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ORIGINAL PAPER

Cupriavidus necator JMP134 rapidly reduces furfural with a Zn-dependent alcohol dehydrogenase

Qunrui Li · L. K. Metthew Lam · Luying Xun

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Abstract Ethanol is a renewable biofuel, and it can be produced from lignocellulosic biomass. The biomass is usually converted to hydrolysates that consist of sugar and sugar derivatives, such as furfural. Yeast ferments sugar to ethanol, but furfural higher than 3 mM is inhibitory. It can take several days for yeast cells to reduce furfural to non-inhibitory furfuryl alcohol before producing ethanol. Bioreduction of furfural to furfuryl alcohol before fermentation may relieve yeast from furfural toxicity. We observed that *Cupriavidus necator* JMP134, a strict aerobe, rapidly reduced 17 mM furfural to less than 3 mM within 14 min with cell turbidity of 1.0 at 600 nm at 50°C. The rapid reduction consumed ethanol. The "furfural reductase" (FurX) was purified, and it oxidized ethanol to acetaldehyde and reduced furfural to furfuryl alcohol with NAD^+ as the cofactor. The protein was identified with mass spectrometry fingerprinting to be a hypothetical protein belonging to Zn-dependent alcohol dehydrogenase family. The *furX*-inactivation mutant of *C. necator* JMP134 lost the ability to rapidly reduce furfural, and *Escherichia coli* producing recombinant FurX gained the ability. Thus, an alcohol dehydrogenase enabled bacteria to rapidly reduce furfural with ethanol as the reducing power.

Keywords Furfural · Furfural reduction · Ethanol · Alcohol dehydrogenase

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Introduction

Lignocellulosic biomass, the structural material of plant, is the most abundant, renewable resource to produce biofuels, e.g. ethanol. One common method for converting lignocellulosic biomass into ethanol consists of three major steps (Klinke et al. 2004). First, lignin is separated from cellulose and hemicellulose by heating in dilute acids. Second, enzymatic digestion is used to convert cellulose and hemicellulose to fermentable sugars. Third, sugars are fermented by yeasts or bacteria into ethanol. The first step often generates toxic compounds, including furfural and 5-hydroxymethylfurfural produced from pentose and hexose at high temperatures (Antal et al. 1991). Other toxic compounds from pretreatment include organic acids and aromatic compounds (Klinke et al. 2004). Furfural is more toxic than 5-hydroxymethylfurfural to yeast (Heer and Sauer 2008), and it is a key inhibitor for yeast fermentation from four different lignocellulosic hydrolysates (Heer and Sauer 2008). The main toxic effect of furfural is to prolong the lag phase of growth, which increases

fermentation time (Almeida et al. 2009). In the prolonged lag phase, furfural is gradually reduced to non-toxic furfuryl alcohol. When furfural concentrations decrease to a threshold level of 2–3 mM, the log phase of (rapid) growth begins (Liu et al. 2004).

Furfural is a strong inhibitor of ethanol fermentation in lignocellulosic hydrolysates to both yeast and bacteria (Taherzadeh et al. 1999; Zaldivar et al. 1999; Thomsen et al. 2009). The effect is partly due to the inhibition of furfural to the yeast alcohol dehydrogenase (YADH1) that is responsible for ethanol production (Modig et al. 2002). An attractive approach to deal with furfural inhibition is to develop resistant strains for ethanol fermentation. Several furfural-resistant yeast strains have been obtained under furfural stress, and the resistant yeast strains can reduce furfural to the threshold level within a day rather than several days by the parent strains (Heer and Sauer 2008; Liu et al. 2008). In addition, numerous chemical and physical methods have been developed to remove furfural (Mussatto and Roberto 2004; Almeida et al. 2009). It has also been proposed to use furfural degrading bacteria to remove furfural (Koopman et al. 2010). The extensive research suggests a continuous effort to search for more efficient and economical methods in dealing with the furfural problem in fermenting lignocellulosic hydrolysates. Here we investigate the biological reduction of furfural to non-inhibitory furfuryl alcohol as a potential alternative method.

Cupriavidus necator (formerly *Ralstonia eutropha*) JMP134 is best known for its ability to degrade 2,4-dichlorophenoxyacetate {Don, 1981 #80}. In this report, *C. necator* JMP134 was observed to rapidly reduce furfural to furfuryl alcohol at the expense of ethanol. The reduction rate was 36 times faster than the best rate reported for resistant yeasts. Since *C. necator* JMP134 is a strict aerobe (unpublished data), it should not interfere with subsequent fermentation under anaerobic conditions. The furfural reductase (FurX) was purified, and it catalyzed ethanol-dependent reduction of furfural to furfuryl alcohol with NAD^+ as the cofactor. FurX was identified with mass spectrometry fingerprinting to be a hypothetical protein in *C. necator* JMP134, belonging to the Zn-dependent alcohol dehydrogenase superfamily. The *furX*-inactivation mutant was unable to use ethanol to reduce furfural, and *Escherichia coli* expressing the cloned *furX* gained the ability. The reaction mechanism is characterized in the companion article (Li et al. 2011).

