Low concentrations of furfural facilitate biohydrogen production in dark fermentation using *Enterobacter aerogenes*

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Abstract

Biomass pretreatments represent a necessary route to overcome the natural physico-chemical barriers of recalcitrant feedstocks. However, current biomass pretreatments generally result in generation of various inhibitors (such as furfural derived from pentose), which could inhibit cell growth and decrease biofuel productivity. This study aims to understand the impact of furfural on hydrogen-producing *Enterobacter aerogenes* in dark fermentation of glucose. When adding 5 mM furfural in fermentation, hydrogen yield unexpectedly increased to 193.7 mL/g compared to 163.5 mL/g (in the absence of furfural); and the associated peak hydrogen production rate increased by 126%. This phenomenon from a thermodynamic perspective was due to the fact that furfural at a low concentration contributes to hydrogen production. A higher concentration of furfural (30 mM) significantly decreased hydrogen yield to 109.1 mL/g owing to severe cell membrane damage. The indicator of half-maximal inhibitory concentration was calculated as 32.5 mM. A postulated metabolic responses of *E. aerogenes* to furfural is that the degradation of low concentrations of furfural (5 mM) involved a reduction reaction of furfural to hydrogen and furfuryl alcohol. However, high concentration of furfural (30 mM) caused significant cell disfunction in normal metabolism, causing increased deformation degree in bacterial surface.

Keywords: *Enterobacter aerogenes*; furfural; hydrogen fermentation; IC50.

1. Introduction

The most recent European Union legislation requires that advanced biofuels must achieve greater than 70% greenhouse gas (GHG) emissions savings as compared to the fossil fuel comparator (gCO_2/MJ basis) [1]. Gaseous fuel (in the form of hydrogen or methane) can offer an opportunity to provide a renewable transport fuel whilst achieving great GHG emissions reductions [2, 3]. The use of hydrogen produces only water instead of GHG and other exhaust pollutants. As compared to traditional hydrogen production through energy intensive steam reforming of methane, biological route (such as dark hydrogen fermentation) presents the possibility of being renewable, carbon neutral and less energy intensive [4-6].

Lignocellulosic biomass is the most abundantly available bioresource with a global yield of approximately 1. 3 billion tons per annum [7]. These feedstocks (such as rice straw and switchgrass) are attractive and promising for the production of biofuels. Pretreatment is a key step for efficient conversion of recalcitrant substrates by microorganisms. Biological pretreatment (such as enzymatic hydrolysis) has been categorized as environmentally friendly and exhibits advantages of low energy consumption, mild treatment conditions and does not generate inhibitors. However, pretreatment using enzymes is not always practical due to relative high cost, long pretreatment time and low efficiencies; therefore, application of enzymatic pretreatment may not always be practical. For example, the effect of enzymes (including cellulase and laccase) on enhancing biogas production from anaerobic digestion of manure waste was found minimal [8]. In comparison, chemical pretreatments are typically more efficient and simpler to scale-up. Chemical pretreatments, such as thermal acid hydrolysis and steam explosion, have been developed as effective approaches for biomass decomposition [6, 9-14]. However, these methods inevitably generate several groups of toxic inhibitors, and pose inhibitory effects on fermentation [15, 16]. Three major types of compounds are recognized as potential inhibitors [6, 17]: (1) furan compounds such as furfural and 5-hydroxymethylfurfural (HMF) arising from the dehydration of sugars; (2) phenolic monomers such as syringaldehyde and vanillin originated from lignin depolymerization; and (3) organic acids such as acetic acid derived from the hydrolysis of acetyl groups in hemicellulose. Among these inhibitors, furfural is one of the major inhibitors found in most hydrolysates of lignocellulose (such as corn stover and wheat straw) [18]. Recent studies also found that thermochemical pretreated algal biomass could also result in furfural generation [18, 19]. The produced concentration of furfural in hydrolysate depends on varous factors primarily including for biomass type, and severity of pretreatment (such as reaction time, pH, pressure and temperature). Gonzales et al. investigated the effect of pretreatment severity on hydrolysis of rice husk, which was treated in 5% (v/v) H_2SO_4 at 10% (w/v) solid/liquid ratio and 121 °C for 30, 60, and 90 min [10].

The authors found that with pretreatment time increased from 30 min to 90 min, the concentration of produced furfural increased significantly from 1.11 g/L to 6.41 g/L [10]. Furans are predominately generated at low pH during thermo-acid pretreatment, while a high pH normally favors lignin degradation, resulting in the formation of lignin monomers [18]. The presence of high concentration of furfural in fermentation typically led to decreased hydrogen production due to its inhibition effect on key cellular enzymes [18, 20].

Furfural has been reported as a potent inhibitor to glycolytic enzymes in various microorganisms, such as ethanologenic yeast *Saccharomyces cerevisiae* and hydrogen-producing bacteria *Thermotoga neapolitana*. In *Saccharomyces cerevisiae*, furfural could cause an accumulation of reactive oxygen species (such as H_2O_2, O_2^- and OH^-) and cellular damage to mitochondria, vacuoles, actin, and nuclear chromatin [21]. The damage was less severe with furfural concentration of 25 mM as compared to that with 50 mM, which completely prevented cell growth [21]. In *Candida tropicalis*, cell growth was inhibited by furfural during xylitol fermentation [22]. For hydrogen fermentation, the respective addition of 1 g/L of furfural and HMF resulted in a decrease in hydrogen yield by 69% and 76% compared to the control [23]. *Clostridium beijerinkii* was found to be more resistant to the inhibitors, making it an ideal candidate for hydrogen production in the presence of inhibitors [23]. Cao et al. investigated the effect of furfural on *Thermoanaerobacterium thermosaccharolyticum* W16 and found a decreased hydrogen production by 50% with 1.0 g/L furfural addition [24]. Siqueira et al. observed that 2.0 g/L furfural led to the total inhibition on hydrogen production using mixed cultures [25]. Furfural and HMF exhibited different inhibition effects on dark fermentation of xylose using mixed consortia; the authors found that furfural (0.1-1 g/L) was completely degraded with no inhibition of hydrogen production, while HMF (0.02-0.19 g/L) was partially degraded exerting strong hydrogen inhibition [26]. Anburajan et al. demonstrated HMF at 2.7 g/L caused the maximum inhibition on hydrogen fermentation of glucose, and the presence of HMF enriched the bacterial abundance of *Lactobacillus* sp. [27]. When galactose was used as substrate, it was found that HMF inhibition was milder to continuous culture than that to batch culture [28]. It can be concluded that the efficiency of hydrogen production in fermentation is highly relate to the nature and concentration of inhibitors; more research is needed to assess the impact of furfural on specific microorganisms during hydrogen fermentation.

