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ARTICLE

Unique glucose oxidation catalysis of *Gluconobacter oxydans* constitutes an efficient cellulosic gluconic acid fermentation free of inhibitory compounds disturbance

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Abstract

Toxic inhibitory compounds from lignocellulose pretreatment are the major obstacle to achieve high bioconversion efficiency in biorefinery fermentations. This study shows a unique glucose oxidation catalysis of *Gluconobacter oxydans* with its gluconic acid productivity free of inhibitor disturbance. The microbial experimentations and the transcriptome analysis revealed that both the activity of the membrane-bound glucose dehydrogenase and the transcription level of the genes in periplasmic glucose oxidation respiratory chain of *G. oxydans* were essentially not affected in the presence of inhibitory compounds. *G. oxydans* also rapidly converted furan and phenolic aldehyde inhibitors into the less toxic alcohols or acids. The synergy of the robust periplasmic glucose oxidation and the rapid inhibitor conversion of *G. oxydans* significantly elevated the efficiency of the oxidative fermentation in lignocellulose hydrolysate. The corresponding genes responsible for the conversion of furan and phenolic aldehyde inhibitors were also mined by DNA microarrays. The synergistic mechanism of *G. oxydans* provided an important option of metabolic modification for enhancing inhibitor tolerance of general fermentation strains.

KEYWORDS
gluconobacter oxydans, inhibitor tolerance, lignocellulose, membrane-bound glucose dehydrogenase (mGDH), oxidative fermentation

1 | INTRODUCTION

Pretreatment for lignocellulose biomass generates various compounds such as 2-furaldehyde (furfural), 5-(hydroxymethyl)-2-furaldehyde (HMF), 4-hydroxybenzaldehyde (HBA), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde), and acetic acid (Klinke, Thomsen, & Ahring, 2004). These compounds harshly inhibit energy generation, enzyme activity, and protein synthesis in central carbon metabolic pathways of microorganisms (Banerjee, Bhatnagar, & Viswanathan, 1981; Hristozova et al., 2006; Palmqvist, Almeida, & Hahn-Hagerdal, 1999; Zaldivar, Martinez, & Ingram, 1999). The direct outcome is the ceased or delayed cell growth (longer lag phase) of fermenting strains in the lignocellulose hydrolysates with similar inhibitor concentrations, such as *Saccharomyces cerevisiae* (more than 7 hr lag phase time; Hawkins & Doran-Peterson, 2011), lactic acid bacteria *Sporolactobacillus inulinus* and *Pediococcus acidilactici* (>20 hr; Bai, Gao, He, & Wu, 2015; Yi et al., 2016), fungus *Aspergillus niger* (>48 hr; Zhang, Zhang, & Bao, 2016b; Zhou, Meng, & Bao, 2017), and oleaginous yeast *Trichosporon cutaneum* (>48 hr; Wang, Gao, Zhang, & Bao, 2016). Besides the negative effect on cell growth, the inhibitory compounds also strongly reduce the enzyme activity in the synthesis pathways of target products, such as lactate dehydrogenase for lactic acid synthesis (Bai et al., 2015), glucose oxidase for sugar acid synthesis (Zhang et al., 2016b), malic enzyme for lipid or succinic acid synthesis (Huang, Wu, Liu, Li, & Zong, 2011; Xu, Wang, Zhou, Chen, & Cai, 2015), and pyruvate dehydrogenase or alcohol dehydrogenase for ethanol

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synthesis (Modig, Liden, & Taherzadeh, 2002). These inhibition actions on central metabolism and target product synthesis are closely correlated and generally occur in a synergistic way on biorefinery fermenting strains.

Our previous studies on a Gram-negative bacterium *Gluconobacter oxydans* showed some surprising results that *G. oxydans* has almost zero lag phase time in the lignocellulose hydrolysate with similar inhibitor concentrations and rapidly converts almost all lignocellulose-derived sugars to the corresponding sugar acids (Yao, Hou, & Bao, 2017; Zhang, Liu, Zhang, & Bao, 2016a). Gluconic acid is one of the top 30 value-added chemicals derived from biomass (Werpy & Petersen, 2004). The sodium salt of gluconic acid (sodium gluconate) is the major cement retarding additive used in construction industry with the huge market (Hou & Bao, 2019). Production of gluconic acid from lignocellulose is more competitive both technically and economically than ethanol production based on the rigorous techno-economic analysis (Zhang et al., 2016a). The high inhibitor tolerance and sugar conversion capacity of the fermenting strain *G. oxydans* are strongly beneficial for efficient production of cellulosic gluconic acid (Yao et al., 2017; Zhang et al., 2016a). Different from the general reactions in the intracellular metabolisms such as glycolysis, Krebs cycle, and pentose-phosphate pathway, glucose oxidation is catalyzed by a membrane-bound glucose dehydrogenase (mGDH; EC 1.1.5.2) in *G. oxydans* coupling with the respiratory chain (Matsushita, Toyama, & Adachi, 1994; Yamada, Elias, Matsushita, Migita, & Adachi, 2003). The contribution of the soluble glucose dehydrogenase (EC 1.1.4.7) in the cytoplasm is negligible (Hanke et al., 2013). When glucose is oxidized into glucono-1,5-lactone (then spontaneously converted to gluconic acid; Figure 1), the electrons from glucose oxidation directly transferred to ubiquinone (UQ) and UQ is reduced into ubiquinol (UQH_2). The terminal UQH_2 oxidases (cytochrome *bo*$_3$ and cytochrome *bd*) oxidize UQH_2 to UQ by transferring the electrons to molecular oxygen (Miura et al., 2013; Prust et al., 2005; Richhardt, Luchterhand, Bringer, Buchs, & Bott, 2013). The energy-consuming transport of glucose to intracellular space or the release of gluconic acid product from intracellular space to the hydrolysate is not required by the glucose oxidation. Besides the unique glucose oxidation catalysis, *G. oxydans* is capable of converting furan or phenolic aldehyde inhibitors such as furfural, HMF, HBA, and vanillin to the less toxic metabolites (Buchert & Niemela, 1991; Zhou, Zhou, Xu, & Chen, 2017).

In this study, we confirmed that the glucose oxidation efficiency of *G. oxydans* was independent of the inhibitors in corn stover hydrolysate. The mechanisms of the high fitness of *G. oxydans* to the inhibitor-containing environment were examined and elucidated by microbial experimentations and transcriptome analysis. This study provides an important model case used for metabolic modification on general fermentation strains to enhance the inhibitors tolerance capacity and fermentability.

2 | MATERIALS AND METHODS

2.1 | Raw materials and reagents

Corn stover was harvested from Dan Cheng (Henan, China) in fall 2013. The preprocessing procedure of corn stover was according to Zhang, Wang, Chu, He, and Bao (2011). Its composition was determined to be 38.7% of cellulose, 20.6% of hemicellulose, 26.5% of lignin, and 2.8% of ash on the dry weight base (w/w) by using ANKOM 220 Cellulose Analyzer (ANKOM Technology, Macedon, NY).