Materials and methods

Chemicals and reagents

All chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Co. (St. Louis, MO). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). PCR was performed with Taq DNA polymerase and primers from Invitrogen (Carlsbad, CA).

Bacterial strains and culture conditions

E. coli strains [TOP10, Nova Blue and BL21(DE3)] and *C. necator* strains (JMP134 and H16) were all grown in Luria–Bertani (LB) medium (Sambrook et al. 1989) at 30°C. When needed, kanamycin was added to 30 μg ml^{-1}.

Detection of furfural, furfuryl alcohol and acetaldehyde

Routine analysis of furfural and furfuryl alcohol was done with a UV–Vis spectrophotometer. Furfural has an absorption peak at 278 nm, and furfuryl alcohol has an absorption peak at 215 nm (Boopathy et al. 1993). Furfural at 100 μM gave an average peak height of 1.72 ± 0.07 at 278 nm. Furfural and furfuryl alcohol was also determined by a high performance liquid chromatography (HPLC) system (Waters, Milford, MA) with a Nova-Pak C18 column (3.9 by 150 mm; Waters). The compounds were eluted by an 11 mM H_3PO_4-acetonitrile gradient and detected by a Waters 996 photodiode array detector (Louie et al. 2002). The compounds were identified by comparing their retention times and absorption spectra to those of authentic standards, and the peak areas were used for quantification. Furfural had a retention time of 6.2 min with an absorption maximum at 276.8 nm, and furfuryl alcohol had a retention time of 5.7 min with an absorption peak at 218 nm.

Acetaldehyde was analyzed with a modified HPLC method (Pellegrino et al. 1999). Briefly, 2 μl of phenylhydrazine solution (3.3 mg·ml^{-1} in distilled

water) was added to a 40-µl sample or a 40-µl standard solution in 40 mM KPi (pH 7). The sample was mixed and incubated at room temperature for 10 min before HPLC analysis as described above. The phenylhydrazine-acetaldehyde adduct gave a retention time of 9.4 min with an absorption peak at 270 nm.

Whole cell assays

Cells from overnight culture were harvested by centrifugation and resupended to a desired optical density at 600 nm (OD_{600nm}) in 40 mM potassium phosphate (KPi) buffer (pH 7). Activities of furfural reduction by cell suspension were measured by following the absorption decrease at 278 nm in 40 mM potassium phosphate (KPi) buffer (pH 7) with 100 µM furfural, 0.1% (ca. 17 mM) ethanol with cell turbidity of 0.1 at 600 nm at 23 ± 1°C unless specified in the text. Furfural stocks were prepared in ethanol or dimethyl sulfoxide (DMSO). When furfural was added from stocks prepared in DMSO, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-amyl alcohol, formaldehyde, or acetaldehyde was added to a final concentration of 0.1% as the reducing power. When the kinetic parameters were determined with whole cells, furfural concentrations varied from 100 to 1,000 µM and ethanol changed from 0.5 to 17 mM at 23°C and 50°C. The reactions were done in 1 ml of 40 mM KPi buffer of pH 7. Hundred-µl samples were taken at 0 and 1 min incubation and were mixed with 900 µl of 1% acetic acid. The furfural concentration was then measured according to absorption at 278 nm. The rates of furfural decrease were used to determine the kinetic parameters.

The effects of pH, ionic strength, and temperature on furfural reduction

The furfural reduction activity with ethanol as the reducing equivalent was measured at pH values ranging from 6.2 to 7.8 in 40 mM KPi buffer and from 7.0 to 9.6 in 40 mM Tris buffer. The activity at ionic strengths ranging from 10 to 200 mM KPi (pH 7.0) was also tested. For temperature tolerance experiments, the cells were re-suspended in 40 mM KPi buffer (pH 7) at OD_{600} of 2. A 1-ml sample was incubated at 65°C, and 50-µl of the heated cell suspension was taken and diluted to 1 ml in the KPi buffer to measure furfural reduction at 23°C. All the above experiment was done with 100 µM furfural. Because the spectrophotometer assay was unable to perform at high temperatures, another method was developed to determine the temperature optimum from 23 to 80°C in 40 mM KPi buffer (pH 7) containing 14 mM furfural and 170 mM ethanol with cell turbidity of 1 at 600 nm. Ten-µl samples were withdrawn at 1-min intervals and mixed with 990 µl of 1% acetic acid to stop the reaction and measure absorption at 278 nm for furfural concentrations. All assays were done at least three times with averages and standard deviations being reported.

Enzyme assays

Furfural reduction was directly monitored at 278 nm in 40 mM KPi buffer (pH 7) containing 100 µM furfural, 10 µM NAD^+, 0.1% ethanol and proteins at 23±1°C.

