Appl Microbiol Biotechnol
DOI 10.1007/s00253-017-8208-6

A new source of resistance to 2-furaldehyde from *Scheffersomyces* (*Pichia*) *stipitis* for sustainable lignocellulose-to-biofuel conversion

Xu Wang[1,2,3] · **Z. Lewis Liu**[2] · **Xiaoping Zhang**[1] · **Menggen Ma**[1,4]

Received: 22 November 2016 / Revised: 15 February 2017 / Accepted: 18 February 2017

Abstract Aldehyde inhibitory compounds derived from lignocellulosic biomass pretreatment have been identified as a major class of toxic chemicals that interfere with microbial growth and subsequent fermentation for advanced biofuel production. Development of robust next-generation biocatalyst is a key for a low-cost biofuel production industry. *Scheffersomyces* (*Pichia*) *stipitis* is a naturally occurring C-5 sugar utilization yeast; however, little is known about the genetic background underlying its potential tolerance to biomass conversion inhibitors. We investigated and identified five uncharacterized putative aryl-alcohol dehydrogenase genes (*SsAAD*s) from this yeast as a new source of resistance against biomass fermentation inhibitor 2-furaldehyde (furfural) by gene expression, gene cloning, and direct enzyme assay analysis using partially purified proteins. All five proteins from *S. stipitis* showed furfural reduction using cofactor NADH. An optimum active temperature was observed at 40 °C for SsAad1p; 30 °C for SsAad3p, SsAad4p, and SsAad5p; and 20 °C for SsAad2p. SsAad2p, SsAad3p, and SsAad4p showed tolerance to a wide range of pH from 4.5 to 8, but SsAad1p and SsAad5p were sensitive to pH changes beyond 7. Genes *SsAAD2*, *SsAAD3*, and *SsAAD4* displayed significantly enhanced higher levels of expression in response to the challenge of furfural. Their encoding proteins also showed higher levels of specific activity toward furfural and were suggested as core functional enzymes contributing aldehyde resistance in *S. stipitis*.

Keywords Aldehyde reduction · Biofuel conversion · Detoxification · Tolerant strain development · Yeast

✉ Z. Lewis Liu
ZLewis.Liu@ars.usda.gov

✉ Menggen Ma
mgen@sicau.edu.cn

[1] Department of Applied Microbiology, College of Resources, Sichuan Agricultural University, Wenjiang, Chengdu, Sichuan, China

[2] BioEnergy Research Unit, USDA-ARS, National Center for Agricultural Utilization Research, 1815 N University St., Peoria, IL 61604, USA

[3] Department of Microbiology, College of Life Sciences, Henan Agricultural University, Zhengzhou, Henan, China

[4] Institute of Natural Resources and Geographic Information Technology, College of Resources, Sichuan Agricultural University, Wenjiang, Chengdu, Sichuan, China

Introduction

Lignocellulosic biomass and agricultural residues are abundant resources for renewable energy including conversion and production of cellulosic biofuels. In order to release fermentable sugars for microbial utilization, a pretreatment procedure is required to decompose cellulose structures of lignocellulosic biomass materials. During the pretreatment especially for the commonly used dilute acid pretreatment procedure, toxic chemical compounds are commonly produced as by-products that inhibit microbial growth and subsequent fermentation. For example, 2-furaldehyde (furfural) and 5-[hydroxymethyl]-2-furaldehyde (HMF) are commonly observed by dehydration of pentoses and hexoses from dilute acid pretreatment. The inhibitory chemical compounds have been classified into three major classes of aldehydes, phenols, and weak organic acids (Larsson et al. 1999; Klinke et al. 2004; Liu and Blaschek 2010). Aldehyde inhibitors represent compounds with a functional aldehyde group including aldehyde compounds attached with furan-, benzene-, or phenol-related structures (Liu and Blaschek 2010). These inhibitors have been reported to damage membrane and cell wall, break down DNA, inhibit protein and RNA synthesis, suppress biological activity, kill microbe cells, and cause cellular damages

Published online: 29 March 2017

in yeast by inducing reactive oxygen species (Sanchez and Bautista 1988; Modig et al. 2002; Liu et al. 2009a; Allen et al. 2010; Liu 2011). Overcoming the toxic compounds derived from lignocellulosic biomass is one of significant challenges for low-cost and sustainable advanced biofuel production.

Remediation of these toxic compounds by chemical or physical means involves complicated methods and is economically impractical (Liu and Blaschek 2010). The economics of fermentation-based bioprocess for biofuel production rely extensively on the performance of microbial biocatalysts. Since current industrial microbial strains are susceptible to the inhibitor stress conditions associated with the biomass conversion process, development of more robust strains that can withstand the presence of inhibitors is vital for a sustainable lignocellulosic biomass-to-biofuel industry. Many tolerance genes were characterized and successfully used in tolerant new strain development. For example, an evolutionary engineered tolerant strain of *Saccharomyces cerevisiae* displayed enhanced expression for many genes with aldehyde reduction functions (Liu et al. 2009a). A furfural reductase gene was genetically engineered into *Escherichia coli* to successfully improve the bacterial resistance to furfural (Wang et al. 2013). Incorporation of numerous genes with aldehyde reduction functions in *Zymomonas mobilis* increased ethanol productivity and the bacterial resistance to several furan aldehydes and phenol aldehydes (Yang et al. 2010; Yi et al. 2015).

Mechanisms of yeast tolerance and in situ detoxification have been intensively studied for *S. cerevisiae.* It is known that aldehyde reduction is a major function to reduce furfural and HMF into less toxic furanmethanol (FM) and furandimethanol (FDM), respectively (Liu et al. 2004, 2005; Liu 2006). Multiple gene mediated NAD(P)H-dependent aldehyde reduction, and reprogrammed pathways facilitated cofactor regeneration balance for improved cell functions (Liu et al. 2008, 2009a). Following characterization of a novel aldehyde reductase gene *ARI1* from *S. cerevisiae* (Liu and Moon 2009; Bowman et al. 2010; Jordan et al. 2011), numerous genes and open reading frames (ORFs) were characterized by direct enzyme assays showing strong aldehyde reductase activities (Larry et al. 2002, 2003; Liu et al. 2008; Moon and Liu 2012, 2015). It appeared that an aldehyde reductase gene family exists in the tolerant strains of *S. cerevisiae* (Moon and Liu 2015).

Scheffersomyces (*Pichia*) *stipitis* is a naturally occurring xylose utilization yeast and has a potential as a candidate for development of the next-generation biocatalyst (Jeffries et al. 2007; Weber et al. 2010; Cho et al. 2011). Stress tolerance in *S. stipitis* was observed (Slininger et al. 2009, 2011); however, little is known about the genetic background underlying the tolerance of *S. stipitis*. Several alcohol dehydrogenases from *S. stipitis* were identified to have aldehyde reductase activity toward furfural and HMF (Ma et al. 2013). The product of a recently characterized gene *GRE2* from *S. stipitis* was also demonstrated to be resistant to aldehyde inhibitors (Wang et al. 2016). During our preliminary study, we observed that a group of putative aryl-alcohol dehydrogenase genes (*AAD*s) in *S. stipitis* showed tolerance gene expression response to furfural and HMF. However, little is known about the function of these uncharacterized ORFs.

Annotation of the current putative *AAD* genes of *S. stipitis* is performed based on computation analysis. Computational annotations serve as a preliminary reference and need to be further validated by biological evidence. Such practices have been commonly observed in the development of Saccharomyces Genome Database and many other microbial genome databases. Evaluation by direct enzyme assay is considered as the most convincing method to assign and validate gene functions. In this study, we investigated five uncharacterized and tentatively assigned aryl-alcohol dehydrogenase genes from *S. stipitis* (*SsAAD*s), through gene expression, gene cloning, and direct enzyme assay analyses against the representative fermentation inhibitor furfural and numerous other aldehyde compounds. Results of this study defined a new source of resistance to furfural from *S. stipitis* for microbial tolerance, which will aid more tolerant strain development for low-cost and sustainable advanced biofuel production.