Commercial cellulase enzyme Youtell #7 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). According to the methods in Adney and Baker (1996), Ghose (1987) and Bradford (1976), the filter paper activity, cellobiase activity, and protein concentration were determined to be 63.0 FPU/g, 344 CBU/g, and 49.5 mg/g, respectively.

Furfural and HMF were purchased from Shanghai Demo Medical Tech Co. (China). HBA, vanillin, and syringaldehyde were purchased from Sangon Biotech (Shanghai) Co. (China). Acetic acid was from Sinopharm Chemical Reagents Co. (Shanghai, China). Phenazine methyl sulfate (PMS) was from Sigma-Aldrich Co. (St. Louis, MO) and $NADPNa_2$ was from Biosharp Co. (Hefei, China). All other chemicals such as glucose, sorbitol, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, NaOH, and H_2SO_4 were from Lingfeng Chemical Reagents Co. (Shanghai, China).

2.2 | Strains and cell cultures

G. oxydans DSM 2003 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). The genome sequence of *G. oxydans* DSM 2003 was obtained from the National Center of Biotechnology Information

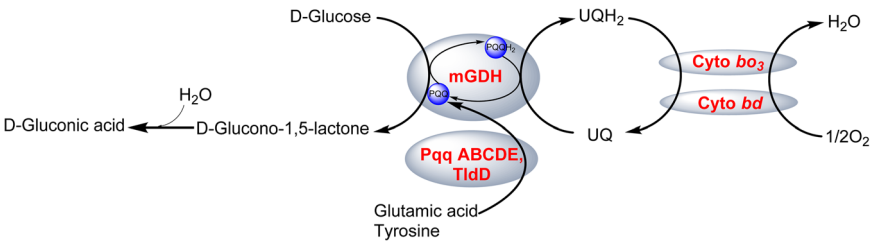

FIGURE 1 Glucose oxidation reaction and the respiratory chain in the cell periplasmic space of *G. oxydans* DSM **2003.** Pqq ABCDE and TldD, PQQ synthesis protein; Cyto *bo*$_3$, Cytochrome *bo*$_3$ ubiquinol oxidase; Cyto *bd*, cytochrome *bd* ubiquinol oxidase; mGDH, membrane-bound glucose dehydrogenase; PQQ, pyrroloquinoline quinone; UQ, ubiquinone; UQH_2, ubiquinol [Color figure can be viewed at wileyonlinelibrary.com]

(NCBI) GenBank with the accession number of AYTY00000000.1 (Sheng, Ni, Gao, Ma, & Xu, 2014). Two synthetic culture media were used, including the seed culture medium contained 80 g of sorbitol, 20 g of yeast extract, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4, and 1.5 g of $(NH_4)_2SO_4$ per liter of deionized water; the fermentation medium contained 80 g of glucose, 20 g of yeast extract, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4, and 1.5 g of $(NH_4)_2SO_4$ per liter of deionized water.

Biodetoxification fungus *Amorphotheca resinae* ZN1 was isolated in our previous work (Zhang et al., 2010) and stored in the China General Microorganism Collection Center (#7452; Beijing, China). The cultured and maintained methods of *A. resinae* ZN1 were used according to Zhang et al. (2010).

2.3 | Pretreatment, biodetoxification, and hydrolysis of lignocellulose

Corn stover was dry acid pretreated according to the pretreatment method in Liu et al. (2018). Briefly, the preprocessed corn stover solid and 5% (w/w) dilute sulfuric acid solution were simultaneously fed into a 20 L helically agitated reactor with the solid-to-liquid ratio of 2:1 (w/w) and the agitation rate of 50 rpm. The pretreatment was carried out at 175 ± 1°C for 5 min to yield the pretreated corn stover with around 50% (w/w) of dry solid matter. The pretreated corn stover contained 39.5% of cellulose, 6.8% of hemicellulose, and 6.6% of ash determined according to the NREL LAP protocol (Sluiter et al., 2008). The inhibitors content in the pretreated corn stover included 5.13 mg of furfural, 3.38 mg of HMF, 0.18 mg of HBA, 1.27 mg of vanillin, 0.67 mg of syringaldehyde, and 16.65 mg of acetic acid per gram of dry solid matter.

The biodetoxification method of pretreated corn stover was according to Liu et al. (2018). Briefly, the pretreated corn stover was neutralized to pH of 5–6 by adding 20% (w/w) $Ca(OH)_2$ suspension slurry, then biodetoxified at 28°C for 5 days by inoculating *A. resinae* ZN1 seeds at 10% (w/w) inoculation ratio. The corn stover hydrolysate was prepared according to Zhang et al. (2016a). Briefly, both the freshly pretreated and the biodetoxified pretreated corn stover were hydrolyzed at 50°C, pH 4.8 for 48 hr in a 5 L bioreactor with proper helical stirring. The cellulase dosage was 15 FPU per gram of dry corn stover matter. Then the hydrolysate slurry was centrifuged at 10,000 rpm for 10 min to remove the solids, autoclaved at 115°C for 20 min, and then filtered by filter paper to yield the hydrolysates. The high inhibitors-containing corn stover hydrolysate was prepared by directly hydrolyzing the freshly pretreated corn stover, while the low inhibitors-containing hydrolysate was prepared by hydrolyzing the biodetoxified and pretreated corn stover. The high inhibitors-containing hydrolysate contained 58.4 g/L of glucose, 27.9 g/L of xylose, 3.00 g/L of acetic acid, 730 mg/L of furfural, 360 mg/L of HMF, 20.0 mg/L of HBA, 6.0 mg/L of vanillin, and 170 mg/L of syringaldehyde. The low inhibitors-containing hydrolysate contained 55.7 g/L of glucose, 29.7 g/L of xylose, 0.86 g/L of acetic acid, 5.20 mg/L of furfural, 8.20 mg/L of HMF, 0.10 mg/L of HBA, 0.10 mg/L of vanillin, and 200 mg/L of syringaldehyde.

2.4 | Whole cell catalysis and fermentation

Resting cells of *G. oxydans* DSM 2003 were harvested according to the method in Meyer, Schweiger, and Deppenmeier (2013). The cells were collected by centrifugation (2,500*g*, 20 min) while the optical density (OD) at 600 nm (OD_{600}) reached 1.0 (approximately 0.5 g dry cells per liter). Then the cells precipitation was washed twice with 40 mM potassium phosphate buffer (KPB, pH 6) and resuspended in the KPB buffer to the OD_{600} of 10.0. The resting cells slurry was added at 10% (v/v) ratio into the 250 ml flask containing 50 ml of corn stover hydrolysate or pure glucose solution (40 g/L) for whole cell catalysis at 30°C and 200 rpm. The pH was maintained at 5–6 by adding 5 M NaOH.

For fermentation experiments, one vial (2 ml) of *G. oxydans* DSM 2003 was inoculated into 20 ml of seed medium in 100 ml flask and cultured at 30°C, 220 rpm for 24 hr. Then the seed broth was inoculated at 10% (v/v) ratio into 50 ml fermentation medium containing different concentrations of inhibitor in 250 ml flask and fermented at 30°C, 220 rpm. The pH was maintained at 5–6 by adding 5 M NaOH.