Purification of furfural reductase from *C. necator* JMP134

About 5 grams of fresh cells were obtained from 1 liter of overnight culture. The cells were suspended in 20 ml of 40 mM Tris buffer (pH 8.0) and disrupted by passing through a French pressure cell three times at 260 MPa. The lysate was centrifuged at 10,000×*g* for 10 min to obtain supernatant, which was then heated in a 65°C water bath for 10 min. The heat-denatured proteins were removed by centrifugation. Solid ammonium sulfate was added to the supernatant to bring it to 0.8 M, and the sample was loaded onto a phenyl agarose (Sigma) column (1.5 by 12.5 cm) equilibrated with 0.8 M ammonium sulfate in the Tris buffer. The proteins were eluted with a linear gradient of ammonium sulfate (0.8 to 0 M, 200 ml) in the Tris buffer at a flow rate of 1 ml min^{-1}. The active fractions were pooled and the proteins were precipitated by adding solid ammonium sulfate to 3.2 M. The samples were centrifuged, and the pellets were resuspended in 40 mM Tris buffer (pH 8.0). The sample was dialyzed against the same buffer at 4°C for 2 h before injected onto a 5-ml High Q column (Bio-Rad, Hercules, Calif.), and proteins were eluted with a 35-ml gradient of 0 to 350 mM NaCl in 40 mM Tris buffer (pH 8.0). The active

fractions were pooled and used for activity assays. For storage, aliquots of 0.1 ml were stored at −80°C. An HPLC system (Waters) with a Biosep Sec-S3000 size-exclusion column (7.8 by 300 mm; Phenomenex, Torrance, Calif.) was used to determine the native molecular weight of the purified protein with protein standards (Bio-Rad, Hercules, CA) for calibration. The running condition was with 100 mM KPi (pH 7.0) at a flow rate of 0.5 ml min^{-1}.

Nano-LC/MS/MS

The furfural reductase protein band was cut from the SDS-PAGE gel. The protein in the gel was digested with trypsin and analyzed by nano-LC/MS/MS (Esquire HCT; Bruker Daltonics, Billerica, MA) as previously described (Tang et al. 2005). The masses of peptide fragments were used to perform searches against the *C. necator* JMP134 genome database using the program MASCOT, licensed in house (version 2.1.0; MatrixScience Ltd., London, United Kingdom). A single protein hit with probability-based Mowse scores exceeding the threshold ($P < 0.05$) was identified by the peptide mass fingerprinting.

Chromosomal disruption of *furX* in *C. necator* JMP134

The *furX* gene (Reut_B3677) is 1029 nucleotides in length. A 648-bp internal fragment of *furX* was PCR amplified from *C. necator* JMP134 DNA by using primer pair FurXiF (GTTTATCCCCGGCCATGAAG)-FurXiR (AAGATCGGCGTGCCGAAATC). The PCR product was directly ligated onto pCR2.1-TOPO (Invitrogen) and transformed into *E. coli* TOP10 cells to obtain plasmid pKO-*furX*. The pKO-*furX* DNA (1 μg) was electroporated into *C. necator* JMP134 cells as reported (Louie et al. 2002). The plasmid, which did not replicate in *C. necator*, was integrated into the chromosome by homologous recombination to result in two truncated copies of *furX* gene: one without the C-terminus and the other without the N-terminus. The mutant was selected on LB agar containing kanamycin and confirmed by colony PCR. The primer pair FurXF (CAAGG AGTCCATATGCCAGCGATG) and FurXR (ACAT CCGAAGCTTCCACGATCAGG) was for the full length *furX* gene in the wide type, and FurXiF and M13 reverse primer (Invitrogen), annealing on pCR2.1-TOPO, was for the detection of the plasmid integration in the mutant. A detailed account of the gene inactivation method in *C. necator* JMP134 has been reported (Louie et al. 2002).

Gene cloning and protein expression

The *furX* gene was amplified from *C. necator* JMP134 DNA with the primer FurXF-FurXR and cloned between the NdeI and HindIII sites of pET-30 LIC vector (Novagen, Madison, WI) as an intact gene. The ligation products were electroporated into *E. coli* Nova Blue. The correct clones were identified by colony PCR. A plasmid with the correct clone was purified and transformed into *E. coli* BL21(DE3) for recombinant protein production after induction with isopropyl-β-D-thiogalactoside.

Protein and cell dry weight analyses

SDS-PAGE was done by the method of Laemmli (1970). Protein concentrations were determined with a Bio-Rad protein dye reagent (Bradford 1976) with bovine serum albumin as the standard. The protein content of 1 ml of cells with OD_{600} of 0.1 was determined by using modified Lowry protein assay (Pierce Biotechnology, Rockford, IL) to be 0.02 mg per ml with bovine serum albumin as the standard. The cell dry weight from 1 ml of cells with OD_{600} of 0.1 was measured to be 0.04 mg per ml by drying the cell at 90°C over night under vacuum.