Materials and methods

Bacterial and yeast strains, plasmids, media, and chemicals

Yeast strain *S. stipitis* CBS 6054 used in this study was obtained from the China Center of Industrial Culture Collection, Beijing, China. *S. cerevisiae* strain INVSc1 (*his3Δ1*/*his3Δ1 leu2*/*leu2 trp1–289*/*trp1–289 ura3–52*/*ura3–52*) was purchased from Invitrogen (Carlsbad, CA, USA) and used to overexpress the targeted proteins. *E. coli* DH5α from Sangon Biotech (Shanghai, China) was used for recombinant DNA manipulation and gene cloning. Strains of *S. stipitis* were cultured on yeast peptone dextrose (YPD) medium (*w*/*v* 1% yeast extract, 2% peptone, and 2% dextrose). The synthetic complete (SC) medium lacking uracil (SC-U) with additional 2% (*w*/*v*) glucose or 2% (*w*/*v*) galactose and 1% (*w*/*v*) raffinose was used for clone selection or induced expression as previously described (Ma et al. 2013). Luria-Bertani (LB) medium (*w*/*v* 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) with a 100 μg/ml ampicillin was used for clone selection. Two-percent agar was applied in the medium when a solid medium was used. All chemicals including various aldehyde-containing compounds were supplied by Best-Reagent (Chengdu, China), Sigma-Aldrich (St. Louis, MO, USA), Difco (Detroit, MI, USA), or Sangon Biotech (Beijing, China).

Cell treatment and RNA isolation

Cells of *S. stipitis* CBS 6054 were incubated in YPD medium at 30 °C with agitation at 200 rpm overnight. Cell growth was monitored by culture density using a UV-2802 spectrophotometer (Unico, Dayton, NJ, USA). Furfural or HMF was added to the media separately at a final concentration of 30 mM when the OD_{600} reading reached approximately 1. The time point of the addition of furfural or HMF was defined as 0 h. Cultures grown on a YPD medium without furfural or HMF served as a control. Culture samples were taken periodically and cells collected by centrifugation at 3645×*g* for 2 min at room temperature. Cell pellets in a tube were freezed immediately in liquid nitrogen and then stored at −80 °C until use. Total RNA was isolated from cell samples following a previously described protocol (Liu and Slininger 2007). RNA samples were purified using a RNA cleaning kit from Tiangen Biotech (Beijing, China). RNA yield, integrity, and purity were measured by denatured gel electrophoresis and spectrophotometry. Samples with RNA purity greater than 1.8 were used. The concentration of purified RNA was determined by a NanoDrop 2000C fluorescence spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA).

Gene expression

Reverse transcription reaction was performed using a set of calibrated spike-in RNA references following procedures as previously described (Liu et al. 2009a). Primers (Table 1) were designed using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Quantitative reverse transcription real-time PCR (qRT-PCR) was performed on a Mastercycler® ep realplex system from Eppendorf (Hamburg, Germany) applying a Real Master Mix kit (SYBR Green) from Tiangen Biotech (Beijing, China). Data were normalized and analyzed using the standard RMA reference and a master equation as described previously (Liu et al. 2009b). Each gene expression experiment was carried out with two biological replications and three technical replications.

Gene cloning

Standard molecular biology techniques were performed using standard protocols as described (Sambrook and Russell 2001). Yeast genomic DNA was prepared using an E.Z.N.A. Yeast DNA kit (Omega Bio-Tek, Norcross, GA, USA). The *AAD* genes were amplified by PCR using genomic DNA from *S. stipitis* CBS 6054 as a template applying corresponding primers. DNA fragments were recovered from the agarose gel applying an E.Z.N.A. Gel Extraction kit (Omega Bio-Tek). The targeted fragment was confirmed by restriction digest and then purified using an E.Z.N.A. Cycle-Pure kit (Omega Bio-Tek). Each purified DNA fragment was verified by sequence analysis performed by Sangon Biotech (Shanghai, China). The DNA fragments were subcloned into *E. coli* DH5α separately using a TOPO TA Cloning® Kit

Table 1 Primers used in this study with endonuclease restriction sites underlined

ID	Sequence (5′–3′)
SsAAD1_F	TCGCGGCCGCATGAAGTCCACAGGACTTTGTCACACCGATA
SsAAD1_R	TCCTCGAGTTAGTACACTAATATTGGCTTCACAACAGTACCTGCCT
SsAAD2_F	TCGCGGCCGCATGTCCTCTTCAATTGAATACAAAAAGCT
SsAAD2_R	TCCTCGAGGGGTAGCTGGAAGCATTATAGTAATTTT
SsAAD3_F	TCGCGGCCGCATGTCTTCTTCAATTGAATACAAAAAGC
SsAAD3_R	TCCTCGAGCTTGAATGAAAACTAAACAAATCCGTAT
SsAAD4_F	TCGCGGCCGCATGTCTTCTTCAATTGAATACAAAAAGCTTGGTGCCTC
SsAAD4_R	TCCTCGAGTGGGTGTAGGCCATTCTCTGTTTGCATCATAA
SsAAD5_F	TCGCGGCCGCATGTCAGCCTCTCCTGTTCAAAA
SsAAD5_R	TCCTCGAGAAAATCAAAGTCATACAACTAATCAAACAA
qSsAAD1_F	TCCCACAGGGTAACTTGGTC
qSsAAD1_R	AACCCCAACCTTGGAGAAAG
qSsAAD2_F	GATGCCGTCAAAGGTTCAGT
qSsAAD2_R	CATCGTGCAAGGCTCTCATA
qSsAAD3_F	TGCTGTCAAGGGTTCAGTTG
qSsAAD3_R	ACGACATCGTTCAAGGCTCT
qSsAAD4_F	CTCCAATTGCCAGAGGTCTT
qSsAAD4_R	TGTCGGCCTCAGTCAAGTTT
qSsAAD5_F	CGTGAAGAGGAGCGTGAAAT
qSsAAD5_R	CAGATTTGGCACCAAGAGGT

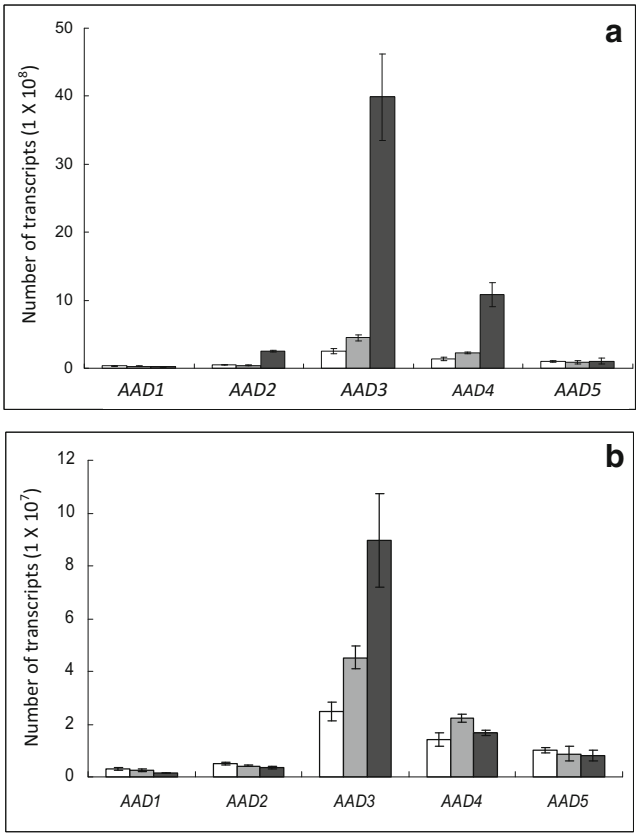


Fig. 1 Gene expression of five *AAD* genes from *S. stipitis* in response to 30 mM of furfural (**a**) and HMF (**b**) on an enriched YPD medium as measured by quantitative transcription analysis using qRT-PCR. A *blank bar* represents expression level at 0 h, a *light gray-shaded bar* represents a non-treated control at 2 h, and a *black bar* represents expression under the inhibitor challenge at 2 h

(Invitrogen, Carlsbad, CA, USA). Subsequently, the gene inserts were cloned into the expression vector pYES2/NT B with a galactose-inducible *GAL1* promoter (Invitrogen, Carlsbad, CA, USA), resulting in a serial of plasmids of pYES2/NT B-*AAD*s.