2.5 | Extraction of crude membrane proteins and enzyme assay of mGDH

Crude membrane proteins of *G. oxydans* DSM 2003 were harvested according to Meyer et al. (2013). Briefly, the seed broth at OD_{600} of 0.8–1.2 was centrifuged at 7,000*g*, 4°C for 10 min, the pellets were resuspended in KPB (40 mM, pH 7) buffer and then lysed by sonication at 400 W for 5.7 min (5 s on and 15 s off). After centrifuged at 1,300*g*, 4°C for 10 min to remove the cell debris, the cell membrane part was harvested from the supernatant above by ultracentrifugation at 150,000*g*, 4°C for 90 min. The cell membrane pellets were washed once with the KPB (40 mM, pH 7) buffer and resuspended in buffer W (100 mM of Tris-HCl, 150 mM of NaCl, 1% (v/v) Triton X-100, pH 8) for the following research. The protein content of the extracted supernatant solution was measured by the Bradford method (Bradford, 1976).

The enzyme activity of the membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (mGDH) was measured by taking PMS as the electron acceptor. One milliliter reaction mixture contained 40 mM of KPB buffer (pH 7), 5 mM of PMS, 6 mM of glucose, and 0.116 mg of crude membrane protein. The reaction was carried out at 25°C for 5 min then terminated at 65°C for 10 min. The relative enzyme activity of mGDH was calculated by measuring the content of gluconic acid produced by 5 min per 1 mg of the crude membrane proteins.

2.6 | Analysis of sugars, gluconic acid, and inhibitors

Glucose was measured using the biosensor SBA-40D (Biology Institute, Shandong Academy of Sciences, Jinan, Shandong, China). Gluconic acid was measured by High Performance Liquid

Chromatography (HPLC; LC-20AD, refractive index detector RID-10A; Shimadzu Co., Kyoto, Japan) equipped with a Shodex RSpak JJ50-4D column (Showa Denko Co., Tokyo, Japan) at 40°C with 12 mM $NaHCO_3$ as mobile phase at a flow rate of 0.5 ml/min. The gluconic acid yield was calculated according to the stoichiometric equation in Zhang et al. (2016a).

Furan and phenolic compounds including furfural, HMF, HBA, vanillin, syringaldehyde, and the corresponding alcohols and acids were measured by HPLC (UV/Vis detector SPD-20A; Shimadzu Co.) equipped with a YMC-Pack ODS-A column (YMC Co., Kyoto, Japan; Wang et al., 2016). Acetic acid was determined by HPLC (LC-20AD; Japan) with refractive index detector RID-10A (Shimadzu Co.) fitted with an Aminex HPX-87H column (Bio-Rad Co., Hercules, CA; Zhang et al., 2010).

2.7 | DNA microarray samples preparation and data analysis

DNA microarray samples were prepared by culturing *G. oxydans* DSM 2003 in a 3 L bioreactor (Baoxing Biotech Co., Shanghai, China) containing 1 L seed medium at 30°C, pH 5.5, 2.5 vvm, and 500 rpm to OD_{600} reached 5.0. Then the seed broth was inoculated at 10% (v/v) ratio into 250 ml flask containing 45 ml synthetic fermentation medium with the addition of 1.2 g/L furfural, 1.5 g/L of HMF, 0.8 g/L of HBA, 0.9 g/L of syringaldehyde, or 0.8 g/L of vanillin, respectively at 30°C, 220 rpm. The cells were harvested at 4 hr by centrifugation at 10,000 rpm, 4°C for 10 min, then quenched by liquid nitrogen and stored at −80°C freezer for RNA extraction.

DNA microarrays were conducted in CapitalBio (Beijing, China) by using the genome sequences of *G. oxydans* 621H (based on NCBI GeneBank accession number of CP000009.1) for designing probes. The symmetrical identity is 95.3% between the genome sequence of *G. oxydans* 621H and *G. oxydans* DSM 2003 (based on NCBI GeneBank accession number of AYTY00000000.1). The hybridized process of DNA microarrays and data analysis methods was similar to Yi, Gu, Gao, Liu, and Bao (2015). Differentially expressed genes were selected using an absolute value of fold change ≥2.0. Significant differentially expressed genes were selected using $p \leq .05$ and an absolute value of fold change ≥2.0 as the threshold. The microarrays data have been deposited in NCBI's Gene Expression Omnibus (GEO) database under the GEO series accession number of GSE125739 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125739).

3 | RESULTS AND DISCUSSIONS

3.1 | Glucose oxidation and inhibitor tolerance of *G. oxydans*

Glucose oxidation by the resting cells of *G. oxydans* DSM 2003 was examined in inhibitors-containing corn stover hydrolysates (Figure S1). Under the catalysis of the whole cell after cell harvesting and thoroughly washing, similar glucose consumption rate (2.4 g/L/hr vs. 2.2 g/L/hr) and gluconic acid yield (75.6% vs. 76.1%) were obtained at the varying inhibitor concentrations. Then the glucose oxidation by the resting cells of *G. oxydans* in the presence of each inhibitor was examined (Figure S2). Six typical inhibitory compounds were selected including two furan aldehydes, furfural, and HMF; three phenolic aldehydes, HBA, vanillin, and syringaldehyde, representing three lignin derivatives p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively; and one weak organic acid, acetic acid. Once again the results show that the gluconic acid productivity was essentially not affected by each inhibitor within the experimental range.

The glucose oxidation of *G. oxydans* under the stress of each inhibitor was further examined in fermentations by using the living cells instead of the resting cells, in which the *G. oxydans* seed was inoculated into the inhibitor-containing medium and the cells still kept a constant growth (Figure 2 and Figure S3–S5). Although the cell growth rate of *G. oxydans* was strongly influenced by the inhibitors at high concentration range, the gluconic acid productivity was almost constant at varying inhibitor concentrations unless the lethal inhibitor concentration was reached such as 2 g/L of furfural.

The results suggest that the gluconic acid productivity of *G. oxydans* was almost free of inhibitors disturbance by either the resting cells in catalysis or the living cells in fermentation. To understand the mechanism of strong inhibitor tolerance of glucose oxidation, we examined the enzyme activity and the transcription level of the genes involved in glucose oxidation of *G. oxydans*.

3.2 | Activity of mGDH and transcription levels of glucose oxidation genes in response to inhibitors

mGDH is the only enzyme with glucose oxidation activity in the membrane proteins of *G. oxydans* (Peters et al., 2013). We extracted the crude membrane proteins of *G. oxydans* and examined the mGDH activity in the crude enzymes in the presence of each inhibitor (Figure 3). The results show that the mGDH activity maintained relatively constant (less than 10% reduction) even at the maximum inhibitor levels of the experimental range. The only exception occurred for the extra-high syringaldehyde (1 g/L), which led to a 22.3% reduction of the mGDH activity. The robust mGDH activity to the inhibitors should be one of the reasons for the highly efficient glucose oxidation of *G. oxydans* in lignocellulose hydrolysate.