Results

Cupriavidus necator JMP134 rapidly reduced furfural

Overnight culture of *C. necator* JMP134 was harvested and re-suspended in 40 mM KPi buffer (pH 7) at OD_{600nm} of 0.1. The cell suspension completely reduced about 90 μM furfural to furfuryl alcohol within 14 min (Fig. 1). The produced furfuryl alcohol was stable for at least 3 h, but it was completely degraded by *C. necator* JMP134 after 24 h. The rate of furfural reduction was then directly measured by following absorption decrease at 278 nm with low

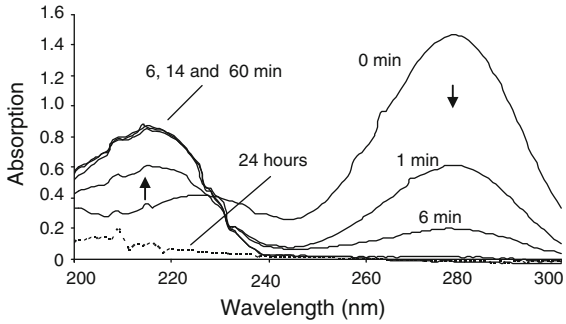

Fig. 1 Furfural reduction by *C. necator* JMP134 cells. The cells at OD_{600nm} of 0.1 rapidly reduced about 90 μM furfural (peak at 278 nm) to furfuryl alcohol (peak at 215 nm) in 40 mM KPi buffer (pH 7). Ethanol was added as solvent for the furfural stock solution. One-ml samples were taken, centrifuged for 1 min, and the supernatants were used to measure absorption spectra. Time 0 was before adding cells

cell turbidity at 23°C. *C. necator* JMP134 reduced 100 μM furfural at 803 ± 24.3 nmol min^{-1} mg^{-1} of cell dry weight; whereas, *C. necator* H16 and *E. coli* BL21(DE3) had marginal activities at 6.0 ± 1.1 and 6.5 ± 2.0 nmol min^{-1} mg^{-1} of cell dry weight, respectively.

Identification of the reducing power

The reducing power was identified to be ethanol, which was used as the solvent to make furfural stock solutions (e.g. 100 mM). When a 100-mM furfural stock solution was prepared in DMSO, *C. necator* JMP134 cells reduced 100 μM furfural at 10.5 ± 1.2 nmol min^{-1} mg^{-1} of cell dry weight, but the rate increased to 803 ± 24.3 nmol min^{-1} mg^{-1} of cell dry weight upon addition of ethanol to a final volume of 0.1% (ca. 17 mM). Thus, ethanol provides the reducing power for furfural reduction by *C. necator* JMP134. Other substrates were also tested (Table 1). Except methanol, all the other tested alcohols stimulated furfural reduction by *C. necator* JMP134 cells. The activity decreased with the increase of chain length from 2-C to 5-C, and primary alcohols were preferred over secondary alcohols. Formaldehyde was a good substrate, but acetaldehyde did not support furfural reduction. Since ethanol supported the fastest rate of furfural reduction among the tested alcohols, further characterization was done with ethanol as the reducing substrate.

Table 1 Substrates for furfural reduction

Substrate	Whole cells[a]	Enzyme[a]
Ethanol	100%	100%
Methanol	0	0
1-Propanol	68 ± 2	71 ± 3
2-Propanol	56 ± 6	24 ± 2
1-Butynol	21 ± 6	42 ± 3
2-Butynol	4.9 ± 1.0	6 ± 1
2-Amyl alcohol	4.2 ± 0.6	4 ± 1
Formaldehyde	127 ± 6	72 ± 4
Acetaldehyde	0	0

[a] The reactions were assayed in 1 ml of 40 mM KPi buffer (pH 7) with either *C. necator* JMP134 cells at a turbidity of 0.1 or the purified furfural reductase at 1.3 μg per ml. The enzyme assay contained 10 μM NAD^+. Furfural from 100 mM stock in DMSO was added to 100 μM, and then a reducing substrate was added to 0.1% to initiate the reaction. The decrease of absorption at 278 nm was monitored for furfural reduction. When ethanol was the substrate, the furfural reduction rates were 803 ± 24.3 nmol min^{-1} mg^{-1} of cell dry weight for the cell and 36.3 μmol min^{-1} mg^{-1} of protein for the purified furfural reductase (FurX)

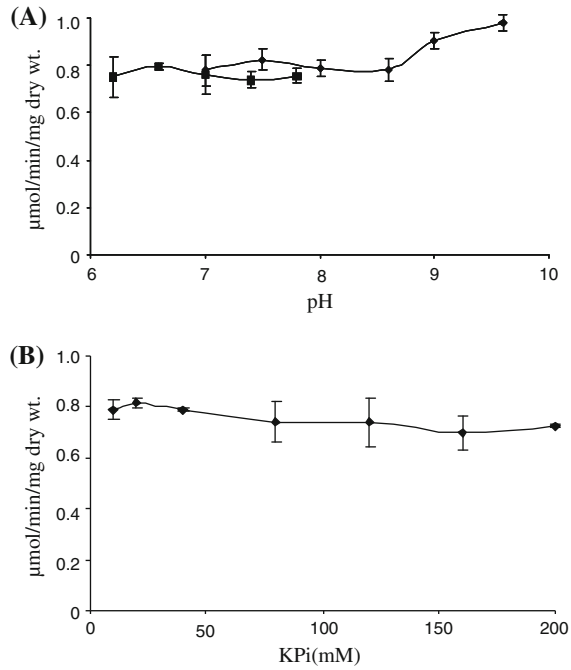

Fig. 2 Effects of pH and ionic strength on furfural reduction by whole cells. **a** The reactions were done in 40 mM KPi (pH 6.2–7.8) and Tris (pH 7.0–9.6). **b** The reactions were done in KPi buffer (pH 7) with varying ionic strength. All the assays were done with 100 μM furfural, 0.1% ethanol and cell turbidity of 0.1. Experiments were done in triplicates and averages were reported with standard deviations as error bars