Progress has been made in optimizing biomass pretreatment methods to achieve higher sugars yield and lower inhibitors generation. Nonetheless, understanding the inhibitory mechanisms is significant in optimizing the production of biofuels. Knowledge on the inhibitions of furfural can contribute to making the use of lignocelluloses a more efficient way to produce fermentative hydrogen. The present study aims to fill the

research gap in the understanding of the impact of furfural on hydrogen-producing bacteria (*Enterobacter aerogenes*) in dark fermentation of a model carbon source (glucose), through comprehensive assessment of the hydrogen production, substrate consumption, metabolic products formation and bacterial morphology. *E. aerogenes* is a promising hydrogen-producing strain for practical hydrogen production owing to several advantageous merits, such as its rapid growth rate, strong adaptability to severe environmental parameters (such as pH, hydrogen partial pressure and dissolved oxygen), and ability to metabolize a wide range of substrates, [29-31]. The specific objectives of this study are to:

(1) Evaluate the hydrogen production in the presence of different concentrations of furfural;

(2) Identify the half-maximal inhibitory concentration of furfural in dark fermentation using *E. aerogenes*;

(3) Propose the metabolism responses of *E. aerogenes* to furfural in dark fermentation.

2. Materials and Methods

2.1. Microorganism and materials

Wild strain *E. aerogenes* ATCC13408 used as seed inoculum was obtained from China General Microbiological Culture Collection Center. The Luria–Bertani medium (LB) was used for bacteria cultivation. The compositions of LB medium consisted of (per liter) 5 g yeast extract, 10 g peptone, and 10 g NaCl. All the chemicals and materials used were purchased from Sigma-Aldrich (USA) and Sinopharm Chemical Reagent Co., Ltd (China).

2.2. Hydrogen fermentation

The 1 mL seed inoculum of *E. aerogenes* ATCC13408 was cultured with 100 mL of LB medium. The cultivations were conducted aerobically in an incubator shaker (working volume 90 L) at a speed of 250 rpm under 37 °C for 12 h to allow the cells for exponential growth. The 100 mL bacteria cultures were then transferred into glass bottles (working volume 417 mL). Afterwards, 200 mL fermentative medium (containing 198 mL deionized water, 3 g glucose, 0.6 g yeast extract, 1.2 g peptone, 0.03 g $FeCl_2$, 1 ml microelement liquid and 1 mL vitamin liquid) were added into the glass bottles. The total liquid solution in the glass bottles was approximately 300 mL for dark fermentation. The initial pH for dark hydrogen fermentation was adjusted to 6.0 ± 0.1. A previous study from the authors demonstrated that 15 mM of furfural induced a significant 28.9% decrease in peak hydrogen production in dark fermentation as compared to that in the absence of furfural [20]. A higher furfural addition of 20.8 mM resulted in a total inhibition of hydrogen production using mixed cultures

[25]. Based on the above, the range of furfural concentration from 0 to 30 mM was selected to investigate the impact of furfural on hydrogen fermentation using *E. aerogenes*. In the experiments, different dosages of furfural were added to each bottle to set the concentrations as 0, 5, 10, 20 and 30 mM during fermentation. The bottles were then purged with nitrogen gas for 5 min, sealed with rubber stoppers, and kept at 37 ± 1.0 °C to initiate fermentation. Sterile conditions were maintained by autoclavation at 100 °C for 30 min. All the experiments were conducted in duplicate.

2.3. Analytical methods

Gaseous products including hydrogen and CO_2 during fermentation were measured using a gas chromatography (GC; Agilent 7820A, USA). The GC system is equipped with a 5A column (Φ 3 mm × 3 m; Agilent, USA) and a thermal conductivity detector [30]. The soluble metabolic products (SMP) in the fermentation solution were quantified using another GC equipped with a DB-FFAP column (Φ 0.32 mm × 50 m; Agilent, USA) and a flame ionization detector [30]. The concentration of glucose was determined using a high-performance liquid chromatography (HPLC; Agilent 1200, USA) equipped with an Aminex HPX-87H column (Bio-Rad, USA) and a refractive index detector. The surface morphologies of *E. aerogenes* ATCC13408 cells collected after hydrogen fermentation were examined by a Field-Emission Scanning Electron Microscopy (FE-SEM SU8010, Japan) with an accelerating voltage of 30 kV.

2.4. Calculations

Hydrogen potential kinetics: The modified Gompertz equation (Eq. 1) was employed to simulate the hydrogen yield. The derived kinetic parameters (H_m, maximum hydrogen potential, mL/g; R_m, peak hydrogen production rate, mL/g/h; and λ, lag-phase time of hydrogen production, h) were calculated using Origin software.

$$H = H_m \exp\left\{-\exp\left[\frac{R_m e}{H_m}(\lambda - t) + 1\right]\right\} \quad \text{(Eq. 1)}$$

***Half-maximal inhibitory concentration analysis*:** The relative H_m for the experiments associated with different furfural concentrations were obtained according to Eq. 2:

$$\text{Relative } H_m = \frac{H_{m\ \text{Furfural}}}{H_{m\ \text{Control}}} \quad \text{(Eq. 2)}$$

in which $H_{m\ \text{Furfural}}$ was derived from the Gompertz model for the experiments with different concentrations of furfural, whereas $H_{m\ \text{Control}}$ was the value achieved in the absence of furfural. These data related the relative hydrogen production to the concentration of furfural. The results were then fitted to an extended Monod kinetics

for inhibition as shown in Eq. 3 [32].

$$H_m = H_{m\ Control} \times \left(1 - \frac{IC}{IC100}\right)^n \quad \text{(Eq. 3)}$$

where IC is inhibitor concentration (mM), IC100 is the lethal inhibitor concentration beyond which the reaction cannot proceed (mM), and n is a constant. IC50, the half-maximal inhibitory concentration, was determined based on Eq. 4.

$$IC_{50} = (1 - 0.5^{\frac{1}{n}}) \times IC100 \quad \text{(Eq. 4)}$$

Bacterial fractal dimension calculation: The changes of bacterial surface roughness were quantified by calculating the bacterial fractal dimension. The concept of fractal geometry was first proposed to study the irregular geometrical morphology [33]. Fractal dimension reflects the irregular degree of complex objects and is a parameter of the effectiveness of space occupation by complex objects. It can be determined by counting the minimum number of small cubes required for covering this irregular object [34]. The fractal dimension was calculated based on Eq. 5 after the SEM images of bacterial cells were binarized using MATLAB software.

$$d = \lim_{\varepsilon \to 0}[\log N(\varepsilon)/\log(1/\varepsilon)] \quad \text{(Eq. 5)}$$

where d is the fractal dimension, N is the number of grids covering the fractal and ε denotes the length of a grid side [35].