The membrane-bound sugar-oxidizing respiratory chain of *G. oxydans* was shown in Figure 1. PQQ serves as a cofactor for mGDH, the PQQ biosynthetic operon includes the gene cluster *pqqABCDE* and the gene *tldD* (Felder et al., 2000; Holscher & Gorisch, 2006). We analyzed the transcription levels of the PQQ biosynthetic operon, the genes encoding mGDH, and two terminal cytochrome oxidases in the glucose oxidation respiratory chain of *G. oxydans* in the presence of each inhibitor by DNA microarrays (Table S1). The results show that these genes were not significantly differentially regulated by furfural, HMF, HBA, syringaldehyde, or acetic acid, except vanillin. Vanillin strongly suppressed the expression of mGDH with 4.2 folds, although mGDH is generally a constitutive enzyme. On the other hand, vanillin significantly upregulated the transcription level of the two cytochrome *bd* oxidase genes

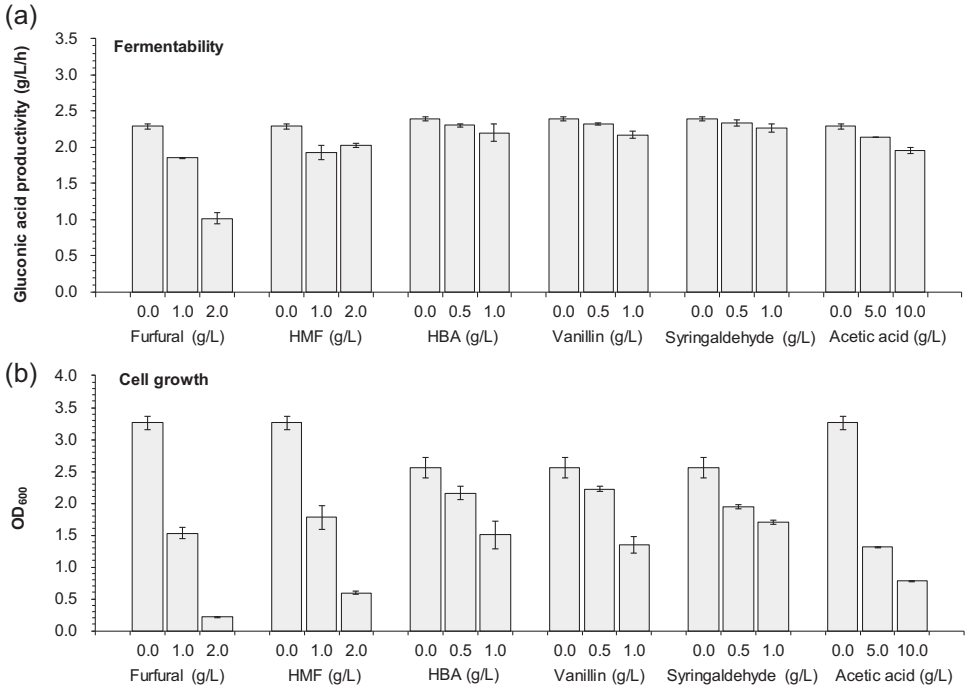

FIGURE 2 Glucose oxidation fermentability and cell growth of *G. oxydans* DSM 2003 in the synthetic medium at different inhibitor contents. (a) Gluconic acid productivity of *G. oxydans* DSM 2003; (b) cell growth of *G. oxydans* DSM 2003. Condition: 30°C, 220 rpm, pH 5–6, 10% (v/v) of inoculum size. The individual inhibitor was added into 50 ml of fermentation medium in a 250 ml flask. Samples were taken at 28 hr for gluconic acid and cell growth determination. Furfural, 2-furaldehyde; HBA, 4-hydroxybenzaldehyde; HMF, 5-(hydroxymethyl)-2-furaldehyde; syringaldehyde, 4-hydroxy-3,5-dimethoxybenzaldehyde; vanillin, 4-hydroxy-3-methoxybenzaldehyde

cydA (5.5 folds) and *cydB* (4.9 folds) and enhanced the PQQ regeneration. The constant gluconic acid productivity in the presence of vanillin (Figures 2a and Figure S5) indicates that the mGDH activity was highly abundant thus the reduced enzyme biosynthesis did not give the obvious repression on gluconic acid oxidation rate. This should be another reason for the highly efficient glucose oxidation of *G. oxydans* in lignocellulose hydrolysate.

3.3 | Inhibitor conversion of *G. oxydans* and the genome mining of the corresponding genes

Rapid conversion of toxic inhibitors into the less toxic metabolites is also considered as an important reason for strong inhibitor tolerance of *G. oxydans* (Buchert & Niemela, 1991; Zhou et al., 2017). Furan and phenolic aldehydes conversions by *G. oxydans* DSM 2003 were

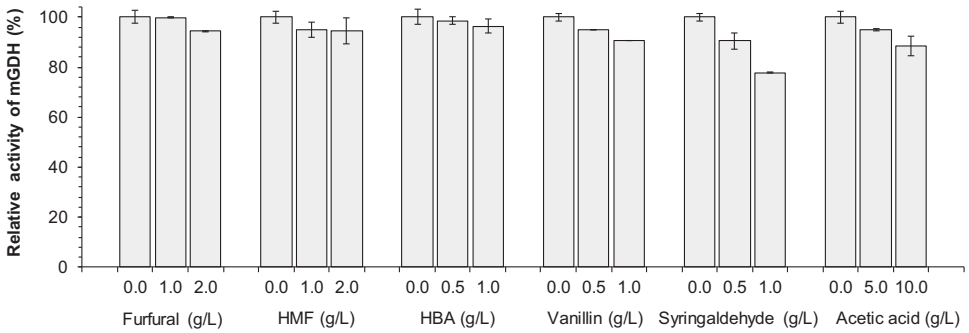

FIGURE 3 Relative activity of membrane-bound glucose dehydrogenase (mGDH) of *G. oxydans* DSM 2003 at different inhibitor contents. Reaction mixture contained 40 mM of KPB buffer (pH 7), 5 mM of PMS, 6 mM of glucose, an 0.116 mg of crude membrane protein. The reaction was carried out at 25°C for 5 min, terminated at 65 °C, 10 min. The activity of mGDH was determined by measuring the gluconic acid produced by 1 mg of the crude membrane proteins in 5 min. The 100% of the relative activity of mGDH was defined as the relative activity of mGDH when no inhibitor was added. The actual activity value of mGDH equal to 100% was 175.1 ± 4.6, 190.6 ± 4.6, 186.8 ± 5.7, 185.0 ± 2.8, 188.4 ± 2.8, or 172.9 ± 4.0 μM/min/mg crude protein without presence of furfural, HMF, HBA, vanillin, syringaldehyde, or acetic acid, respectively. Furfural, 2-furaldehyde; HBA, 4-hydroxybenzaldehyde; HMF, 5-(hydroxymethyl)-2-furaldehyde; syringaldehyde, 4-hydroxy-3,5-dimethoxybenzaldehyde; vanillin, 4-hydroxy-3-methoxybenzaldehyde