Heterologous overexpression

The recombinant construct plasmids were verified and transformed into *S. cerevisiae* strain INVSc1 (Invitrogen, Carlsbad, CA, USA) by the conventional lithium acetate method as previously described (Daniel et al. 1995). The transformants were selected on solid selective SC-U plates, supplemented with 2% (*w*/*v*) glucose at 30 °C. Cell cultures of each *S. cerevisiae* INVSc1 transformant were transferred onto SC-U medium supplemented with 2% (*w*/*v*) glucose and incubated at 30 °C with agitation at 200 rpm overnight. Then, 2% (*w*/*v*) galactose and 1% (*w*/*v*) raffinose were added into the SC-U medium, and the culture was incubated at 30 °C with agitation at 200 rpm overnight for protein expression. An INVSc1 transformant containing pYES2/NT B without a gene insert served as a control.

Protein isolation and purification

Fresh cells were harvested and lysed using Y-PER® Plus dialyzable yeast protein extraction reagent from Thermo Scientific (Rockford, IL, USA) with Protease Inhibitor Cocktail (Sangon Biotech, Shanghai, China) following the instructions of the manufacturers. Protein expression was induced by galactose for each gene clone. To facilitate efficiency of protein purification, a polyhistidine tag with 65 extra amino acid residues was added, accounting approximately additional 5.37 kDa. Crude protein extract was purified using a Ni-NTA Sefinose™ Kit (Sangon Biotech, Shanghai, China). Samples of the purified proteins were kept on ice and used within 24 h. Concentrations of partially purified proteins were determined applying a standard curve established using bovine serum albumin applying a Modified Bradford Protein Assay Kit (Sangon Biotech, Shanghai, China). Molecular weight of the partially purified proteins was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 1.8 to 2.2 μg/well protein loaded on the gel.

Enzyme assay

Specific activity of each enzyme was measured by monitoring NAD(P)H oxidation at 340 nm using a UV-2802 spectrophotometer (Unico, Dayton, NJ, USA) as previously described (Liu et al. 2008). Briefly, the final concentration of 10 mM furfural or HMF substrate was added in 500 μl of 100 mM potassium phosphate buffer (pH 7.2) including 100 μM NADH or NADPH. One enzyme unit was defined as 1 μmol of NAD(P)H oxidized per minute, and the specific activity was described as the units per milligram of protein as previously described. In addition to furfural and HMF, nine other aldehyde substrates were also evaluated including formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, isovaleraldehyde, glutaraldehyde, hexaldehyde, benzaldehyde, and phenylacetaldehyde. All reagents were prewarmed and reactions carried out at 30 °C using a water bath. Assays were carried out in duplicate for each defined condition. The optimal pH of specific activity for each enzyme was tested in

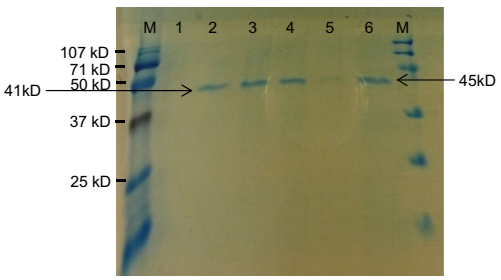


Fig. 2 An SDS-PAGE gel showing partially purified proteins encoded by *AAD* genes from *S. stipitis*. *Lanes*: *M*, molecular weight marker; *1*, a buffer control without protein; *2*, SsAad1p; *3*, SsAad2p; *4*, SsAad3p; *5*, SsAad4p; and *6*, SsAad5p

Table 2 Estimated molecular weights of expressed AAD proteins from *S. stipitis*

Protein	SsAad1p	SsAad2p	SsAad3p	SsAad4p	SsAad5p
SsAAD protein (kDa)	36	40	40	40	40
His-tag (kDa)	5.37	5.37	5.37	5.37	5.37
Fusion protein (kDa)	41	45	45	45	45

the range of 4.5–9.0 and the optimal temperature estimated between 20 and 60 °C. Kinetic parameters were estimated with the double-reciprocal Lineweaver-Burk transformation of the Michaelis-Menten equation.

Sequence analysis

Amino acid sequence analysis was performed using the ClustalW method of the MegAlign program 5.0 (DNAStar, Inc. Madison, WI, USA). Sequences of the five AADs from *S. stipitis* (SsAADs) were analyzed in alignment with previously reported sequences of AADs from *Phanerochaete chrysosporium* (PcAAD, NCBI accession: L08964) and *Taiwanofungus camphoratus* (TcAAD, NCBI accession: HQ453361); AAD3 from *S. cerevisiae* (ScAAD3, NCBI accession: NM_001178814); ADH4 from *S. stipitis* (SsADH4, NCBI accession: XM_001387085); and aldehyde reductases YGL039W (NCBI accession: AY692765), YGL157W (NCBI accession: EF059032), and YDR541C (NCBI accession: AY692675) from *S. cerevisiae*. A phylogenetic analysis was also performed for 30 proteins using the neighbor-joining method from MEGA 5.10 (Tamura et al. 2011). In order to access relationships for more proteins possessing aldehyde reductase activity, additional proteins that demonstrated various levels of aldehyde reduction activity were also included in the analysis, including four AADs from *S. cerevisiae* (ScAAD4, ScAAD10, ScAAD14, and ScAAD15), six ADHs from *S. stipitis* (SsADH1, SsADH2, SsADH3, SsADH5, SsADH6, and SsADH7), seven ADHs from *S. cerevisiae* (ScADH1, ScADH2, ScADH3, ScADH4, ScADH5, ScADH6, and ScADH7), and YOL151W from *S. cerevisiae* (ScYOL151W).

Results

Gene expression

Gene expression response of *S. stipitis* cells was analyzed under the challenge of furfural and HMF separately. Under normal cell growing conditions without the chemical treatment, all genes showed relatively lower levels of expression and a slightly increased number of transcripts 2 h after incubation (Fig. 1a, b). For 30 mM furfural-treated cells, *AAD2*, *AAD3*, and *AAD4* displayed a significantly greater increase of transcription compared with the non-treated control at 2 h (Fig. 1a). The increased expression levels ranged from 5- to 12-fold compared with a non-treated control. *AAD1* and *AAD5* did not show a significant change of expression in response to the challenge of furfural. Most genes were not sensitive in response to HMF, except for *AAD3* which showed about 2-fold increased expression than the non-treated control with 30 mM HMF at 2 h (Fig. 1b).