The effects of pH and ionic strength on furfural reduction by *C. necator* JMP134 cells

Cupriavidus necator JMP134 cells were active in a wide range of pH values in 40 mM KPi or Tris buffers (Fig. 2a). The activity was similar between pH 6.2 to 8.6 at 793 ± 19 nmol min^{-1} mg^{-1} of cell dry weight, but increased to 902 ± 38 nmol min^{-1} mg^{-1} of cell dry weight at pH 9 and 977 ± 35 nmol min^{-1} mg^{-1} of cell dry weight at pH 9.6. High ionic strength slightly decreased the activity between 10 to 200 mM KPi buffers (pH 7) (Fig. 2b). At 10, 20 and 40 mM KPi buffer, the activities were 800 ± 16 nmolmin^{-1} mg^{-1} of cell dry weight. At 80 and 120 mM KPi buffer, the activities were 742 ± 9 nmol min^{-1} mg^{-1} of cell dry weight. At 160 and 200 mM KPi buffer, the activities were 712 ± 15 nmol min^{-1} mg^{-1} of cell dry weight. The changes were noticeable, but not dramatic.

The effects of temperature on furfural reduction by *C. necator* JMP134 cells

The effects of incubating *C. necator* JMP134 cells at 65°C before testing furfural reduction at 23°C was performed. The activity (799 ± 9 nmol min^{-1} mg^{-1} of cell dry weight) was heat-stable at 65°C for the first 16 min, and then the activities slowly decreased to 453 ± 19 nmol min^{-1} mg^{-1} of cell dry weight at 36 min. The furfural reduction by whole cells at different temperatures was determined by a modified method (Fig. 3 legend and method section). The activity increased gradually from 23 to 67°C with an optimum around 67°C (Fig. 3). The activity was then rapidly decreased to zero at 80°C. The furfural reduction rate was 19.8 ± 0.7 μmol min^{-1} mg^{-1} of cell dry weight at 67°C.

Kinetic parameters for furfural reduction by *C. necator* JMP134 cells

Using the initial rates, the kinetic parameters were determined with *C. necator* JMP134 cells. The apparent K_m values were 708 ± 54 μM for ethanol and 133 ± 20 μM for furfural, and the V_{max} was 2.3 ± 0.1 μmol min^{-1} mg^{-1} of cell dry weight at 23°C. The apparent K_m value was 180 ± 5 μM furfural, and V_{max} was 7.2 ± 0.3 μmol min^{-1} mg^{-1} of cell dry weight at 50°C.

Rapid reduction of high concentrations of furfural and end products

At 50°C, *C. necator* JMP134 cells at turbidities of 1.0 and 0.5 (600 nm) reduced 17 mM furfural to less than 3 mM within 14 and 25 min, respectively (Fig. 4). The cells at OD_{600} of 0.1 reduced furfural to about 5 mM after 80 min incubation (Fig. 4). At 80 min, the cell suspensions at turbidities of 0.1, 0.5, and 1.0 consumed 11.1, 14.3 and 14.3 mM furfural and accumulated 10.0, 11.3, and 11.4 mM acetaldehyde, respectively. Since acetaldehyde has a boiling point of 20.8°C, some evaporation was expected when caps were opened for sampling. Alternatively, some acetaldehyde was consumed by the cells. At 67°C,

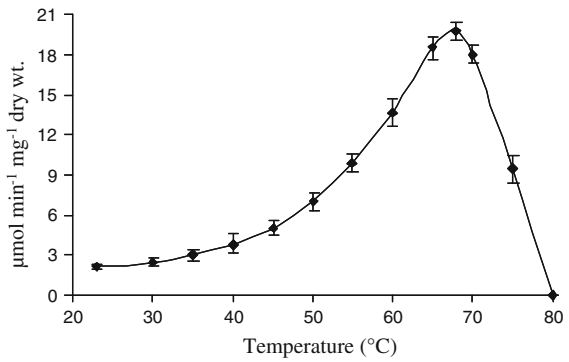

Fig. 3 Effects of temperature on furfural reduction by *C. necator* JMP134 cells. The reactions were done in 40 mM KPi buffer (pH 7) containing 14 mM furfural and 170 mM ethanol with cell at OD_{600} of 0.1. Ten-μl samples were taken at 1-min intervals and added to 990 μl of ice-cold 1% acetic acid for immediate measurement of absorption at 278 nm