3. Results and discussion

3.1. Hydrogen production from glucose during dark fermentation in the presence of furfural

Fig. 1 presents the results of hydrogen production from glucose in the presence of different concentrations of furfural ranging from 0 to 30 mM. As shown in Fig. 1a, the hydrogen yield from glucose in the absence of furfural was found as 163.5 mL/g after fermentation of 72 h. When adding 5 mM and 10 mM furfural in fermentation, the hydrogen yield significantly increased to 193.7 mL/g and 177.5 mL/g, respectively. The presence of 5 mM furfural boosted peak hydrogen production rate to the highest value of 10.4 mL/g/h as compared to a value of 4.6 mL/g/h in the absence of furfural, as shown in Fig. 1b. This is an interesting phenomenon because furfural has been traditionally considered a potent biological inhibitor in fermentation processes (such as hydrogen fermentation and ethanol fermentation) [17, 18]. A previous study showed that the presence of 15 mM furfural slightly decreased hydrogen production by 2.3% when using anaerobic sludge as inoculum, but significantly prolonged fermentation lag-phase time by 94.9% [20]. This suggested that 15 mM

197 furfural was not the lethal concentration to the microorganism in the sludge for hydrogen fermentation [20].
198 Further increasing inhibitor concentration, hydrogen fermentation was severely inhibited [24, 36]. In the present
199 study, when increasing furfural concentration to 20 mM, the hydrogen yield decreased to 121.4 mL/g;
200 equivalent to a decrease of 25.7% compared to no furfural addition. Further increasing furfural concentration to
201 30 mM resulted in a hydrogen yield of only 109.1 mL/g; equivalent to a decrease of 33.3% compared to no
202 furfural addition. The inhibition effect was previously ascribed to the fact that the degradation of inhibitors
203 required a significant consumption of reductive power in the form of reduced nicotinamide adenine dinucleotide
204 (NADH) generated in glycolysis [20]. The reductive capability of fermentative bacteria was a key driving force
205 to convert protons to hydrogen in fermentation [37]. The consumption of reductive power thereby led to
206 decreased hydrogen production.
207 The kinetic parameters of the hydrogen production curves were calculated by fitting the data to the
208 modified Gompertz model (Table 1). The correlation coefficient (R^2) values of all groups were higher than 0.98,
209 suggesting a satisfactory match with the experimental data. A hydrogen potential of 179.1 mL/g and peak
210 hydrogen production rate of 4.2 mL/g were observed in the absence of furfural. In a similar trend with
211 experimental data, the addition of 5 mM and 10 mM furfural significantly enhanced hydrogen potential and
212 peak hydrogen production rate. The inhibition effects on fermentation in the presence of high concentrations of
213 furfural (20 mM and 30 mM) were observed in terms of hydrogen potential, lag-phase time, and peak
214 production time. These results were consistent with the experimental data.
215 Fig. 2 shows the effects of furfural concentration on the relative hydrogen yield from glucose. The
216 correlations between relative hydrogen yield and furfural concentration fitted well with the model ($R^2 = 0.8693$).
217 The estimations of IC50 and IC100 were derived using the extended Monod kinetics model (Eq. 3 and 4). IC50
218 (the half-maximal inhibitory concentration) was calculated using the hydrogen potential obtained from the
219 modified Gompertz model at each furfural concentration. The simulation results showed that the values of IC50
220 and IC100 were 32.5 mM and 45.2 mM, respectively. Table 2 summarizes the values of IC50 in hydrogen
221 fermentation using different hydrogen-producing bacteria [25, 38, 39]. It shows that the IC50 values of furfural
222 in hydrogen fermentation varied significantly in literature ranging from 1.4 mM to 41.6 mM. For example,
223 Siqueira et al. found that 6.5 mM furfural inhibited 50% of hydrogen production from glucose in mesophilic
224 condition [25], whereas a relatively higher IC50 value of 10.7 mM was observed by using mixed sugars as the
225 substrate [39]. Under thermophilic condition, the IC50 of 5.2 mM was observed [39]. This result was likely due
226 to the fact that mesophilic and thermophilic fermentation systems had different microbial communities, leading

to different levels of tolerance to inhibitor. These results suggest that the inhibitory concentration is associated with various factors, such as the type of microorganism, fermentation temperature, and whether there is a synergy effect with other compounds [18].

3.2. Glucose consumption during dark fermentation in the presence of furfural

Fig. 3 presents the consumption of glucose during dark fermentation with different furfural additions. As shown in Fig. 3a, the overall degradation efficiency of glucose in all conditions reached 100% after fermentation of 36−72 h regardless of furfural concentrations. The addition of 5 mM furfural resulted in a complete glucose consumption within 36 h approximately. The presence of low concentrations of furfural enabled a much quicker glucose consumption process throughout fermentation. The addition of 5 mM furfural led to the highest glucose consumption rate of 0.40 g/L/h as compared to 0.37 g/L/h without furfural addition. In comparison, the presence of 30 mM furfural significantly prolonged the consumption of glucose to 72 h with a decreased peak consumption rate of 0.37 g/L/h. This result is similar to a previous study, in which the presence of furfural adversely affected the bioconversion of glucose in hydrogen fermentation [20]. The authors found that the consumption of glucose by mixed bacteria decreased from 96.9% to 79.5% in 48 h [20].

3.3. Soluble metabolic products formation during dark fermentation in the presence of furfural

Fig. 4 presents the effect of furfural concentration on SMP distribution after dark fermentation. Hydrogen production via dark fermentation always accompanies with diverse SMP production, such as acetic acid and butyric acid. Previous research has summarized the metabolic pathways of glucose in dark hydrogen fermentation [40, 41]. The metabolic pathways via ethanol, acetate and butyrate are shown in Eq. 6, 7 and 8 as follows.

Ethanol pathway: $C_6H_{12}O_6 + 2H_2O = 2CH_3CH_2OH + 2H^+ + 2HCO_3^-$, $\Delta G'_0 = -225.3$ kJ/mol (Eq. 6)

Acetate pathway: $C_6H_{12}O_6 + 4H_2O = 2CH_3COO^- + 4H^+ + 2HCO_3^- + 4H_2$, $\Delta G'_0 = -215.7$ kJ/mol (Eq. 7)

Butyrate pathway: $C_6H_{12}O_6 + 2H_2O = CH_3CH_2CH_2COO^- + 3H^+ + 2HCO_3^- + 2H_2$, $\Delta G'_0 = -254.8$ kJ/mol (Eq. 8)

During fermentation, glucose is converted by bacteria into pyruvate coupled with NADH generation. NADH in the intracellular system is associated with the further reaction which reduces protons into hydrogen. 1 mol glucose can metabolically produce 4 mol NADH via the acetate pathway [42]. Therefore, 1 mol glucose can maximumly produce 4 mol hydrogen (Eq. 7). However, the production of other SMP (such as ethanol and

butyrate) requires the consumption of NADH, leading to the decrease in hydrogen yield (Eq. 6 and 8). SMP analysis revealed that ethanol, acetate and butyrate were the major components, comprising over 85% in all groups as shown in Fig. 4. The control group without furfural addition yielded the SMP of 98.3 mM, of which ethanol, acetate and butyrate comprised 85.6% of total SMP. The addition of 5 mL furfural increased total SMP to 100.9 mM, of which ethanol, acetate and butyrate comprised 90.6% of total SMP. This suggests that the increased hydrogen production stimulated by furfural did not significantly affect the metabolic pathways during fermentation. The overdose of furfural (30 mM), however, greatly reduced SMP to 69.7 mM, due to the inhibition effect. This is in agreement with the decreased hydrogen yield in the presence of high concentrations of furfural. Monlau et al. reported that the presence of high concentrations of inhibitors resulted in no hydrogen production; and this was accompanied with a shift from hydrogen-producing pathways (such as acetate and butyrate) to non-hydrogen-producing pathways (such as lactate and ethanol) [43]. This was also concomitant with a microbial population shift from *Clostridium* sp. to *Sporolactobacillus* sp. [43].