examined (Figure 4). The volatilization of these aldehydes could be negligible (Ran, Zhang, Gao, Lin, & Bao, 2014; Yi et al., 2015). Furfural was rapidly converted into furfuryl alcohol and furoic acid by *G. oxydans*, then furfuryl alcohol was decreased and completely converted into furoic acid (Figure 4a). HMF, HBA, and vanillin were also converted into the corresponding alcohols (7–23%) and the acids (77–93%; Figure 4b–d). However, the conversion of the three alcohols to the corresponding acids was not directly observed as in the case of furfuryl alcohol conversion. Syringaldehyde was only partially converted into syringic alcohol with no syringate detection (Figure 4e). As an acetic acid bacterium, *G. oxydans* DSM 2003 was not capable of converting acetic acid to other metabolites, but strongly tolerant to acetic acid (Data not shown). Although the presence of inhibitors delayed the cell growth, the rapid conversion of toxic aldehyde inhibitors into the less toxic alcohols and acids relieved the delayed cell growth of *G. oxydans*. This should also be an

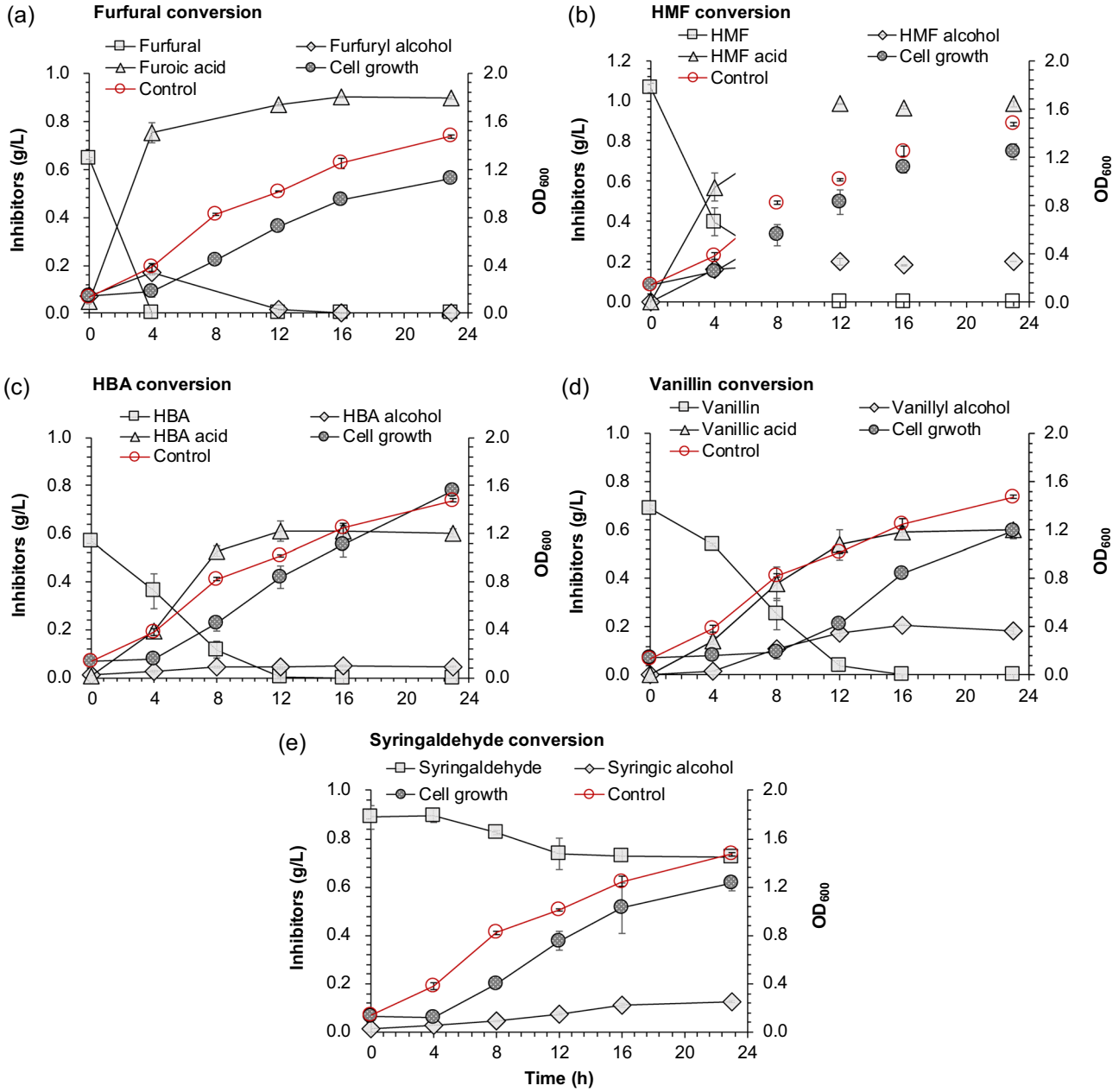

FIGURE 4 Furan and phenolic aldehydes conversions by *G. oxydans* DSM 2003. (a) Furfural; (b) HMF; (c) HBA; (d) vanillin; (e) syringaldehyde. Cell growth: cell growth of *G. oxydans* DSM 2003 with aldehyde addition. Control: cell growth of *G. oxydans* DSM 2003 without aldehyde addition. Synthetic fermentation medium: 80 g of glucose, 20 g of yeast extract, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4, and 1.5 g of $(NH_4)_2SO_4$ per liter of deionized water, and separately addition of the inhibitory compound. Condition: 30°C, 220 rpm, pH 5–6, 10% (v/v) of inoculum size, and 50 ml synthetic fermentation medium containing individual inhibitor in 250 ml flask. Furfural, 2-furaldehyde; HBA, 4-hydroxybenzaldehyde; HMF, 5-(hydroxymethyl)-2-furaldehyde; syringaldehyde, 4-hydroxy-3,5-dimethoxybenzaldehyde; vanillin, 4-hydroxy-3-methoxybenzaldehyde [Color figure can be viewed at wileyonlinelibrary.com]

important reason for the highly efficient glucose oxidation of *G. oxydans*.