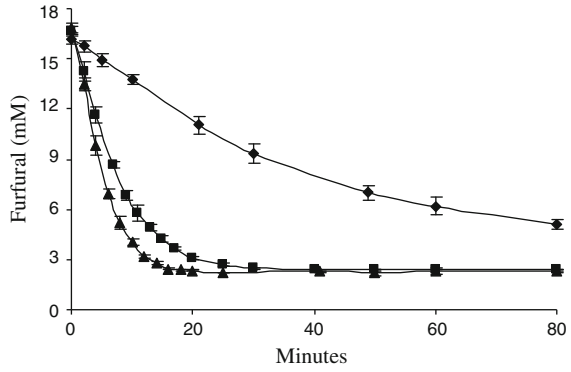

Fig. 4 The time course of furfural reduction by *C. necator* JMP134 cells. The experiments were done at 50°C as described in Fig. 3 legend, but sampled at longer time intervals. Three cell turbidities at 600 nm were tested: 0.1 (*filled diamond*), 0.5 (*filled square*), and 1.0 (*filled triangle*)

the initial rate was the highest (Fig. 3), but the reaction was incomplete, perhaps due to the cell or enzyme inactivation in the presence of 17 mM furfural and 170 mM ethanol.

Purification of furfural reductase from *C. necator* JMP134

Ethanol-dependent furfural reduction was detected in the cell extract of *C. necator* JMP134. Since the furfural reductase activity of the whole cell was thermal stable at 65°C for 15 min, the extract was heated at 65°C for 10 min. As expected, the heating remove significant portion of the total protein, but did not decrease the total furfural reductase activity. In fact, it increased the total activity by 20% (Table 2). The activity increase could be due to inactivating competing enzymes or releasing the required cofactor. After the phenyl agarose column, no apparent activity was detected with individual fractions. The activity was detected after combining the first fraction that did not bind to the column (0.8 M ammonium sulfate) and the fraction eluted around 0.5 M ammonium sulfate. When the first fraction was heated to 95°C for 10 min, the supernatant still supported the furfural reduction by the fraction around 0.5 M ammonium sulfate. Thus, the first fraction contained a cofactor for the furfural reductase. The supernatant of 95°C-treated first fraction was then used for activity assays to purify the reductase. Further purification of the furfural reductase through a high Q column generated a major protein peak around 150 mM NaCl, and furfural reductase activity was only associated with the peak. An SDS-PAGE analysis of the proteins from each purification step clearly pointed to the enrichment and purification of the furfural reductase (Fig. 5).

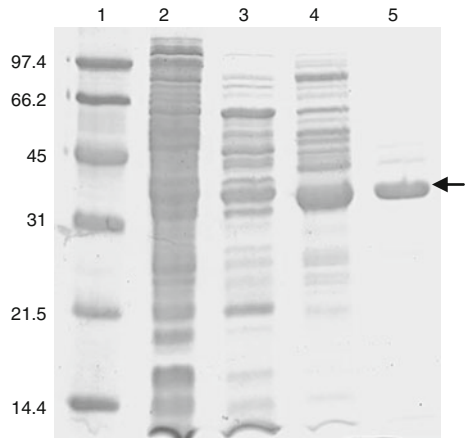

Fig. 5 SDS-PAGE analysis of *C. necator* JMP134 furfural reductase. *Lane 1*, Protein markers; *lane 2*, 3 µl of cell extracts; *lane 3*, 3 µl of heat-treated extracts; *lane 4*, 1.5 µl of protein after phenyl agarose; *lane 5*, 3 µl of protein after High-Q. The corresponding protein concentrations were given in Table 2

Properties of the purified furfural reductase (FurX)

The purified furfural reductase was named as FurX that gave a denatured molecular mass of about 36 kDa on SDS-PAGE (Fig. 5), and the native molecular mass was estimated by gel filtration to be 130 ± 10 kDa, suggesting the enzyme was a homotetramer. The purified FurX was colorless, and it catalyzed ethanol-dependent furfural reduction that required the supernatant of 95°C-treated first fraction from the phenyl agarose column. The cofactor in the 95°C-treated sample was shown to be NAD^+ because 10 µM NAD^+, but not $NADP^+$, completely reconstituted the furfural reduction by the purified protein. The purified FurX required ethanol and NAD^+ for furfural reduction. There was no activity without ethanol, and the activity was marginal without NAD^+ (Fig. 6). With excess ethanol, it quantitatively converted 98 ± 2 nmol of furfural to 95 ± 4 nmol of

Table 2 Purification of furfural reductase from *C. necator* JMP134

Purification step	Vol (ml)	Protein (mg)	Sp act (U)[a]	Total act (mg × U)	Recovery (%)
Cell extract	18.3	223.7	1.9	429.8	100
Heat at 65°C	15.8	30.1	17.2	516.7	120
Phenyl agarose	2.2	13.3	20.1	268.1	62
High Q	3.3	4.3	36.3	157.3	37

[a] Specific activity (µmol min^{-1} mg^{-1}) was measured with 10 µM NAD^+ in 1 ml of 40 mM KPi buffer (pH 7) containing 100 µM furfural, 0.1% ethanol and proteins at 23°C

