. It was proved that there were many poisonous substances inhibiting yeast growth in the corn stover hydrolysates. The possible one reason was that

adh2 deletion increased poisonous substances tolerance of the mutant strains. The other reason was that some genes of sugar-metabolizing enzymes were up-regulated expression due to the *adh2* deletion; however these hypotheses needed to be verified in the further study.

The fermentation result for the corn stover hydrolysate also shows that the heterozygote ND had higher ethanol yield than the homozygote MC **(Table 2).** The possible reason for increased productivity with the mutants is reduction in consumption of produced ethanol. Theoretically, the utilization of ethanol would become lower due to *adh2* deletion, but the ethanol metabolism in *P. tannophilus* was a complex process involving various enzymes. It has been proved that only the *adh2* deletion from *S. cerevisiae* cannot have a very good effect for improving ethanol production [33].

Figure 5A shows that the heterozygote ND strain had the highest ADH1 activity, and the homozygote MC strain also had higher ADH1 activity than the wild strain P-01. This result suggests that the deletion of ADH2 could be a main factor for increasing the ADH1 activity, but the relationship between ADH1 and ADH2 remains to be elucidated. In general, ADH1 can catalyze acetaldehyde into ethanol in the ethanol metabolic pathway, and the ADH1 activity of heterozygote ND is higher than that of homozygote MC, which may be one of the reasons for the higher ethanol yield of heterozygote. However, as shown in **Figure 5B**, a slight ADH2 activity could be detected in the homozygote MC, probably due to the existence of isoenzymes or allele of ADH2 in *P. tannophilus*, but one thing is certain: ADH2 deletion reduced the ADH2 activity either in the homozygote or in the heterozygote.

Future studies should focus on the identification of differentially expressed proteins among strains MC, ND and P-01 using two-dimensional electrophoresis/mass spectrometry, or the development of more efficient gene alteration strategies for analyzing the key enzymes in metabolism affecting ethanol production. Additionally, the ethanol production from the corn stover hydrolysate can be improved by introducing the target genes into the MC and ND strains with gene deletion [34]. For example, *P. tannophilus* cannot utilize the L-arabinose present in the corn stover hydrolysate, but the enzyme using the L-arabinose can be introduced into *P. tannophilus* to improve the ethanol yield by fermenting lignocellulosic hydrolysates.

CONCLUSION

In this study, we cloned the *adh2* from yeast *P. tannophilus* by using the RACE method. Sequences were submitted to NCBI database under the accession numbers of EU570211 and EU590042. Using gene deletion technology SFH-PCR, the *adh2* gene was deleted from *P. tannophilus*, and two recombinants (heterozygote ND and homozygote MC) were obtained. The ADH2 activities of both ND and MC strains were decreased during the fermentation of the pentose and hexose medium. Stable G418 resistance was observed in the ND and MC strains after more than 20-generation cultivation. The result of corn stover hydrolysate fermentation indicated that the ethanol yields of the ND (57.8%) and MC (53.2%) strains were higher than that of the initial strain P-01 (43.9%). The overall findings from this research provide useful information for improving ethanol production from corn stover hydrolysate by gene deletion.

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