The key genes responsible for aldehydes reduction to alcohols or oxidation to acids by *G. oxydans* were mined using the whole genome microarrays in the presence of each inhibitor. The conversion pathways of furan and phenolic aldehydes at molecular level by *G. oxydans* DSM 2003 were illustrated in Figure 5 based on the inhibitor conversion experiments, the transcriptional analysis, and the relevant studies (Jozefczuk et al., 2010; Li, Metthew Lam, & Xun, 2011; Liu, 2011; Wang, Gao, & Bao, 2015). Total 4, 6, 11, 40, and 5 significantly upregulated expression genes with putative functions on aldehydes bioconversion were screened under the stress of furfural, HMF, HBA, vanillin, and syringaldehyde, respectively. Among the upregulated genes, *W826_RS0106015* and *W826_RS0106020*, *W826_RS0110490*, and *W826_RS0110495* are in the purine biosynthetic operon; *W826_RS0102840* and *W826_RS0102845* are in the operon involved in the pentose phosphate pathway. Total nine genes were membrane-bound oxidoreductases according to the genome annotation of *G. oxydans* DSM 2003 (GeneBank accession number AYTY00000000.1) and *G. oxydans* 621H (GeneBank accession number CP000009.1; Prust et al., 2005; Sheng et al., 2014). In addition to four membrane-bound dehydrogenase genes (*W826_RS0102840* encoding glycerol dehydrogenase large subunit; *W826_RS0102845* encoding glycerol dehydrogenase small subunit; *W826_RS0110195* encoding dehydrogenase and *W826_RS0109170* encoding D-lactate dehydrogenase) have been reported (Peters et al., 2013), another five putative oxidoreductase genes (*W826_RS0100105* encoding NADPH: quinone reductase; *W826_RS0105635* encoding NAD [FAD]-dependent dehydrogenase; *W826_RS0110615* encoding NAD-dependent dehydratase; *W826_RS14555* and *W826_RS0110240* encoding oxidoreductase) also encoded membrane-bound proteins. Among the upregulated membrane-bound oxidoreductase genes, *W826_RS0100105*, *W826_RS0102840*, *W826_RS0102845*,

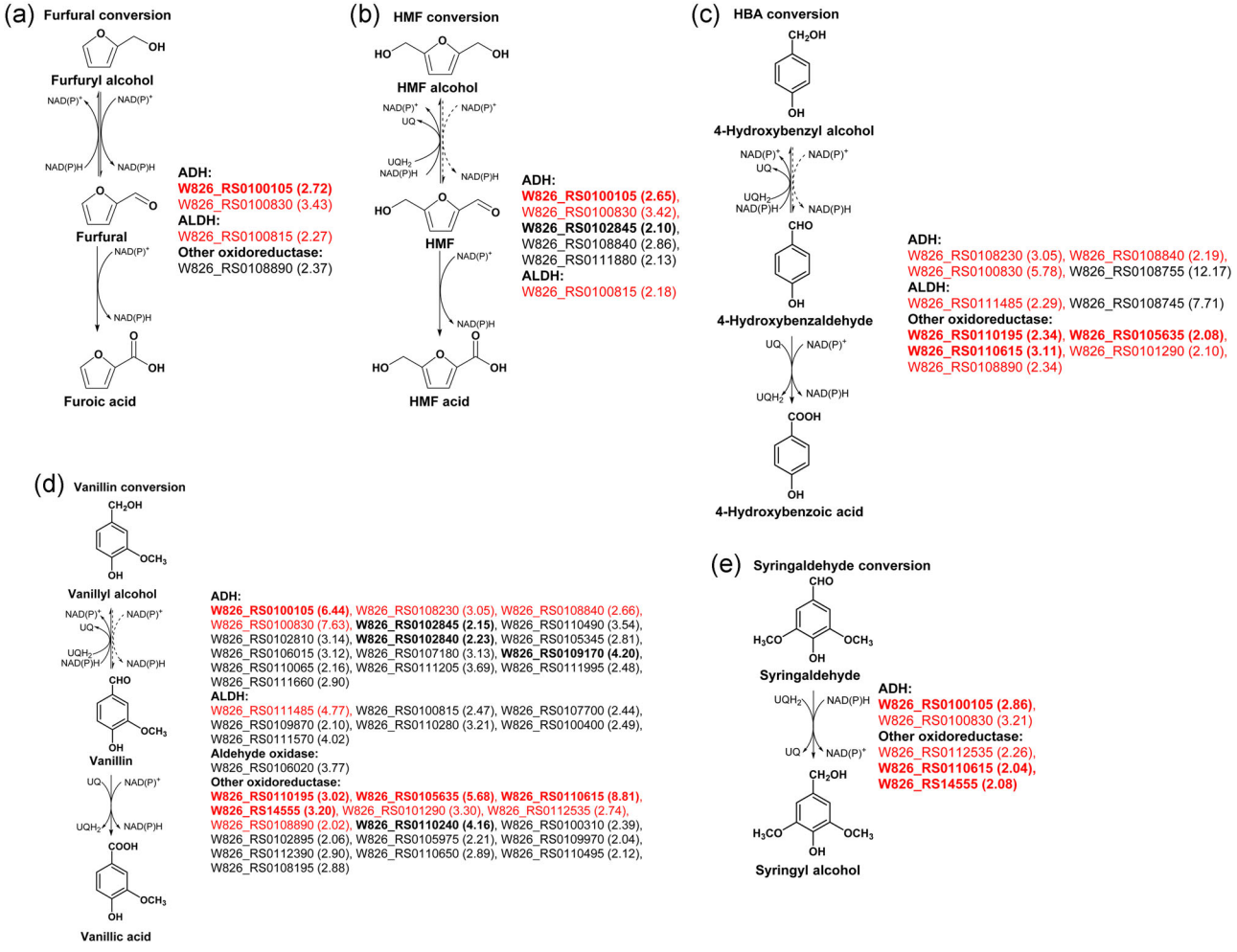

FIGURE 5 Furan and phenolic aldehydes conversion pathways of *G. oxydans* DSM 2003. (a) Furfural conversion; (b) HMF conversion; (c) HBA conversion; (d) vanillin conversion; (e) syringaldehyde conversion. The dashed line represents the hypothetical reaction. Genes in red color, the differentially expressed more than two-fold under two furan aldehydes or at least two phenolic aldehydes; black, the differentially expressed more than two-fold only by the corresponding aldehyde; bold, encoding the membrane-bound proteins. Furfural, 2-furaldehyde; HBA, 4-hydroxybenzaldehyde; HMF, 5-(hydroxymethyl)-2-furaldehyde; syringaldehyde, 4-hydroxy-3,5-dimethoxybenzaldehyde; vanillin, 4-hydroxy-3-methoxybenzaldehyde [Color figure can be viewed at wileyonlinelibrary.com]

W826_RS0109170, *W826_RS0110615*, and *W826_RS14555* were involved in the reduction of furan or phenolic aldehydes to the corresponding alcohols; *W826_RS0110195*, *W826_RS0105635*, and *W826_RS0110240* were responsible for the oxidation of furan or phenolic aldehydes to the corresponding acids. The membrane-bound oxidoreductases might play a more important role than the cytoplasmic oxidoreductases in relieving the toxicity of aldehydes by *G. oxydans*, because the membrane-bound enzymes have the priority of aldehydes conversion before the transport of inhibitors into intracellular space. On the other hand, two ADH genes and one ALDH gene were shared by the two furan aldehydes for their conversion, 12 genes (four ADH genes, one ALDH gene, and seven other oxidoreductase genes) were shared by at least two phenolic aldehydes. These significant oxidoreductase genes responsible for the conversion of single or multiple aldehyde inhibitors could be used as the candidate gene source for metabolic modification or synthetic biology circuits for enhancing inhibitors tolerance of various biorefinery fermentation strains.