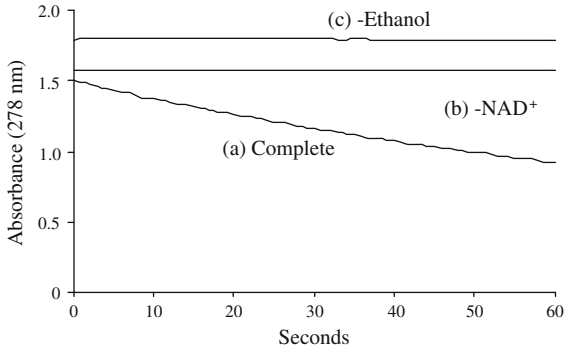

Fig. 6 Furfural reduction by purified FurX from *C. necator* JMP134. (*a*) The reaction was performed in 40 mM KPi buffer (pH 7) containing 1.2 μg of FurX, 10 μM NAD^+, approximately 100 μM furfural and 0.1% ethanol, (*b*) The reaction did not contain NAD^+, (*c*) The reaction did not contain ethanol

furfuryl alcohol and produced 96 ± 2 nmol of acetaldehyde. Besides ethanol, FurX also used many alcohols and formaldehyde for furfural reduction with varying efficiencies (Table 1). Unlike the cell, the enzyme had a neutral pH preference with the rates similar between pH 6.5 and 8. In addition, the enzyme had a lower temperature optimum between 50 and 55°C with a rate of 250 ± 16 μmol min^{-1} mg^{-1} of protein and completely lost activity at 70°C. It appears that the whole cell environment provided some thermal protection for the enzyme. The optimal ionic strength was from 0.1 to 1 M KPi (pH 7.0) with an average (6 samples) specific activity of 39.0 ± 1.1 μmol min^{-1} mg^{-1} of protein. When NaCl was added to 0.1 M KPi buffer, the enzyme showed 100, 72, 72, 54, 22, 4% of activity at 0, 1, 2, 3, 4, and 5 M NaCl, respectively. Thus, the enzyme was robust under a wide range of conditions.

Identification of the gene coding for the furfural reductase in *C. necator* JMP134

The protein band of the purified FurX on SDS-PAGE (Fig. 5) was cut, digested, and analyzed by nano-LC-MS-MS. Peptide mass fingerprinting identified a single protein encoded by gene Reut_B3677 in *C. necator* JMP134 genome. The encoded protein belonged to the Zn-containing alcohol dehydrogenase superfamily. The calculated molecular mass of FurX was 35,954.23, in agreement with the SDS-PAGE result (Fig. 5). The *furX* gene in *C. necator* JMP134 was inactivated by homologous recombination of a plasmid containing an internal fragment of *furX*, resulting in two truncated copies of the gene. The integration of the plasmid was confirmed by PCR. The *furX* mutant grew similarly as the wild type *C. necator* JMP134 in LB medium. The mutant cells were harvested from overnight cultures and analyzed for ethanol-dependent furfural reduction. The *furX* mutant reduced furfural very slowly at 12.5 ± 1.0 nmol min^{-1} mg^{-1} of cell dry weight, which was 1.5% of the activity of the wild type *C. necator* JMP 134. The *furX* mutant also reduced furfural prepared in DMSO at 12.5 ± 1.0 nmol min^{-1} mg^{-1} of cell dry weight, and ethanol addition did not stimulate the reduction. Thus, FurX was responsible for the ethanol-dependent furfural reduction in *C. necator* JMP134. The *furX* gene was cloned into pET30-LIC as a non-fusion gene, the protein was over produced in *E. coli* BL21(DE) cells in the presence of 0.2 mM isopropyl-*β*-D-thiogalactoside and various amount of $ZnSO_4$. Most FurX proteins formed inclusion bodies in *E. coli*, and the addition of $ZnSO_4$ to 0.5 mM in the medium appeared to be helpful. The induced cells reduced 100 μM furfural at 356, 470, 590, 922, 1,019, 918, and 254 nmol min^{-1} mg^{-1} of cell dry when the cells were induced by isopropyl-*β*-D-thiogalactoside in the presence of 0, 0.1, 0.2, 0.4, 0.5, 0.7, and 1 mM $ZnSO_4$, respectively. The reduction required ethanol (0.1%). Without ethanol, the cells reduced furfural at an average of 6.5 ± 2.0 nmol min^{-1} mg^{-1} of cell dry weight. The control of *E. coli* BL21(DE3) cells reduced furfural at 6.5 ± 2.0 nmol min^{-1} mg^{-1} of cell dry weight with or without ethanol.

Discussion

Several furfural-resistant strains of yeast and *E. coli* have been selected to overcome furfural inhibition. The mutants apply different strategies. A resistant *E. coli* has mutations that silence two NADPH-dependent furfural reductase genes (Miller et al. 2009). The two enzymes have low K_m values for NADPH, and they are assumed to deplete cellular NADPH via furfural reduction, prolonging lag phase in the parent yeast strain. A mutant reprograms glucose metabolism so that the pentose phosphate pathway is more active for the increased production

of NADPH to facilitate NADPH-dependent furfural reduction (Liu et al. 2009). Nonetheless, furfural is still inhibitory to the resistant strains.