3.4. Effect of furfural on bacterial morphology of *Enterobacter aerogenes*

Fig. 5 shows the cell morphology of *E. aerogenes* ATCC13408 in response to different levels of furfural addition. The original bacterial cells exhibited a smooth and integrated cell membrane (Fig. 5a). In the presence of 5 mM furfural in fermentation, the surface of bacterial cells remained integrated, but a large amount of "extracellular dots" appeared on the surface of the cell membrane (Fig. 5b). This was presumably due to the stress response of bacteria to furfural, which can attack the hydrophobic sites of the cell membrane. When 30 mM furfural was added in fermentation, the structure of the cell membrane system was significantly damaged (Fig. 5c), thereby leading to abnormal microbial function during fermentation. To further quantify these differences in cell surface morphology, the concept of fractal dimension was introduced and calculated (Fig. 5d). With furfural concentration increased from 0 to 5 mM, the fractal dimension of bacterial cells increased from 1.21 to 1.38. When furfural concentration increased to 30 mM, the fractal dimension of bacterial cells was 1.54. This result suggested that a high concentration of furfural resulted in the increase of deformation and corrugation degree of cells. It has been found that ethanologenic cells were sensitive to furfural, and it could lead to membrane leakage of *Escherichia coli* [44]. Huang et al. studied the influence of aldehyde inhibitors (such as furfural and HMF) on the cell membrane integrity of oleaginous yeast, and found that the cell membrane integrity was well related to the hydrophobicity property of inhibitors [45]. These inhibitors could effectively attack hydrophobic sites of the cell membrane, leading to further disintegration.

3.5. Proposed response mechanism of *E. aerogenes* to furfural in dark fermentation

Hydrogen is produced from glucose by *E. aerogenes* ATCC13408 mainly via two pathways [41]: formate pathway and NADH pathway. In the formate pathway, formate is readily converted to hydrogen gas by formate hydrogen lyase (FHL); in the NADH pathway, excess intracellular NADH produced is involved in hydrogen production catalyzed by hydrogenase [41]. Fig. 6 provides the proposed metabolic responses of *E. aerogenes* ATCC13408 to furfural addition in dark fermentation. When glucose is used as the substrate in fermentation, it is first converted to pyruvate with the release of NADH [42]. Pyruvate can be further transformed to acetylcoenzyme A (acetyl-CoA), CO_2 and hydrogen [42]. Acetyl-CoA is finally converted to different soluble metabolites such as ethanol, acetate and butyrate [42]. Some NADH are consumed by the generation of ethanol and butyrate, while the excess NADH can be converted to hydrogen with re-generation of NAD^+ ($NADH + H^+ = NAD^+ + H_2$) [46]. When a low concentration of furfural (5 mM) was added in fermentation, it could be transported through the cell membrane into intracellular metabolism. The degradation of furfural in fermentation involved a reduction reaction, which converted furfural to furfuryl alcohol [11]. Haroun et al. suggested the thermodynamic reaction of furfural degradation to acetate at a very low concentration of furfural (Eq. 9) [47]. From a thermodynamic perspective, furfural can be degraded into acetate and hydrogen in the acidogenic phase of fermentation. This provides the theoretical evidence that explains why hydrogen production increased in the presence of a small amount of furfural.

$$C_5H_4O_2 + 6H_2O = CH_3COO^- + 2H^+ + 2HCO_3^- + 6H_2,\ \Delta G'_0 = -152.0\ kJ/mol \quad \text{(Eq. 9)}$$

The high concentration of furfural (30 mM) caused a significant cell membrane damage and the disfunction of normal metabolism, thus leading to decreased hydrogen production. It was reported that furfuran derivatives could cause detrimental effects on microbial cells by inducing DNA damage, inhibiting cell growth and inhibiting key cellular enzymes [20]. The detoxification of aldehyde inhibitors by microorganisms was attributed to the reduction activities of multiple enzymes coupled with $NADH/NAD^+$ [48, 49]. Lin et al. proposed that the reduction of aldehyde inhibitors (such as furfural and vanillin) in hydrogen fermentation required the consumption of NADH ($R\text{-}CHO + 2NADH = R\text{-}CH_2OH + 2NAD^+$) [20], and further experiments demonstrated that adding reducing chemical (such as sodium borohydride) in fermentation could ease the inhibition effect and recover the most of hydrogen potential [50]. Wang et al. reported that the ratio of $NADH/NAD^+$ in yeast *Candida tropicalis* decreased significantly with furfural addition; this indicated that the reduction of furfural in fermentation consumed NADH and adversely affected the redox balance [51]. Another

study demonstrated that xylitol accumulation from xylose decreased with furfural addition, due to the fact that NADH was oxidized to NAD^+ in the reduction of furfural to furfuryl alcohol [52].

4. Conclusions

This study found that low concentrations (5 mM and 10 mM) of furfural could facilitate hydrogen production from glucose using *E. aerogenes* ATCC13408. In a thermodynamic view, this phenomenon was presumably due to the bioconversion of low concentration of furfural to hydrogen. However, a high concentration of furfural (30 mM) significantly decreased hydrogen yield because of severe cell membrane damages. The fractal dimension calculation suggested the increase of deformation degree of cells with increasing furfural concentrations. The half-maximal inhibitory concentration was obtained as 32.5 mM, which serves as an indicator of inhibition levels in fermentation. The findings from this study can advance the understanding of furfural's impact on hydrogen fermentation, and contribute to effective optimization of biomass pretreatment.

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References

1. *European Commission. Proposal for a DIRECTIVE OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on the promotion of the use of energy from renewable sources (recast) 2017; 382.* European Commission.
2. Lin, R., Cheng, J., Ding, L., and Murphy, J.D., *Improved efficiency of anaerobic digestion through direct interspecies electron transfer at mesophilic and thermophilic temperature ranges.* Chemical

Engineering Journal, 2018. **350**: 681-691.

3. Bhatia, S.K., Kim, S.-H., Yoon, J.-J., and Yang, Y.-H., *Current status and strategies for second generation biofuel production using microbial systems.* Energy Conversion and Management, 2017. **148**: 1142-1156.

4. Chaubey, R., Sahu, S., James, O.O., and Maity, S., *A review on development of industrial processes and emerging techniques for production of hydrogen from renewable and sustainable sources.* Renewable and Sustainable Energy Reviews, 2013. **23**: 443-462.

5. Sambusiti, C., Bellucci, M., Zabaniotou, A., Beneduce, L., and Monlau, F., *Algae as promising feedstocks for fermentative biohydrogen production according to a biorefinery approach: A comprehensive review.* Renewable and Sustainable Energy Reviews, 2015. **44**: 20-36.