Gene cloning

Constructs of positive gene clones were selected and verified by restriction digests and sequencing analysis, resulting in

Table 3 Enzyme units and specific activities of the partially purified AAD proteins from *S. stipitis* in reduction of furfural and HMF using NADH or NADPH as a cofactor

Substrate	Enzyme	NADH mU (μmol cofactor/min)	Specific activity (U/mg protein)	NADPH mU (μmol cofactor/min)	Specific activity (U/mg protein)
Furfural	SsAad1p	1.90 ± 0.00	2.42 ± 0.00	ns	ns
	SsAad2p	7.65 ± 0.32	10.20 ± 0.45	ns	ns
	SsAad3p	5.71 ± 0.00	6.59 ± 0.00	ns	ns
	SsAad4p	4.64 ± 0.03	3.93 ± 0.14	ns	ns
	SsAad5p	3.49 ± 0.06	4.38 ± 0.64	ns	ns
HMF	SsAad1p	ns	ns	ns	ns
	SsAad2p	ns	ns	ns	ns
	SsAad3p	ns	ns	1.75 ± 0.08	2.42 ± 0.26
	SsAad4p	ns	ns	ns	ns
	SsAad5p	ns	ns	ns	ns

Data are presented by mean values ± standard deviations (n = 3), and ns stands for not significant

Table 4 Kinetic parameters of partially purified AAD proteins from *S. stipitis* in reduction of furfural using NADH as a cofactor

Enzyme	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
SsAad2p	34.12 ± 0.15	49.63 ± 0.16	1361.00 ± 0.81	39.77 ± 0.87
SsAad3p	20.55 ± 0.33	28.24 ± 0.46	1467.57 ± 0.98	71.43 ± 0.82
SsAad4p	3.24 ± 0.03	4.35 ± 0.16	165.93 ± 0.18	51.27 ± 0.55
SsAad5p	58.33 ± 0.45	80.23 ± 0.59	4579.72 ± 2.78	78.51 ± 1.82

pYES2/NT-*SsAAD1*, pYES2/NT-*SsAAD2*, pYES2/NT-*SsAAD3*, pYES2/NT-*SsAAD4*, and pYES2/NT-*SsAAD5*, respectively. Each newly constructed plasmid was transformed into strain INVSc1 of *S. cerevisiae* separately and confirmed by heterologous expression.

Isolation and purification of protein

Partially purified proteins were obtained separately each for SsAad1p, SsAad2p, SsAad3p, SsAad4p, and SsAad5p. Molecular weight was estimated by SDS-PAGE gel electrophoresis. After subtraction of the His-tag, the net molecular weight was estimated at approximately 36 kDa for SsAad1p and 40 kDa for SsAad2p, SsAad3p, SsAad4p, and SsAad5p (Fig. 2 and Table 2). This estimate was consistent with the theoretical molecular weight calculated using protein molecular weight software (http://www.bioinformatics.org/sms/prot_mw.html).

Enzyme activity and cofactor preference

All AAD proteins from *S. stipitis* demonstrated a strong specific activity against furfural using cofactor NADH. Among which, SsAad2p showed the highest level of activity, followed by SsAadp3p, SsAad4, SsAadp5p, and SsAadp1p (Table 3). When cofactor NADPH was used, no significant activity was observed against furfural (data not shown). Most proteins showed no significant reduction activity for HMF, except for SsAadp3, which showed HMF reduction activity using NADPH.

Using furfural as substrate, kinetic parameters of SsAadp2, SsAadp3, SsAadp4, and SsAadp5 were determined applying cofactor NADH. SsAadp4 displayed the strongest binding affinity for furfural with a low K_m of 3.24 mM. SsAad5p and SsAad3p showed relatively higher levels of catalytic efficiency and substrate preference as indicated by the measurement of catalytic constant over the Michaelis constant (K_{cat}/K_m) (Table 4).

Optimal temperature and pH

Using cofactor NADH and furfural as substrate, an optimal temperature of 40 °C was observed for SsAad1p, and 30 °C was recorded for SsAad3p, SsAad4p, and SsAad5p (Fig. 3a, c, d, e). SsAad2p seemed sensitive to higher temperatures, and the highest specific activity was shown at 20 °C (Fig. 3b). While the enzyme activity remained at 30 °C, its specific activity declined sharply with increased temperatures. SsAad1p, SsAad4p, and SsAad5p displayed the best specific activity at pH 7 (Fig. 3f, i, j) while SsAad2p and SsAad3p preferred pH 6 (Fig. 3g, h). SsAad2p appeared to be relatively stable to a wide range of pH from 4.5 to 8. In contrast, SsAad1p and SsAad5p were more sensitive and their specific activity declined sharply when the pH value was changed beyond 7.

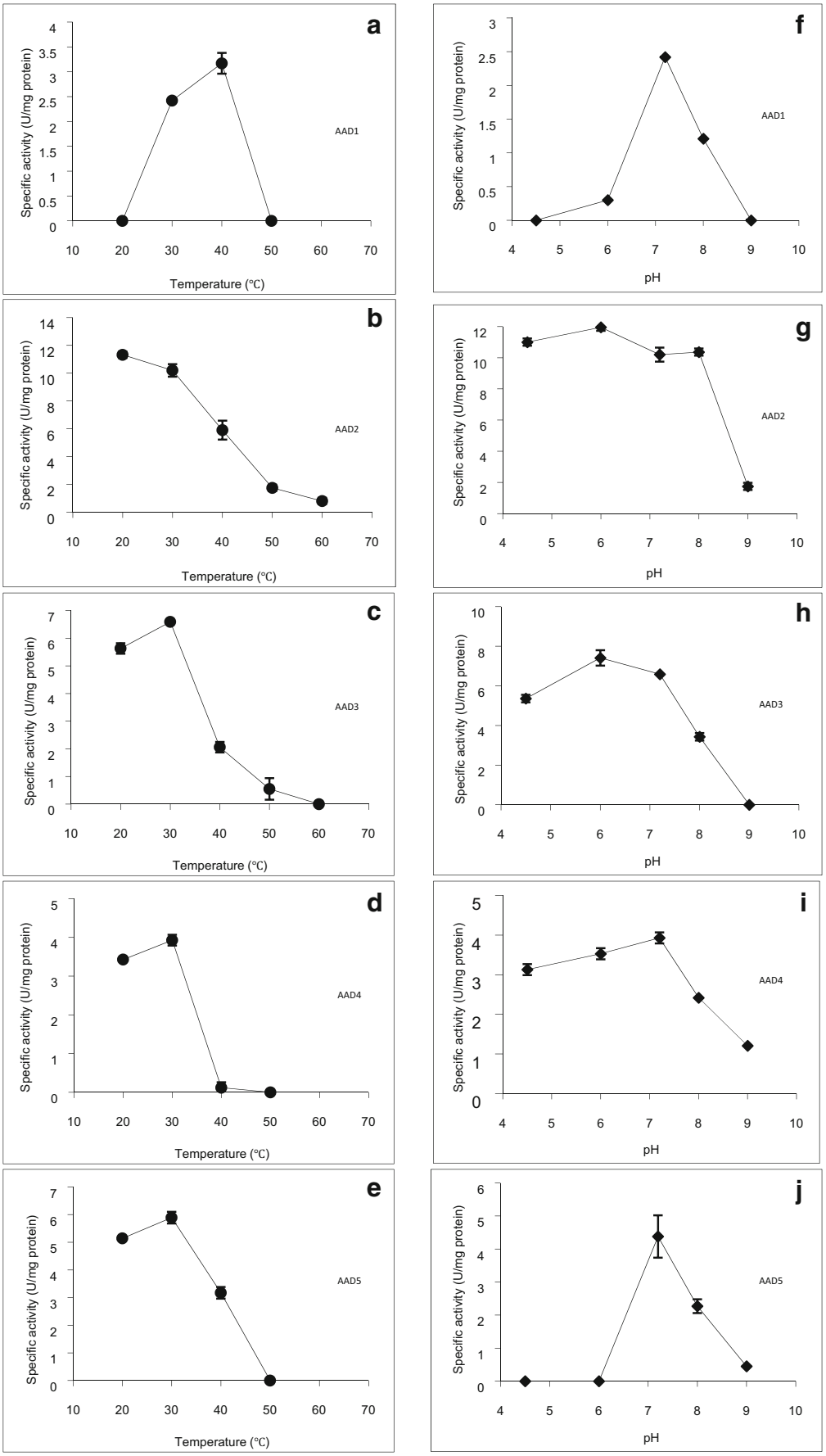
Substrate specificity

In addition to furfural, we evaluated enzyme specific activity of five AAD proteins toward other nine aldehyde substrates using cofactor NADH and NADPH. It was interesting to find these proteins showing activity toward a broad range of substrate using both NADH and NADPH. For the nine substrates tested in this study, SsAad2p and SsAad3p preferred NADH in general, and SsAad4p preferred cofactor NADPH against most aldehydes tested (Table 5). Both SsAad1p and SsAad5p showed a decent specific activity using either cofactor NADH or NADPH toward various aldehydes. The highest specific activity was recorded for SsAad2p in reduction of acetaldehyde and formaldehyde using NADH.