Yeast can reduce furfural in the lag phase of growth, but the reduction rates are considerably slow. *Saccharomyces cerevisiae* strain CBS 8066 reduced furfural at 0.1 μmol min^{-1} mg^{-1} of cell dry weight (Taherzadeh et al. 1999). *S. cerevisiae* strain TMB3001 and its resistant strain reduced furfural at 0.065 and 0.13 μmol min^{-1} mg^{-1} of cell dry weight, respectively (Martín et al. 2007). TMB3400, a robust half-industrial strain, reduces furfural at 0.2 μmol min^{-1} mg^{-1} of cell dry weight (Heer and Sauer 2008). In contrast, *C. necator* JMP134 reduced furfural with maximal rates of 2.4 and 7.2 μmol min^{-1} mg^{-1} of cell dry weight at 23 and 50°C, respectively, which were 12 and 36 times faster than the maximal reported rate for yeast.

The fast reduction of furfural by *C. necator* JMP134 is powered by ethanol. *C. necator* JMP134 furfural reductase used ethanol directly as the reducing power to reduce furfural. The previously reported furfural reduction in *E. coli* and *S. cerevisiae* is mainly NADPH-dependent (Gutierrez et al. 2006; Petersson et al. 2006; Miller et al. 2009). In order for the reaction to occur, cells have to generate NADPH via metabolism, and then the enzymes use NADPH to reduce furfural. The use of ethanol to directly reduce furfural offers a bypass of the cellular metabolism, allowing faster furfural reduction by *C. necator* JMP134 cells.

The *C. necator* furfural reductase (FurX) belonged to the Zn-dependent alcohol dehydrogenase superfamily. The overproduction of FurX in *E. coli* BL21(DE3) resulted mainly in inactive proteins in inclusion bodies. *E. coli* produced more soluble and active FurX when $ZnSO_4$ was added, showing the Zn requirement. FurX was highly homologous to several structurally characterized alcohol dehydrogenases, including *Pseudomonas aeruginosa* alcohol dehydrogenase (PaADH) (Levin et al. 2004) with 77% identity and *Bacillus stearothermophilus* alcohol dehydrogenase (BsADH) (Ceccarelli et al. 2004) with 59% identify. Although FurX was a predominant protein in the cell extract of *C. necator* JMP134 cells grown in LB medium (Fig. 5), the *furX* inactivation mutant appeared normal in growth except the loss of rapid reduction for furfural to furfuryl alcohol. *C. necator* JMP134 has the genes to degrade furfuryl alcohol under aerobic conditions (Koopman et al. 2010). When *C. necator* JMP134 reduced furfural to furfuryl alcohol, the latter was stable for several hours before being consumed (Fig. 1). It appears that the expression of the furfuryl alcohol degrading genes requires induction in the presence of furfuryl alcohol. *E. coli* has a FurX homolog (EcADH) that catalyzes ethanol-dependent reduction of furfural (Li et al. 2011). The low furfural reduction activity reported in this report is likely due to the minimal gene expression under the culturing conditions.

In summary, both *C. necator* JMP134 cells and purified FurX catalyzed ethanol-dependent furfural reduction with the production of acetaldehyde and furfuryl alcohol (Fig. 7). The furfural reduction by *C. necator* JMP134 was much faster than that by any other cells reported to date. The ethanol-dependent furfural reductase was identified to be FurX that requires NAD^+ as a cofactor in *C. necator* JMP134. Both the whole cell and the enzyme are heat tolerant and able to function at relatively high temperatures over wide ranges of pH and ionic strength. These properties may potentially enable to detoxify furfural during or right after pretreatment of lignocellulosic biomass. Since *C. necator* JMP134 did not grow by fermentation in LB medium (data not shown), it should not interfere with subsequent ethanol fermentation under anaerobic conditions. It may seem paradoxical to use ethanol for furfural reduction before ethanol production (fermentation); however, the amount of ethanol consumed (0.1% = 17.1 mM)

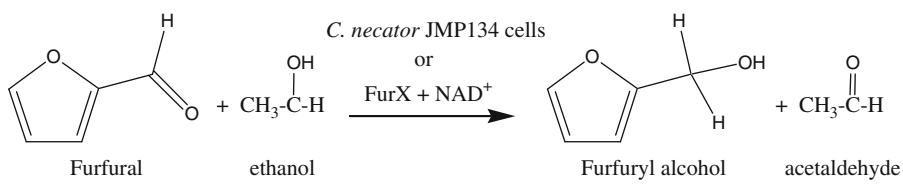

Fig. 7 The ethanol-dependent furfural reduction catalyzed by *C. necator* JMP134 cell or purified FurX protein. FurX required added NAD^+ for catalysis, and *C. necator* JMP134 cells did not

is relatively small and it may be economically feasible, especially when ethanol is readily available at ethanol producing facilities. In addition, both products, acetaldehyde and furfuryl alcohol, may be recovered as value added products. The biochemical mechanism of ethanol-dependent reduction of furfural and physiological function of FurX in *C. necator* JMP134 are discussed in the companion article (Li et al. 2011).

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