6. Sivagurunathan, P., Kumar, G., Mudhoo, A., Rene, E.R., Saratale, G.D., Kobayashi, T., Xu, K., Kim, S.-H., and Kim, D.-H., *Fermentative hydrogen production using lignocellulose biomass: An overview of pre-treatment methods, inhibitor effects and detoxification experiences.* Renewable and Sustainable Energy Reviews, 2017. **77**: 28-42.

7. Baruah, J., Nath, B., Sharma, R., Kumar, S., Deka, R., Baruah, D., and Kalita, E., *Recent Trends in the Pretreatment of Lignocellulosic Biomass for Value-Added Products. Front.* Energy Res, 2018. **6**: 141.

8. Bruni, E., Jensen, A.P., and Angelidaki, I., *Comparative study of mechanical, hydrothermal, chemical and enzymatic treatments of digested biofibers to improve biogas production.* Bioresource Technology, 2010. **101**(22): 8713-8717.

9. Chen, H., Rao, Y., Cao, L., Shi, Y., Hao, S., Luo, G., and Zhang, S., *Hydrothermal conversion of sewage sludge: Focusing on the characterization of liquid products and their methane yields.* Chemical Engineering Journal, 2019. **357**: 367-375.

10. Gonzales, R.R., Sivagurunathan, P., and Kim, S.-H., *Effect of severity on dilute acid pretreatment of lignocellulosic biomass and the following hydrogen fermentation.* International Journal of Hydrogen Energy, 2016. **41**(46): 21678-21684.

11. Behera, S., Arora, R., Nandhagopal, N., and Kumar, S., *Importance of chemical pretreatment for bioconversion of lignocellulosic biomass.* Renewable and sustainable energy reviews, 2014. **36**: 91-106.

12. Ruiz, H.A., Rodríguez-Jasso, R.M., Fernandes, B.D., Vicente, A.A., and Teixeira, J.A., *Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: A review.* Renewable and Sustainable Energy Reviews, 2013. **21**: 35-51.

13. Riau, V., De la Rubia, M.A., and Pérez, M., *Upgrading the temperature-phased anaerobic digestion of waste activated sludge by ultrasonic pretreatment.* Chemical Engineering Journal, 2015. **259**: 672-681.
14. Aguilar-Reynosa, A., Romani, A., Rodriguez-Jasso, R.M., Aguilar, C.N., Garrote, G., and Ruiz, H.A., *Microwave heating processing as alternative of pretreatment in second-generation biorefinery: an overview.* Energy Conversion and Management, 2017. **136**: 50-65.
15. Lin, R., Cheng, J., and Murphy, J.D., *Unexpectedly low biohydrogen yields in co-fermentation of acid pretreated cassava residue and swine manure.* Energy Conversion and Management, 2017. **151**(Supplement C): 553-561.
16. Lin, R., Cheng, J., and Murphy, J.D., *Inhibition of thermochemical treatment on biological hydrogen and methane co-production from algae-derived glucose/glycine.* Energy Conversion and Management, 2018. **158**: 201-209.
17. Jönsson, L.J., Alriksson, B., and Nilvebrant, N.-O., *Bioconversion of lignocellulose: inhibitors and detoxification.* Biotechnology for biofuels, 2013. **6**(1): 16.
18. Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.-P., and Carrère, H., *Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review.* Biotechnology advances, 2014. **32**(5): 934-951.
19. Jung, K.-W., Kim, D.-H., and Shin, H.-S., *Fermentative hydrogen production from Laminaria japonica and optimization of thermal pretreatment conditions.* Bioresource Technology, 2011. **102**(3): 2745-2750.
20. Lin, R., Cheng, J., Ding, L., Song, W., Zhou, J., and Cen, K., *Inhibitory effects of furan derivatives and phenolic compounds on dark hydrogen fermentation.* Bioresource Technology, 2015. **196**: 250-255.
21. Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., and Gorsich, S.W., *Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae.* Biotechnology for Biofuels, 2010. **3**(1): 2.
22. Wang, L., Tang, P., Fan, X., and Yuan, Q., *Effect of selected aldehydes found in the corncob hemicellulose hydrolysate on the growth and xylitol fermentation of Candida tropicalis.* Biotechnology progress, 2013. **29**(5): 1181-1189.
23. Quéméneur, M., Hamelin, J., Barakat, A., Steyer, J.-P., Carrère, H., and Trably, E., *Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures.* international journal of hydrogen energy, 2012. **37**(4): 3150-3159.

24. Cao, G.-L., Ren, N.-Q., Wang, A.-J., Guo, W.-Q., Xu, J.-F., and Liu, B.-F., *Effect of lignocellulose-derived inhibitors on growth and hydrogen production by Thermoanaerobacterium thermosaccharolyticum W16.* international journal of hydrogen energy, 2010. **35**(24): 13475-13480.
25. Siqueira, M.R. and Reginatto, V., *Inhibition of fermentative H2 production by hydrolysis byproducts of lignocellulosic substrates.* Renewable Energy, 2015. **80**: 109-116.
26. Muñoz-Páez, K.M., Alvarado-Michi, E.L., Buitrón, G., and Valdez-Vazquez, I., *Distinct effects of furfural, hydroxymethylfurfural and its mixtures on dark fermentation hydrogen production and microbial structure of a mixed culture.* International Journal of Hydrogen Energy, 2019. **44**(4): 2289-2297.
27. Anburajan, P., Pugazhendhi, A., Park, J.-H., Kumar, G., Choi, C.-S., and Kim, S.-H., *Inhibitory effect of 5-hydroxymethylfurfural on continuous hydrogen fermentation by mixed culture in a fixed bed reactor.* International Journal of Hydrogen Energy, 2017. **42**(45): 27570-27576.
28. Anburajan, P., Pugazhendhi, A., Park, J.-H., Sivagurunathan, P., Kumar, G., and Kim, S.-H., *Effect of 5-hydroxymethylfurfural (5-HMF) on high-rate continuous biohydrogen production from galactose.* Bioresource Technology, 2018. **247**: 1197-1200.
29. Zhang, C., Lv, F.-X., and Xing, X.-H., *Bioengineering of the Enterobacter aerogenes strain for biohydrogen production.* Bioresource technology, 2011. **102**(18): 8344-8349.
30. Lin, R., Cheng, J., Ding, L., Song, W., Liu, M., Zhou, J., and Cen, K., *Enhanced dark hydrogen fermentation by addition of ferric oxide nanoparticles using Enterobacter aerogenes.* Bioresource Technology, 2016. **207**: 213-219.
31. Batista, A.P., Gouveia, L., and Marques, P.A., *Fermentative hydrogen production from microalgal biomass by a single strain of bacterium Enterobacter aerogenes–Effect of operational conditions and fermentation kinetics.* Renewable Energy, 2018. **119**: 203-209.
32. Han, K. and Levenspiel, O., *Extended Monod kinetics for substrate, product, and cell inhibition.* Biotechnology and bioengineering, 1988. **32**(4): 430-447.
33. Mandelbrot, B.B., *On the geometry of homogeneous turbulence, with stress on the fractal dimension of the iso-surfaces of scalars.* Journal of Fluid Mechanics, 1975. **72**(3): 401-416.
34. Li, J., Du, Q., and Sun, C., *An improved box-counting method for image fractal dimension estimation.* Pattern Recognition, 2009. **42**(11): 2460-2469.
35. Cheng, J., Sun, J., Huang, Y., Zhou, J., and Cen, K., *Fractal microstructure characterization of wet*

microalgal cells disrupted with ultrasonic cavitation for lipid extraction. Bioresource technology, 2014. **170**: 138-143.