Amino acid sequence analysis

Amino acid sequences of all AAD proteins from *S. stipitis*, except for SsAAD1, were found to be highly conserved. These proteins consisted of approximately 350 residues of amino acids. A typical zinc-containing signature sequence motif [$GXSEX_2$(V,I)LGKFLKK] for catalytic reaction was identified for SsAAD2, SsAAD3, SsAAD4, and SsAAD5 as indicated in segment 67 to 79 near the N-terminus in SsAAD2 (Fig. 4). A sequence region of GX_2GX_9G involved in cofactor binding (Kavanagh et al. 2008) was observed in position from 229 to 242 as shown in SsAAD2. A conserved catalytic domain (YX_5K) (Bottoms et al. 2002) positioned from 337 to 342 in SsAAD2 was also identified for these proteins. Based on these characteristics, most AAD proteins from *S. stipitis*

Fig. 3 Specific enzyme activity observed for partially purified proteins encoded by *AAD* genes from *S. stipitis* under varied temperature (**a**–**e**) and pH conditions (**f**–**j**) against furfural using cofactor NADH


a
Specific activity (U/mg protein)
AAD1
Temperature (℃)
f
Specific activity (U/mg protein)
AAD1
pH
b
Specific activity (U/mg protein)
AAD2
Temperature (℃)
g
Specific activity (U/mg protein)
AAD2
pH
c
Specific activity (U/mg protein)
AAD3
Temperature (℃)
h
Specific activity (U/mg protein)
AAD3
pH
d
Specific activity (U/mg protein)
AAD4
Temperature (℃)
i
Specific activity (U/mg protein)
AAD4
pH
e
Specific activity (U/mg protein)
AAD5
Temperature (℃)
j
Specific activity (U/mg protein)
AAD5
pH

investigated in this study were identified as members of the zinc-dependent medium-chain dehydrogenase/reductase (MDR) superfamily. As indicated in SsAAD2, a NADH-specific binding residue Asp152 was also observed for these proteins. Asp152 was reported to be bonded to oxygen atoms of the adenine ribose moiety of NADH in a reduction reaction (Fan et al. 1991). Many conserved sequence groups were also identified following procedures of Biological Workbench v3.2 (http://workbench.sdsc.edu/). These sequences were highly consistent with AADs observed from other species such as TcAAD, PcAAD, and ScAAD3. In contrast, the amino acid sequence of SsAAD1 was distinct from other AADs in *S. stipitis* and SsAAD1 appeared to be a member of the short-chain dehydrogenase/reductase (SDR) superfamily.

A phylogenetic analysis was performed for 30 proteins with various levels of aldehyde reductase activity. Based on similarity of amino acid sequences, two major groups were obtained each containing 15 proteins (Fig. 5). Except for SsAAD1 falling into group I, all the rest AAD proteins from *S. stipitis* were classified in group II. Among four SsAAD proteins, SsAAD2, SsAAD4, and SsAAD3 were clustered together and found to be closely related to each other (Fig. 5). These proteins were distantly related to SsAAD5 and distinct from other ADHs from *S. stipitis* and *S. cerevisiae* in group II. In group I, three subgroups can be recognized. Five aldehyde reductases from *S. cerevisiae* were closely related to each other as a subgroup and distinct from other proteins. ScADH6 and ScADH7 from *S. cerevisiae*, including SsADH7 from *S. stipitis*, were also separated from other proteins. SsAAD1 from *S. stipitis* fell into this subgroup and was related to ScAAD4 from *S. cerevisiae*. Four AAD proteins including ScAAD14 and ScAAD10 from *S. cerevisiae*, the earlier reported TcAAD from *T. camphoratus*, and PcAAD from *P. chrysosporium* were found to be related to each other forming another subgroup.

Discussion

In this study, we reported a new source of resistance from *S. stipitis* against toxic furfural by gene expression analysis, gene cloning, and characterization of partially purified proteins using direct enzyme assay analysis. Evaluation by direct enzyme assay is considered the most reliable evidence to sign and validate functions of unknown genes. All five AAD proteins showed strong aldehyde reduction activity toward various aldehyde compounds coupled with either cofactor NADH or NADPH. Genes *SsAAD2*, *SsAAD3*, and *SsAAD4* displayed significantly higher levels of expression in response to the challenges of furfural, a commonly observed toxic furan aldehyde derived from lignocellulosic biomass pretreatment. Their encoding proteins also showed higher levels of specific activity toward furfural reduction. The close relatedness of their amino

Table 5 Specific activities of partially purified AAD proteins from *S. stipitis* against selective aldehydes using NADH or NADPH as a cofactor

Substrate	SsAad1p		SsAad2p		SsAad3p		SsAad4p		SsAad5p	
	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH
Formaldehyde	ns	1.81 ± 0.03	11.16 ± 0.34	ns	5.90 ± 0.19	2.06 ± 0.19	ns	1.31 ± 0.14	ns	ns
Acetaldehyde	ns	1.51 ± 0.02	208.45 ± 5.36	ns	0.82 ± 0.00	ns	ns	1.01 ± 0.03	ns	ns
Propionaldehyde	3.93 ± 0.12	ns	0.64 ± 0.00	ns	1.92 ± 0.13	ns	ns	1.01 ± 0.00	2.42 ± 0.08	ns
Butyraldehyde	ns	ns	2.23 ± 0.08	ns	5.77 ± 0.19	ns	ns	0.61 ± 0.00	ns	ns
Glutaraldehyde	ns	ns	1.92 ± 0.06	ns	1.92 ± 0.09	1.92 ± 0.08	ns	ns	ns	2.12 ± 0.10
Hexaldehyde	ns	ns	6.37 ± 0.15	ns	1.37 ± 0.04	ns	ns	1.92 ± 0.14	ns	1.81 ± 0.05
Isovaleraldehyde	3.02 ± 0.09	ns	ns	ns	ns	ns	ns	ns	4.53 ± 0.11	1.51 ± 0.06
Benzaldehyde	ns	2.12 ± 0.08	0.96 ± 0.04	ns	ns	0.55 ± 0.00	ns	ns	ns	ns
Phenylacetaldehyde	0.60 ± 0.00	3.93 ± 0.18	0.64 ± 0.00	1.91 ± 0.03	1.37 ± 0.02	1.37 ± 0.03	3.33 ± 0.14	1.01 ± 0.02	ns	Ns