This study illustrated a unique and efficient membrane-bound glucose oxidation of *G. oxydans* free of inhibitors disturbance. Comparing with general fermentations by multiple metabolic pathways occurring inside the cytoplasmic space, gluconic acid fermentation of *G. oxydans* is a direct glucose oxidation reaction occurring in the periplasmic space and no energy-consuming transport of glucose substrate into the cells or the products out of the cells was required. Both the strong tolerance of the periplasmic glucose oxidation to the inhibitors and the rapid conversion of inhibitors all strengthened the gluconic acid fermentation. Understanding the mechanisms of *G. oxydans* in periplasmic conversion by membrane-bound enzymes provided an important option for elevating the performance of inhibitors sensitive lignocellulose bioconversions.

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REFERENCES

Adney, B., & Baker, J. (1996). *Measurement of Cellulase Activities. AP-006. NREL. Analytical Procedure*. Golden, CO: National Renewable Energy Laboratory.

Bai, Z., Gao, Z., He, B., & Wu, B. (2015). Effect of lignocellulose-derived inhibitors on the growth and D-lactic acid production of *Sporolactobacillus inulinus* YBS1-5. *Bioprocess & Biosystems Engineering*, *38*(10), 1993–2001. https://doi.org/10.1007/s00449-015-1440-5

Banerjee, N., Bhatnagar, R., & Viswanathan, L. (1981). Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. *European Journal of Applied Microbiology and Biotechnology*, *11*(4), 226–228. https://doi.org/10.1007/BF00505872

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Buchert, J., & Niemela, K. (1991). Oxidative detoxification of wood-derived inhibitors by *Gluconobacter oxydans*. *Journal of Biotechnology*, *18*(1-2), 1–12. https://doi.org/10.1016/0168-1656(91)90231-J

Felder, M., Gupta, A., Verma, V., Kumar, A., Qazi, G. N., & Cullum, J. (2000). The pyrroloquinoline quinone synthesis genes of *Gluconobacter oxydans*. *FEMS Microbiology Letters*, *193*(2), 231–236. https://doi.org/10.1111/j.1574-6968.2000.tb09429.x

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry*, *59*(2), 257–268. https://doi.org/10.1351/pac198759020257

Hanke, T., Noh, K., Noack, S., Polen, T., Bringer, S., Sahm, H., … Bott, M. (2013). Combined fluxomics and transcriptomics analysis of glucose catabolism via a partially cyclic pentose phosphate pathway in *Gluconobacter oxydans* 621H. *Applied and Environmental Microbiology*, *79*(7), 2336–2348. https://doi.org/10.1128/AEM.03414-12

Hawkins, G. M., & Doran-Peterson, J. (2011). A strain of *Saccharomyces cerevisiae* evolved for fermentation of lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. *Biotechnology for Biofuels*, *4*(1), 49. https://doi.org/10.1186/1754-6834-4-49

Holscher, T., & Gorisch, H. (2006). Knockout and overexpression of pyrroloquinoline quinone biosynthetic genes in *Gluconobacter oxydans* 621H. *Journal of Bacteriology*, *188*(21), 7668–7676. https://doi.org/10.1128/JB.01009-06

Hou, W., & Bao, J. (2019). Evaluation of cement retarding performance of cellulosic sugar acids. *Construction and Building Materials*, *202*, 522–527. https://doi.org/10.1016/j.conbuildmat.2019.01.025

Hristozova, T., Angelov, A., Tzvetkova, B., Paskaleva, D., Gotcheva, V., Gargova, S., & Pavlova, K. (2006). Effect of furfural on carbon metabolism key enzymes of lactose-assimilating yeasts. *Enzyme and Microbial Technology*, *39*(5), 1108–1112. https://doi.org/10.1016/j.enzmictec.2006.02.015

Huang, C., Wu, H., Liu, Q. P., Li, Y. Y., & Zong, M. H. (2011). Effects of aldehydes on the growth and lipid accumulation of oleaginous yeast *Trichosporon fermentans*. *Journal of Agricultural & Food Chemistry*, *59*(9), 4606–4613. https://doi.org/10.1021/jf104320b

Jozefczuk, S., Kile, S., Catchpole, G., Szymanski, J., Cuadros-Inostroza, A., Steinhauser, D., … Willmitzer, L. (2010). Metabolomic and transcriptomic stress response of *Escherichia coli*. *Molecular Systems Biology*, *6*, 364–380. https://doi.org/10.1038/msb.2010.18

Klinke, H. B., Thomsen, A. B., & Ahring, B. K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Applied Microbiology and Biotechnology*, *66*(1), 10–26. https://doi.org/10.1007/s00253-004-1642-2

Li, Q., Metthew Lam, L. K., & Xun, L. (2011). *Cupriavidus necator* JMP134 rapidly reduces furfural with a Zn-dependent alcohol dehydrogenase. *Biodegradation*, *22*(6), 1215–1225. https://doi.org/10.1007/s10532-011-9476-y

Liu, G., Zhang, Q., Li, H. X., Qureshi, A. S., Zhang, J., Bao, X. M., & Bao, J. (2018). Dry biorefining maximizes the potentials of simultaneous saccharification and cofermentation for cellulosic ethanol production. *Biotechnology and Bioengineering*, *115*(1), 60–69. https://doi.org/10.1002/bit.26444

Liu, Z. L. (2011). Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. *Applied Microbiology and Biotechnology*, *90*(3), 809–825. https://doi.org/10.1007/s00253-011-3167-9

Matsushita, K., Toyama, H., & Adachi, O. (1994). Respiratory chains and bioenergetics of acetic acid bacteria. *Advances in Microbial Physiology*, *36*(6), 247–301. https://doi.org/10.1016/S0065-2911(08)60181-2

Meyer, M., Schweiger, P., & Deppenmeier, U. (2013). Effects of membrane-bound glucose dehydrogenase overproduction on the respiratory chain of *Gluconobacter oxydans*. *Applied Microbiology & Biotechnology*, *97*(8), 3457–3466. https://doi.org/10.1007/s00253-012-4265-z

Miura, H., Mogi, T., Ano, Y., Migita, C. T., Matsutani, M., Yakushi, T., … Matsushita, K. (2013). Cyanide-insensitive quinol oxidase (CIO) from *Gluconobacter oxydans* is a unique terminal oxidase subfamily of cytochrome bd. *The Journal of Biochemistry*, *153*(6), 535–545. https://doi.org/10.1093/jb/mvt019

Modig, T., Liden, G., & Taherzadeh, M. J. (2002). Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochemical Journal*, *363*(3), 769–776. https://doi.org/10.1042/0264-6021:3630769