36. Veeravalli, S.S., Chaganti, S.R., Lalman, J.A., and Heath, D.D., *Effect of furans and linoleic acid on hydrogen production.* International Journal of Hydrogen Energy, 2013. **38**(28): 12283-12293.

37. Oh, Y.-K., Raj, S.M., Jung, G.Y., and Park, S., *Current status of the metabolic engineering of microorganisms for biohydrogen production.* Bioresource Technology, 2011. **102**(18): 8357-8367.

38. De Vrije, T., Bakker, R.R., Budde, M.A., Lai, M.H., Mars, A.E., and Claassen, P.A., *Efficient hydrogen production from the lignocellulosic energy crop Miscanthus by the extreme thermophilic bacteria Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana.* Biotechnology for biofuels, 2009. **2**(1): 12.

39. Akobi, C., Hafez, H., and Nakhla, G., *Impact of furfural on biological hydrogen production kinetics from synthetic lignocellulosic hydrolysate using mesophilic and thermophilic mixed cultures.* International Journal of Hydrogen Energy, 2017. **42**(17): 12159-12172.

40. Hallenbeck, P.C. and Ghosh, D., *Advances in fermentative biohydrogen production: the way forward?* Trends in biotechnology, 2009. **27**(5): 287-297.

41. Lu, Y., Zhao, H., Zhang, C., and Xing, X.-H., *Insights into the global regulation of anaerobic metabolism for improved biohydrogen production.* Bioresource technology, 2016. **200**: 35-41.

42. Xia, A., Cheng, J., Song, W., Su, H., Ding, L., Lin, R., Lu, H., Liu, J., Zhou, J., and Cen, K., *Fermentative hydrogen production using algal biomass as feedstock.* Renewable and Sustainable Energy Reviews, 2015. **51**: 209-230.

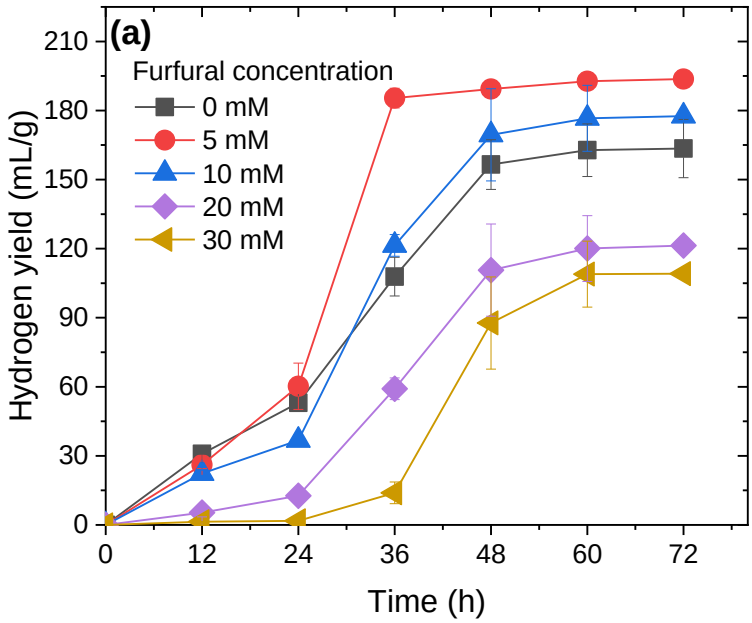
43. Monlau, F., Aemig, Q., Trably, E., Hamelin, J.m., Steyer, J.-P., and Carrere, H., *Specific inhibition of biohydrogen-producing Clostridium sp. after dilute-acid pretreatment of sunflower stalks.* international journal of hydrogen energy, 2013. **38**(28): 12273-12282.

44. Almeida, J.R., Modig, T., Petersson, A., Hähn‐Hägerdal, B., Lidén, G., and Gorwa‐Grauslund, M.F., *Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae.* Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology, 2007. **82**(4): 340-349.

45. Huang, C., Zhu, D.-h., Wu, H., Lou, W.-y., and Zong, M.-h., *Evaluating the influence of inhibitors present in lignocellulosic hydrolysates on the cell membrane integrity of oleaginous yeast Trichosporon fermentans by flow cytometry.* Process Biochemistry, 2014. **49**(3): 395-401.

46. Wang, J. and Wan, W., *Factors influencing fermentative hydrogen production: a review.* International journal of hydrogen energy, 2009. **34**(2): 799-811.
47. Haroun, B.M., Nakhla, G., Hafez, H., and Nasr, F.A., *Impact of furfural on biohydrogen production from glucose and xylose in continuous-flow systems.* Renewable Energy, 2016. **93**: 302-311.
48. Nilsson, A., Gorwa-Grauslund, M.F., Hahn-Hägerdal, B., and Lidén, G., *Cofactor dependence in furan reduction by Saccharomyces cerevisiae in fermentation of acid-hydrolyzed lignocellulose.* Applied and environmental microbiology, 2005. **71**(12): 7866-7871.
49. Liu, Z.L., *Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates.* Applied microbiology and biotechnology, 2011. **90**(3): 809-825.
50. Lin, R., Cheng, J., Ding, L., Song, W., Zhou, J., and Cen, K., *Sodium borohydride removes aldehyde inhibitors for enhancing biohydrogen fermentation.* Bioresource Technology, 2015. **197**: 323-328.
51. Wang, S., He, Z., and Yuan, Q., *Xylose enhances furfural tolerance in Candida tropicalis by improving NADH recycle.* Chemical Engineering Science, 2017. **158**: 37-40.
52. Ask, M., Bettiga, M., Mapelli, V., and Olsson, L., *The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing Saccharomyces cerevisiae.* Biotechnology for biofuels, 2013. **6**(1): 22.