ns not significant

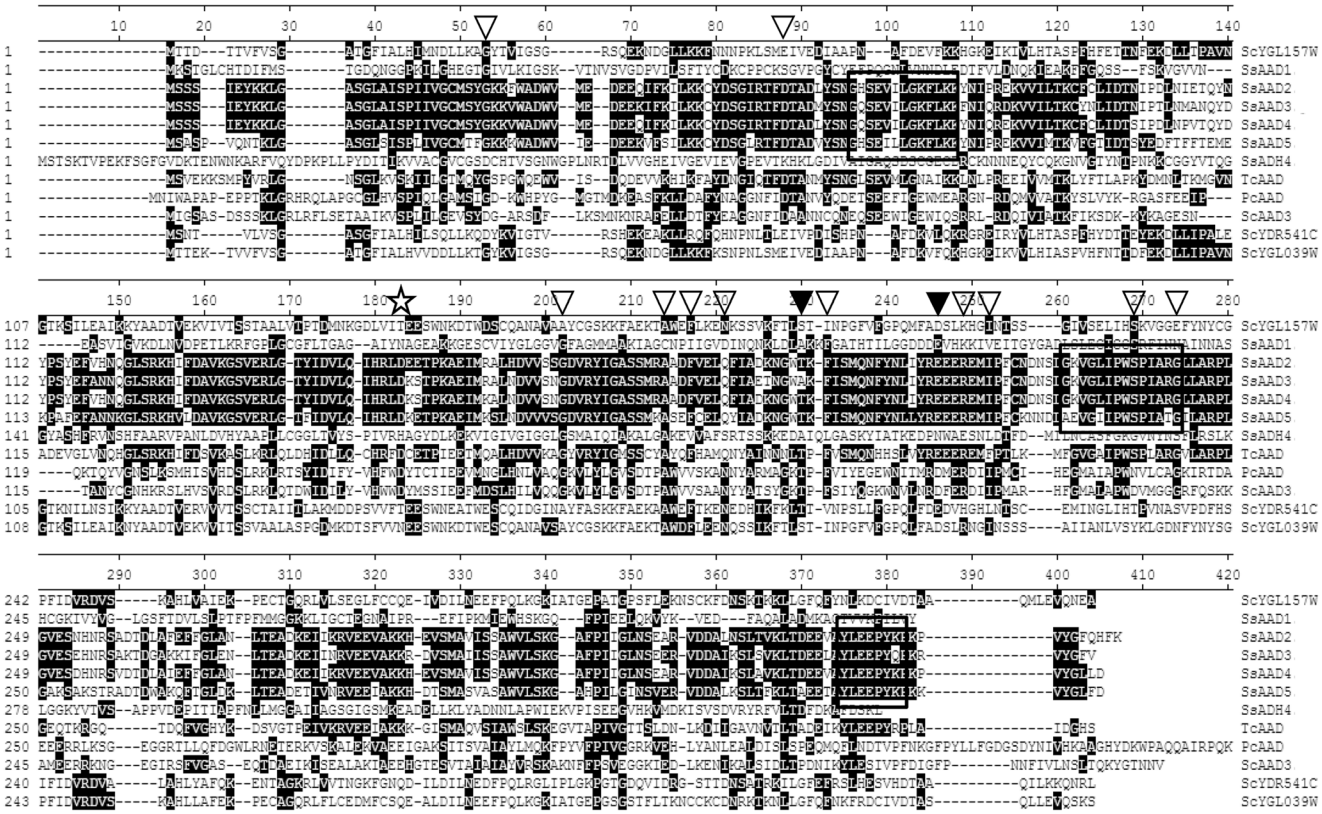


Fig. 4 Comparisons of amino acid sequences of five putative aryl-alcohol dehydrogenases from *S. stipitis* (SsAAD1, SsAAD2, SsAAD3, SsAAD4, and SsAAD5) with AAD proteins from other species including *Phanerochaete chrysosporium* (PcAAD, NCBI accession: L08964), *Taiwanofungus camphoratus* (TcAAD, NCBI accession: HQ453361), and *Saccharomyces cerevisiae* (ScAAD3, NCBI Accession: NM_001178814); alcohol dehydrogenase from *S. stipitis* (SsADH4, XM_001387085); and aldehyde reductases from *S. cerevisiae* (ScYGL157W, ScYDR541C, and ScYGL039W with NCBI accession numbers EF059032, AY692675, and AY692765, respectively) using the ClustalW method form the MegAlign program 5.0. A typical zinc-containing signature sequence motif [$GXSEX_2$(V,I)LGKFLKK] as indicated in segment from 67 to 79 near the N-terminus in SsAAD2 is *boxed*. A sequence region of GX_2GX_9G involved in cofactor binding from position 229 to 242 as shown in SsAAD2 and a conserved catalytic domain (YX_5K) positioned from 337 to 342 as in SsAAD2 are *boxed*. A NADH-specific binding residue as exampled at Asp152 in SsAAD2 is marked with a *star*. Strongly conserved groups and weak groups are marked with *solid* and *open triangles*, respectively

acid sequences suggests *SsAAD2*, *SsAAD3*, and *SsAAD4* as the core function genes for aldehyde reduction in *S. stipitis*. Discovery of this new source of resistance directly impacts more tolerant strain development for low-cost and sustainable advanced biofuel production from lignocellulosic materials.

Mechanisms of tolerance against toxic chemicals for the industrial yeast *S. cerevisiae* have been studied intensively; however, little is known about the genetic background associated with potential tolerance in *S. stipitis*. Selective strains of *S. stipitis* were able to survive and grow on an enriched medium with modest concentrations of furfural or HMF. Some ADH proteins in *S. stipitis* showed aldehyde reductase activities and were suggested as tolerance genes such as ADH4 (Ma et al. 2013). Previously, we designated an uncharacterized ORF from *S. stipitis* tentatively as *GRE2* and its encoding protein showed strong reduction activity against furfural and HMF (Wang et al. 2016). These genes were considered as candidate genes involved in the tolerance of *S. stipitis*. In this study, we present another group of *AAD* genes in *S. stipitis* possessing aldehyde reduction functions that may contribute to the tolerance against furfural in this yeast. Yeast tolerance to aldehyde inhibitors was demonstrated to be involved in multiple genes in *S. cerevisiae* (Liu et al. 2008). We observed numerous genes with strong aldehyde reduction activity in *S. stipitis* that is consistent with those previously reported in *S. cerevisiae*. Genetics and molecular biology of *S. stipitis* have not been well studied. Currently, many genes putatively assigned by computation analysis are not validated with biological evidence. In this study, we reported the function of five *SsAAD* genes using direct enzyme assay evidence. Our results suggest that these *AAD* genes, in addition to the previously reported *GRE2* and *ADH* genes from *S. stipitis*, can be considered as candidate genes contributing to the tolerance of this yeast against the toxic chemicals.

Unlike most ADH proteins in *S. stipitis*, such as ADH4, showing strong affinity with furan aldehydes using cofactor