Palmqvist, E., Almeida, J. S., & Hahn-Hagerdal, B. (1999). Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. *Biotechnology and Bioengineering*, *62*(4), 447–454. https://doi.org/10.1002/(SICI)1097-0290(19990220)62:4<447::AID-BIT7>3.3.CO;2-S

Peters, B., Mientus, M., Kostner, D., Junker, A., Liebl, W., & Ehrenreich, A. (2013). Characterization of membrane-bound dehydrogenases from *Gluconobacter oxydans* 621H via whole-cell activity assays using multideletion strains. *Applied Microbiology and Biotechnology*, *97*(14), 6397–6412. https://doi.org/10.1007/s00253-013-4824-y

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W. F., Ehrenreich, A., … Deppenmeier, U. (2005). Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nature Biotechnology*, *23*(2), 195–200. https://doi.org/10.1038/nbt1062

Ran, H., Zhang, J., Gao, Q., Lin, Z., & Bao, J. (2014). Analysis of biodegradation performance of furfural and 5-hydroxymethylfurfural by *Amorphotheca resinae* ZN1. *Biotechnology for Biofuels*, *7*(1), 51. https://doi.org/10.1186/1754-6834-7-51

Richhardt, J., Luchterhand, B., Bringer, S., Buchs, J., & Bott, M. (2013). Evidence for a key role of cytochrome bo_3 oxidase in respiratory energy metabolism of *Gluconobacter oxydans*. *Journal of Bacteriology*, *195*(18), 4210–4220. https://doi.org/10.1128/JB.00470-13

Sheng, B. B., Ni, J., Gao, C., Ma, C. Q., & Xu, P. (2014). Draft genome sequence of the *Gluconobacter oxydans* strain DSM 2003, an important biocatalyst for industrial use. *Genome Announcements*, *2*(2), e00417–14. https://doi.org/10.1128/genomeA.00417-14

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). *Determination of sugars, byproducts, and degradation products in liquid fraction process samples. NREL/TP-510-42623*. Golden CO: National Renewable Energy Laboratory.

Wang, J., Gao, Q. Q., Zhang, H., & Bao, J. (2016). Inhibitor degradation and lipid accumulation potentials of oleaginous yeast *Trichosporon cutaneum* using lignocellulose feedstock. *Bioresource Technology*, *218*, 892–901. https://doi.org/10.1016/j.biortech.2016.06.130

Wang, X., Gao, Q. Q., & Bao, J. (2015). Transcriptional analysis of *Amorphotheca resinae* ZN1 on biological degradation of furfural and 5-hydroxymethylfurfural derived from lignocellulose pretreatment. *Biotechnology for Biofuels*, *8*(1), 136. https://doi.org/10.1186/s13068-015-0323-y

Werpy, T., & Petersen, G. (2004). Top value added chemicals from biomass: Volume I—results of screening for potential candidates from sugars and synthesis gas. Office of Scientific and Technical Information (OSTI). *Office of Scientific and Technical Information*, *69*, 1–67. https://doi.org/10.2172/15008859

Xu, H. T., Wang, C., Zhou, Z. H., Chen, Z. J., & Cai, H. (2015). Effects of lignocellulose-derived inhibitors on growth and succinic acid accumulation by *Corynebacterium glutamicum*. *Biotechnology & Bioprocess Engineering*, *20*(4), 744–752. https://doi.org/10.1007/s12257-015-0201-2

Yamada, M., Elias, M. D., Matsushita, K., Migita, C. T., & Adachi, O. (2003). *Escherichia coli* PQQ-containing quinoprotein glucose dehydrogenase: Its structure comparison with other quinoproteins. *Biochimica Et Biophysica Acta-Proteins and Proteomics*, *1647*(1-2), 185–192. https://doi.org/10.1016/S1570-9639(03)00100-6

Yao, R. M., Hou, W. L., & Bao, J. (2017). Complete oxidative conversion of lignocellulose derived nonglucose sugars to sugar acids by *Gluconobacter oxydans*. *Bioresource Technology*, *244*, 1188–1192. https://doi.org/10.1016/j.biortech.2017.08.078

Yi, X., Gu, H. Q., Gao, Q. Q., Liu, Z. L., & Bao, J. (2015). Transcriptome analysis of *Zymomonas mobilis* ZM4 reveals mechanisms of tolerance and detoxification of phenolic aldehyde inhibitors from lignocellulose pretreatment. *Biotechnology for Biofuels*, *8*(1), 153. https://doi.org/10.1186/s13068-015-0333-9

Yi, X., Zhang, P., Sun, J. E., Tu, Y., Gao, Q. Q., Zhang, J., & Bao, J. (2016). Engineering wild-type robust *Pediococcus acidilactici* strain for high titer L- and D-lactic acid production from corn stover feedstock. *Journal of Biotechnology*, *217*, 112–121. https://doi.org/10.1016/j.jbiotec.2015.11.014

Zaldivar, J., Martinez, A., & Ingram, L. O. (1999). Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. *Biotechnology and Bioengineering*, *65*(1), 24–33. https://doi.org/10.1002/(SICI)1097-0290(19991005)65:1<24::AID-BIT4>3.0.CO;2-2

Zhang, H. S., Liu, G., Zhang, J., & Bao, J. (2016a). Fermentative production of high titer gluconic and xylonic acids from corn stover feedstock by *Gluconobacter oxydans* and techno-economic analysis. *Bioresource Technology*, *219*, 123–131. https://doi.org/10.1016/j.biortech.2016.07.068

Zhang, H. S., Zhang, J., & Bao, J. (2016b). High titer gluconic acid fermentation by *Aspergillus niger* from dry dilute acid pretreated corn stover without detoxification. *Bioresource Technology*, *203*, 211–219. https://doi.org/10.1016/j.biortech.2015.12.042

Zhang, J., Wang, X. S., Chu, D. Q., He, Y. Q., & Bao, J. (2011). Dry pretreatment of lignocellulose with extremely low steam and water usage for bioethanol production. *Bioresource Technology*, *102*(6), 4480–4488. https://doi.org/10.1016/j.biortech.2011.01.005

Zhang, J., Zhu, Z. N., Wang, X. F., Wang, N., Wang, W., & Bao, J. (2010). Biodetoxification of toxins generated from lignocellulose pretreatment using a newly isolated fungus, *Amorphotheca resinae* ZN1, and the consequent ethanol fermentation. *Biotechnology for Biofuels*, *3*(1), 26. https://doi.org/10.1186/1754-6834-3-26

Zhou, P. P., Meng, J., & Bao, J. (2017). Fermentative production of high titer citric acid from corn stover feedstock after dry dilute acid pretreatment and biodetoxification. *Bioresources Technology*, *224*, 563–572. https://doi.org/10.1016/j.biortech.2016.11.046

Zhou, X. L., Zhou, X., Xu, Y., & Chen, R. R. (2017). *Gluconobacter oxydans* (ATCC 621H) catalyzed oxidation of furfural for detoxification of furfural and bioproduction of furoic acid. *Journal of Chemical Technology & Biotechnology*, *92*(6), 1285–1289. https://doi.org/10.1002/jctb.5122

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