List of Figures and Tables



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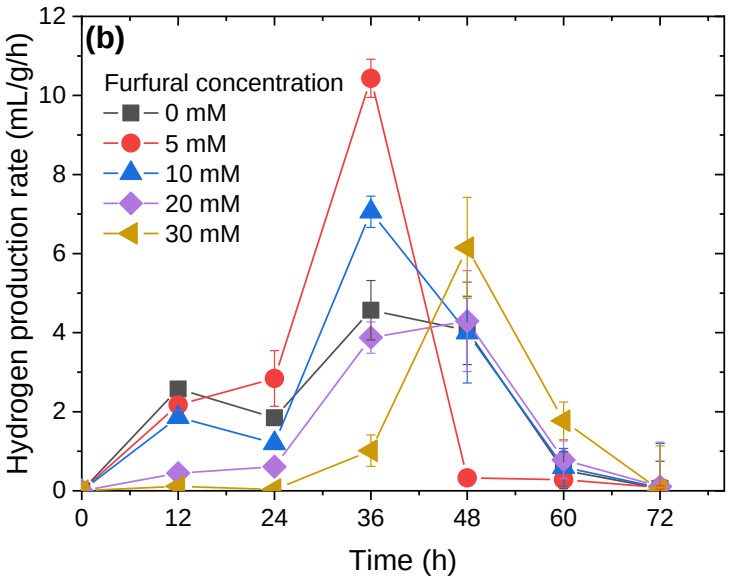
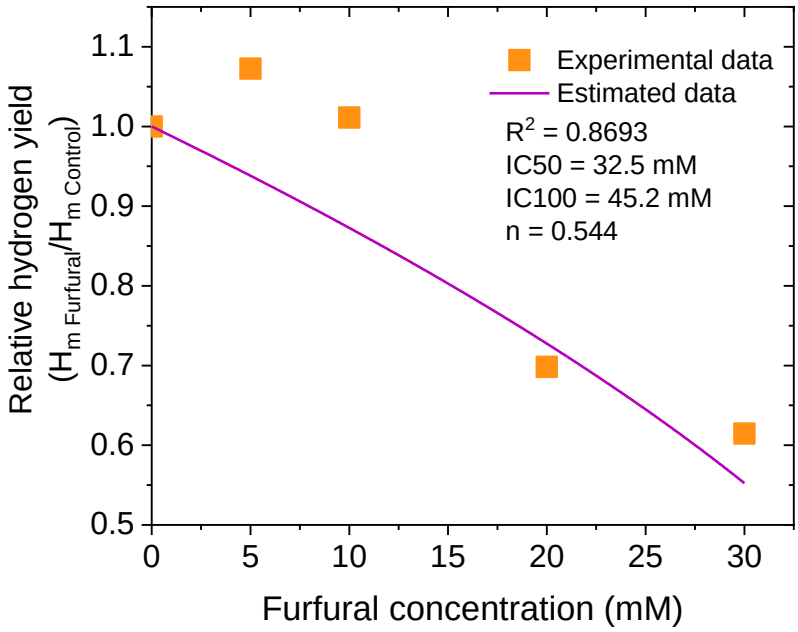


504

505 **Figure 1. Hydrogen production from glucose in the presence of furfural: (a) hydrogen yield, and (b)**

506 **hydrogen production rate.**

507

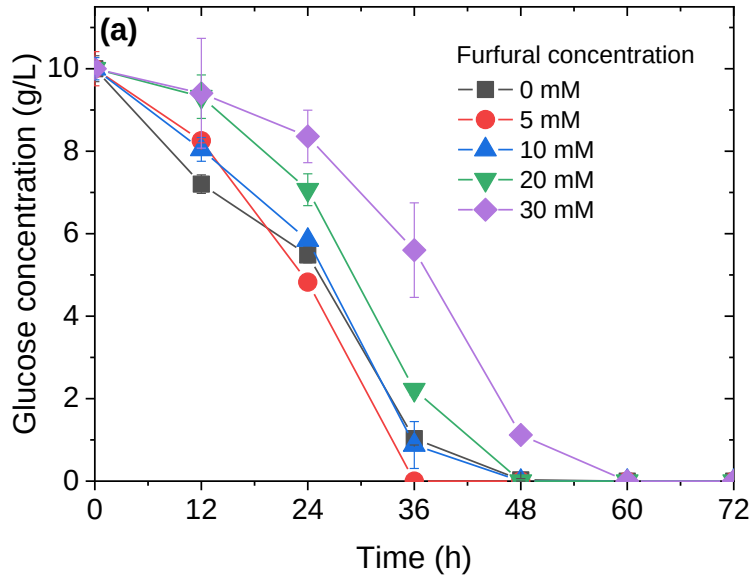


508

509 **Figure 2. Relative hydrogen yield in the presence of furfural. Parameters including IC50, IC100 and n are**

510 **derived from the extended Monod fitting.**

511



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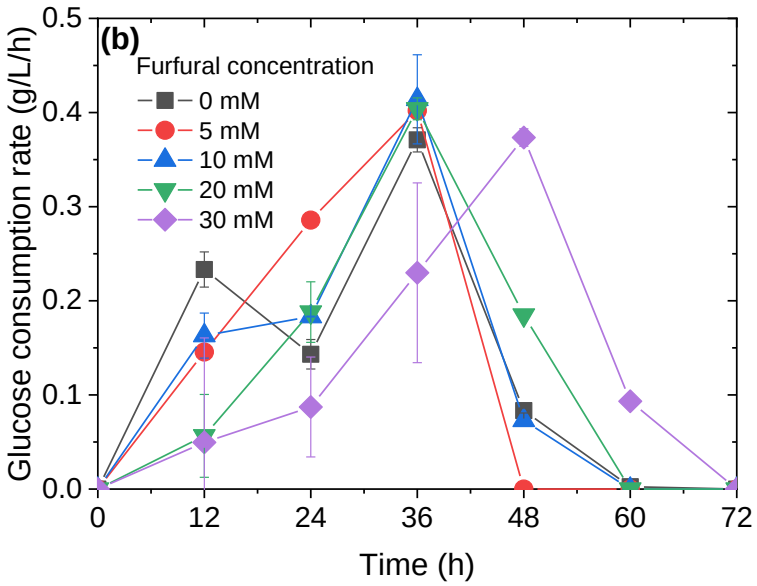
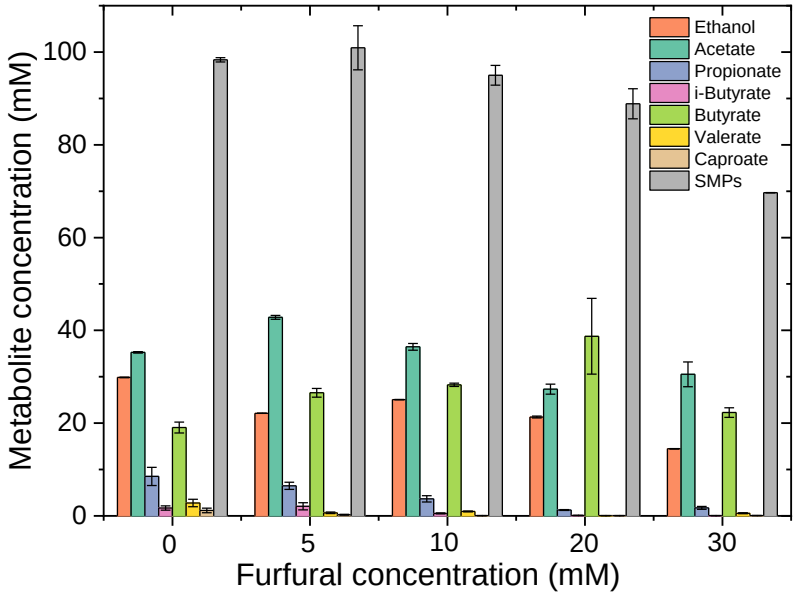