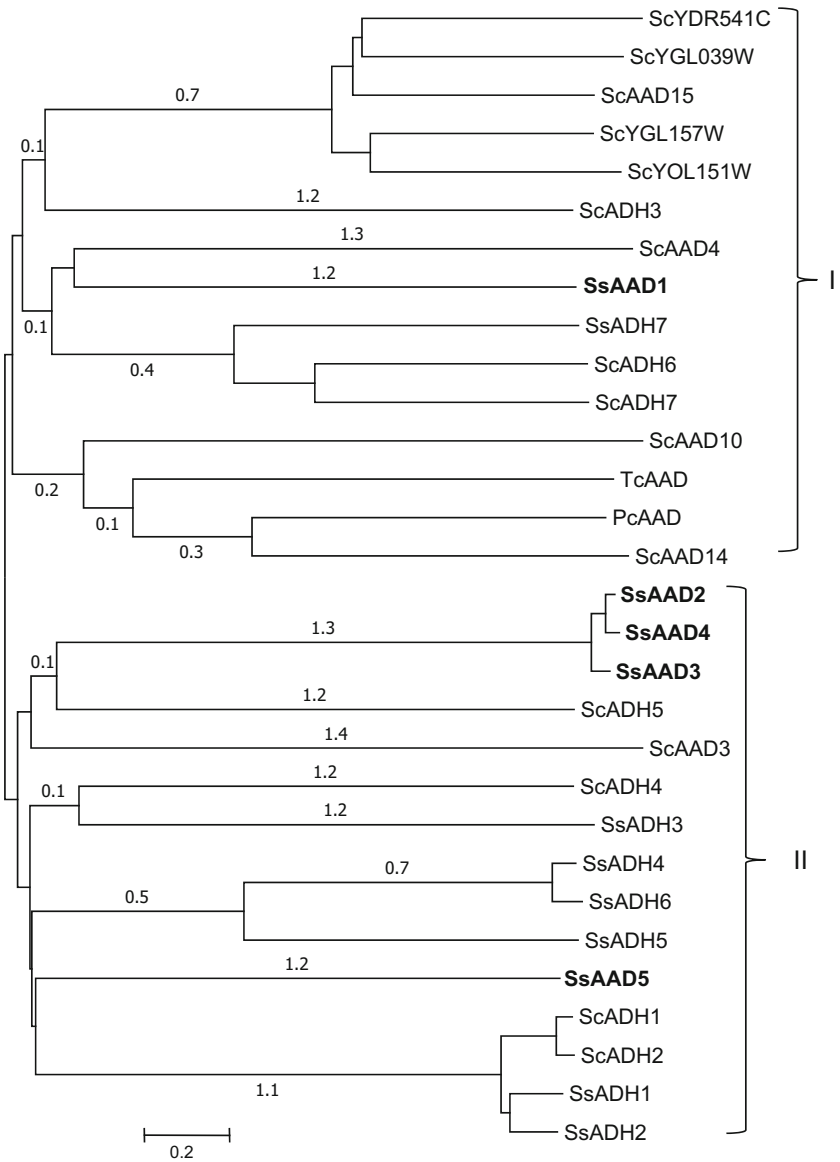


Fig. 5 A phylogenetic tree showing relationships of five putative aryl-alcohol dehydrogenases from *S. stipitis* (SsAAD1, SsAAD2, SsAAD3, SsAAD4, and SsAAD5) with AAD proteins from other species, including *Phanerochaete chrysosporium* (PcAAD), *Taiwanofungus camphoratus* (TcAAD), and *Saccharomyces cerevisiae* (ScAAD3, ScAAD4, ScAAD10, ScAAD14, and ScAAD15); alcohol dehydrogenase from *S. stipitis* (SsADH1, SsADH2, SsADH3, SsADH4, SsADH5, SsADH6, and SsADH7); alcohol dehydrogenase from *S. cerevisiae* (ScADH1, ScADH2, ScADH3, ScADH4, ScADH5, ScADH6, and ScADH7); and aldehyde reductases from *S. cerevisiae* (ScYGL157W, ScYDR541C, ScYOL151W, and ScYGL039W). The phylogenetic analysis was performed using the neighbor-joining method from MEGA 5.10 (Tamura et al. 2011)

NADPH (Ma et al. 2013), all SsAADs from *S. stipitis* reported in this study preferred cofactor NADH in reduction of furfural. GRE2 from *S. stipitis* also preferred NADH in furfural reduction reactions (Wang et al. 2016). It is commonly observed that many enzymes use both cofactors in aldehyde reductase reactions. However, the use of NADH is more economic for catabolic reactions since NADPH will require additional energy in the cofactor regeneration process (Modig et al. 2002). Thus, the use of cofactor NADH in an enzyme reaction in yeast is more desirable under stress conditions. In this regard, SsGRE2 and SsAADs can be more efficient for in situ detoxification of furan aldehydes in *S. stipitis*. It was interesting to note that in addition, to reduce furfural using NADH, SsAAD3 also showed specific activity against HMF using NADPH. Mechanisms of reduction function by SsAAD3 involved in both cofactors are currently unknown. A stereochemistry mechanism study for an aldehyde reductase from *S. cerevisiae* revealed that Ari1p directed hydrate transfer from the *si* face of NADPH to the *re* face of furfural reduction using NADPH (Bowman et al. 2010). A recent investigation using reverse engineering and site-directed mutagenesis demonstrated that a single amino acid substitution of Val285 to Asp285 enabled GRE2 from *S. cerevisiae* to utilize cofactor NADPH in addition to its major cofactor NADH (Moon and Liu 2012). Nonetheless, SsAAD3 appeared to be one of the strong candidates toward furfural reduction due to its higher level of enzyme activity observed in this study.

Under stress conditions, excessive amino acids in cells were also observed to form various aldehydes through the Ehrlich pathway (Hazelwood et al. 2008). In addition, damaged or misfolded proteins are highly toxic once aggregated together (Goldberg 2003). Degradation of the damaged and

misfolded proteins was considered to be critical maintaining cell viability (Wang et al. 2010). Pathway analysis suggested that numerous tolerance genes with aldehyde reduction functions in *S. cerevisiae* may contribute to detoxification of endogenous intermediate aldehydes (Ma and Liu 2010; Liu 2011). It is expected that the newly defined aldehyde reduction functions from *S. stipitis* reported in this study can be used in detoxification of toxic chemicals and intermediate aldehydes from both endogenous and exogenous sources.

In general, most SsAAD proteins showed higher levels of specific activity at relatively lower temperatures in the range of 20 to 30 °C, except for SsAAd1p which showed the highest specific activity at 40 °C. Some of aldehyde reductases in *S. cerevisiae* were able to tolerate relatively higher temperatures and function well even at 45 °C such as Ydr541cp (Moon and Liu 2015). To function at a higher temperature close to the fermentation, condition benefits practical applications for in situ detoxification. Except that SsAad1p and SsAad5p were sensitive to pH conditions, SsAad2p, SsAad3p, and SsAad4p were tolerant to a wide range of pH from 4.5 to 8, which is desirable for potential industrial applications.

The strain of *S. stipitis* used in this study showed resistance to furfural on an enriched medium as previously reported (Ma et al. 2013). However, it is not as robust as the highly tolerant strains from *S. cerevisiae* such as NRRL Y-50049. Most tolerant strains of *S. cerevisiae* were developed on synthetic minimum medium through environmental engineering and genetic engineering (Liu et al. 2005, 2009a; Ma et al. 2012; Moon et al. 2013). Such strains can be applied for in situ detoxification and ethanol production (Liu and Wang 2015). It is important to point out that the current strain from *S. stipitis* requires additional adaptation and engineering efforts to improve its overall resistance and robustness. However, with the aid of an enriched medium in this study, we were able to identify and define functions of the new *AAD* genes from this yeast that showed resistance against furfural.

An aryl-alcohol dehydrogenase in the white-rot fungus *P. chrysosporium*, also known as aryl-aldehyde reductase, was previously confirmed to reduce varied aldehyde-containing compounds (Muheim et al. 1991). The defined aryl-dehydrogenase is considered as one of functional enzymes in lignin biodegradation. Another lignin degradation fungus *Trametes versicolor* (*Coriolus versicolor*) was found to have a protein sequence similar to AAD amino acid sequences from *P. chrysosporium* and *S. cerevisiae* (Ichinose et al. 2002). Its encoding gene was observed to have higher levels of gene expression response to the chemical stress caused by 4-methyldibenzothiophene-5-oxide or dibenzothiophene-5-oxide. These results suggested that *AAD* genes are able to reduce certain phenol compounds. In our study, SsAad1p, SsAad2p, SsAad3p, and SsAad4p also showed reduction capability to phenylacetaldehyde that is in agreement with the previously reported (Ichinose et al. 2002). In addition, most SsAAD proteins demonstrated relatively higher levels of specific activity toward an array of aldehyde substrates as observed in this study. These results suggest that the defined *AAD* genes by this study may have reduction functions toward a wide range of substrate of aldehydes.

Our amino acid sequence analysis indicated that AAD proteins and genes encoding aldehyde reductase activity from different species are highly diversified. Among the two groups recognized, SsAAD2, SsAAD3, and SsAAD4 are highly conserved and closely related to each other. They fell into group II along with SsAAD5, which was distantly related. In contrast, SsAAD1 was distinct from all other AAD proteins in *S. stipitis*. It was grouped in group I and more closely related to SDR proteins from *S. cerevisiae*. SsAAD1 also showed relatively lower levels of specific activity against furfural than other proteins. It is possible that SsAAD1 may have additional functions beyond the current observations by this study. The identity and function of the putative gene *SsAAD1* remain to be clarified by additional investigations.

Acknowledgements This study was supported in part by the National Natural Science Foundation no. 31570086, the Talent Introduction Fund of Sichuan Agricultural University No. 01426100, China Scholar Council, and USDA-ARS National Program 213. The funding sources are not involved in the study design; the collection, analysis, and interpretation of data; the writing of the report; and the decision to submit this manuscript for publication. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Allen SA, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger PJ, Liu ZL, Gorsich SW (2010) Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol Biofuels 3:2

Bottoms CA, Smith PE, Tanner JJA (2002) Structurally conserved water molecule in Rossmann dinucleotide-binding domains. Protein Sci 11:2125–2137

Bowman MJ, Jordan DB, Vermillion KE, Braker JD, Moon J, Liu ZL (2010) Stereochemistry of furfural reduction by an aldehyde reductase from *Saccharomyces cerevisiae* that contributes to in situ furfural detoxification. Appl Environ Microbiol 76:4926–4932

Cho DH, Shin SJ, Bae Y, Park C, Kim YH (2011) Ethanol production from acid hydrolysates based on the construction and demolition wood waste using *Pichia stipitis*. Bioresour Technol 102:4439–4443

Daniel GR, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360

Fan F, Lorenzen JA, Plapp BV (1991) An aspartate residue in yeast alcohol dehydrogenase I determines the specificity for coenzyme. Biochemist 30:6397–6401

Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426:895–899

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 74:2259–2266

Ichinose H, Wariishi H, Tanaka H (2002) Molecular analysis of arylalcohol dehydrogenase of *Coriolus versicolor* expressed against exogenous addition of dibenzothiophene derivatives. J Basic Microbiol 42:327–336

Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H, Jin Y-S, Passoth V, Richardson PM (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nat Biotechnol 25:319–326

Jordan DB, Braker JD, Bowman MJ, Vermillion KE, Moon J, Liu ZL (2011) Kinetic mechanism of an aldehyde reductase of *Saccharomyces cerevisiae* that relieves toxicity of furfural and 5-hydroxymethylfurfural. BBA 1814:1686–1694

Kavanagh KL, Persson JH, Oppermann U (2008) The SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. Cell Mol Life Sci 5:3895–3906

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Larry C, Pares X, Biosca JA (2002) Characterization of a *Saccharomyces cerevisiae* NAD(P)H-dependent alcohol dehydrogenase (ADHVII), a member of the cinnamyl dehydrogenase family. Eur J Biochem 269:5738–5745

Larry C, Rosario FM, Gonzalez E, Pares X, Biosa JA (2003) Properties and functional significance of *Saccharomyces cerevisiae* ADHVI. Chem Biol Interact 143-144:229–238

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzy Microb Technol 24:151–159

Liu ZL (2006) Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. Appl Microbiol Biotechnol 73:27–36

Liu ZL (2011) Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Appl Microbiol Biotechnol 90:809–825

Liu ZL, Blaschek HP (2010) Biomass conversion inhibitors and in situ detoxification. In: Vertes A, Qureshi N, Yukawa H, Blaschek H (eds) Biomass to biofuels: strategies for global industries. Wiley Ltd, London, pp 233–258

Liu ZL, Moon J (2009) A novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 446:1–10

Liu ZL, Slininger PJ (2007) Universal external RNA controls for microbial gene expression analysis using microarray and qRT-PCR. J Microbiol Methods 68:486–496

Liu ZL, Wang X (2015) A reference model system of industrial yeast *Saccharomyces cerevisiae* is needed for development of the next-generation biocatalyst toward advanced biofuels production. J Microbial Biochem Technol 7:e125

Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 31:345–352

Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121-124: 451–460

Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S (2008) Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxyfurfural by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81:743–753

Liu ZL, Ma M, Song M (2009a) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Gen Genomics 282:233–244

Liu ZL, Palmquist DE, Ma M, Liu J, Alexander N (2009b) Application of a master equation for quantitative mRNA analysis using qRT-PCR. J Biotechnol 143:10–16

Ma M, Liu ZL (2010) Comparative transcriptome profiling analyses during the lag phase uncover *YAP1*, *PDR1*, *PDR3*, *RPN4*, and *HSF1* as key regulatory genes in genomic adaptation in the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 11:660

Ma M, Liu ZL, Moon M (2012) Genetic engineering of inhibitor-tolerant *Saccharomyces cerevisiae* for improved xylose utilization in ethanol production. Bioenerg Res 5:459–469

Ma M, Wang X, Zhang X (2013) Alcohol dehydrogenases from *Scheffersomyces stipitis* involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Appl Microbiol Biotechnol 97:8411–8425

Modig T, Liden G, Taherzadeh MJ (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem J 363:769–776

Moon J, Liu ZL (2012) Protein engineering of GRE2 from *Saccharomyces cerevisiae* for enhanced detoxification of 5-hydroxymethylfurfural. Enzyme Microbial Technol 50:115–120

Moon J, Liu ZL (2015) Direct enzyme assay evidence confirms aldehyde reductase function of Ydr541cp and Ygl039wp from *Saccharomyces cerevisiae*. Yeast 32:399–407

Moon J, Liu ZL, Ma M, Slininger PJ (2013) New genotypes of industrial yeast *Saccharomyces cerevisiae* engineered with YXI and heterologous xylose transporters improved xylose utilization and ethanol production. Biocatal Agri Biotechnol 2: 247–254

Muheim A, Waldner R, Sanglard D, Reiser J, Schoemaker HE, Letsola MSA (1991) Purification and properties of an aryl-alcohol dehydrogenase from the white-rot fungus *Phanerochaete chrysosporium*. Eur J Biochem 195:369–375

Sambrook J, Russell D (2001) A laboratory manual 3rd ed. Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanchez B, Bautista J (1988) Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. Enzym Microb Technol 10:315–318

Slininger PJ, Gorsich SW, Liu ZL (2009) Culture nutrition and physiology impact the inhibitor tolerance of the yeast *Pichia stipitis* NRRL Y-7124. Biotechnol Bioeng 102:778–790

Slininger PJ, Thompson SR, Weber SC, Liu ZL, Moon J (2011) Repression of xylose-specific enzymes by ethanol in *Scheffersomyces* (*Pichia*) *stipitis* and utility of repitching xylose-grown populations to eliminate diauxic lag. Biotechnol Bioeng 108:1801–1815

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Wang X, Xu H, Ha SW, Ju D, Xie Y (2010) Proteasomal degradation of Rpn4 in *Saccharomyces cerevisiae* is critical for cell viability under stressed conditions. Genetics 184:335–342

Wang X, Yomano LP, Lee JY, York SW, Zheng H, Mullinnix MT, Shanmugam KT, Ingram LO (2013) Engineering furfural tolerance in *Escherichia coli* improves the fermentation of lignocellulosic sugars into renewable chemicals. PNAS 110:4021–4026

Wang X, Ma M, Liu ZL, Xiang Q, Li X, Liu N, Zhang X (2016) GRE2 from *Scheffersomyces stipitis* as an aldehyde reductase contributes tolerance to aldehyde inhibitors derived from lignocellulosic biomass. Appl Microbiol Biotechnol 100:6671–6682

Weber C, Farwick A, Benisch F, Brat D, Dietz H, Subtil T, Boles E (2010) Trend and challenges in the microbial production of lignocellulosic bioethanol fuels. Appl Microbiol Biotechnol 87:1303–1315

Yang S, Pelletier DA, Lu TY, Brown SD (2010) The *Zymomonas mobilis* regulator *hfg* contributes to tolerance against multiple lignocellulosic pretreatment inhibitors. BMC Microbiol 10:135

Yi X, Gu H, Gao Q, Liu ZL, Bao J (2015) Transcriptome analysis of *Zymomonas mobilis* ZM4 reveals mechanisms of tolerance and detoxification of phenolic inhibitors derived from lignocellulosic biomass pretreatment. Biotechnol Biofuels 8:153