513

514 **Figure 3. Glucose consumption in the presence of furfural: (a) glucose concentration, and (b) glucose**

515 **consumption rate.**

516



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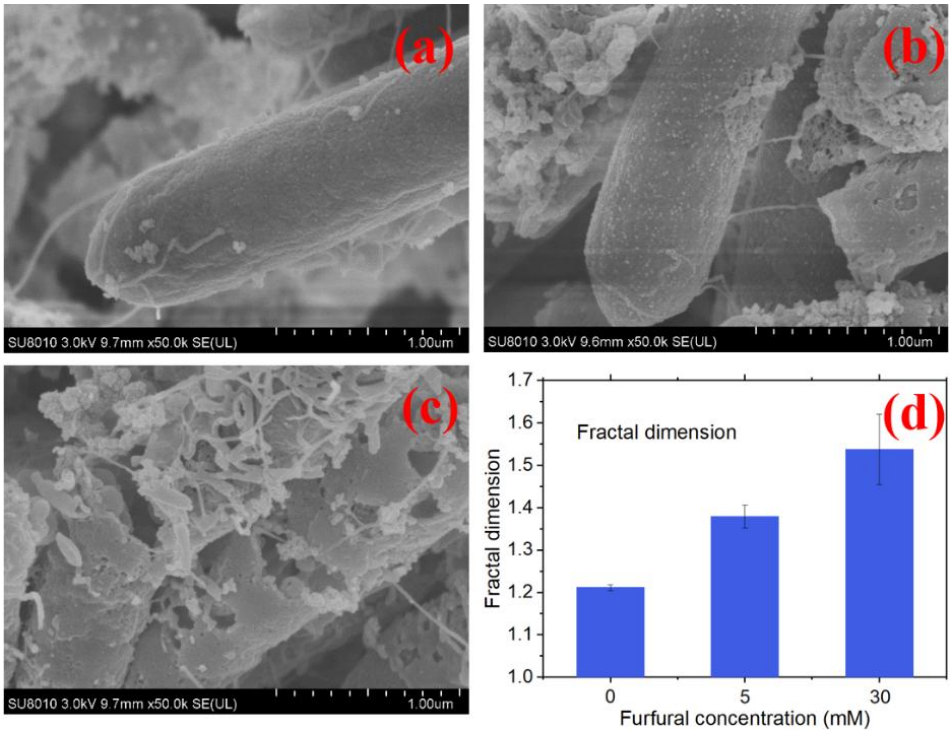
518 **Figure 4. Effects of furfural concentration on the distribution of soluble metabolic products.**

519



520

521 **Figure 5. Effects of furfural concentration on the cell morphology of *Enterobacter aerogenes* after**

522 **hydrogen fermentation: (a) 0 mM furfural ×50k; (b) 5 mM furfural ×50k; (c) 30 mM furfural ×50k; and**

523 **(d) fractal dimension.**

524

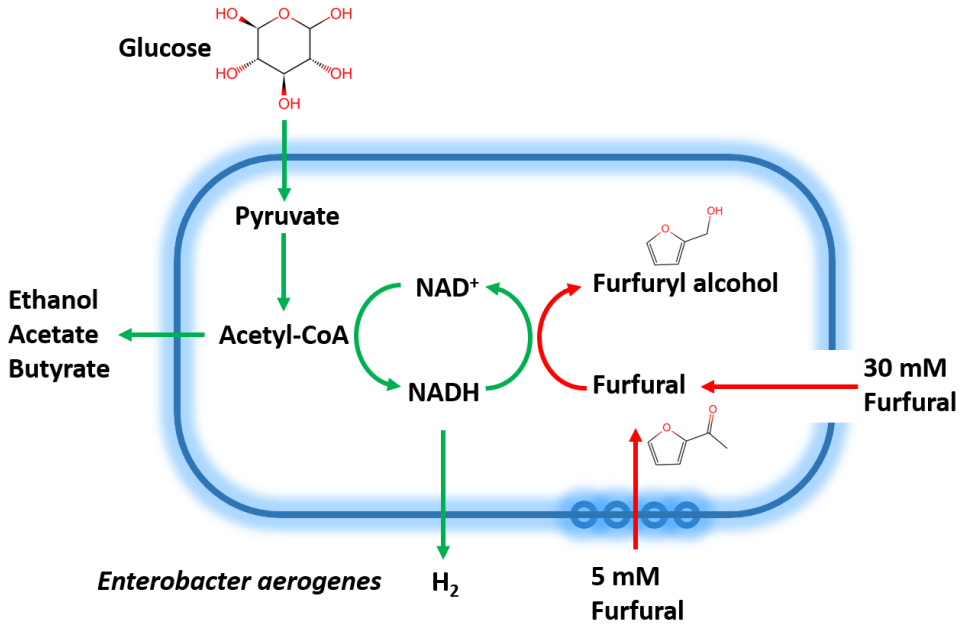


Figure 6. Proposed metabolic responses of *Enterobacter aerogenes* to furfural in dark fermentation. Green lines indicate the metabolic conversion of glucose, and red lines indicate the metabolic conversion of furfural.

530 Table 1 Kinetic parameters of hydrogen production from glucose in the presence of furfural.

Furfural (mM)	H_2 yield (mL/g)	Peak H_2 production rate (mL/g/h)	Kinetic model parameters					SMP [a] (mM)
			H_m (mL/g)	R_m (mL/g/h)	λ (h)	T_m (h)	R^2	
0	163.5 ± 12.7	4.6 ± 0.8	179.1	4.2	8.9	24.5	0.983	69.9±1.16
5	193.7 ± 1.5	10.4 ± 0.5	192.0	20.4	21.0	24.5	0.985	61.1±1.80
10	177.6 ± 0.9	7.1 ± 0.4	181.0	7.9	19.5	27.9	0.986	63.6±0.32
20	121.5 ± 0.9	4.3 ± 1.3	125.6	5.2	23.8	32.6	0.995	58.6±5.36
30	109.1 ± 0.9	6.1 ± 1.3	110.4	7.5	34.5	40.0	0.999	61.1±7.88

531 [a] SMPs = soluble metabolic products.

532

533 Table 2 The half-maximal inhibitory concentration (IC50) of furfural in hydrogen fermentation of sugars.

Substrate	Inoculum	Temp (°C)	IC50 [b] (mM)	Ref.
Glucose	Mixed culture from anaerobic sludge	37	6.5	[25]
Glucose	*Caldicellulosiruptor saccharolyticus*	72	1.4-20.8	[38]
Glucose	*Thermotoga neapolitana*	80	20.8-41.6	[38]
Mixed sugars [a]	Mixed culture from anaerobic sludge	37	10.7	[39]
Mixed sugars [a]	Mixed culture from anaerobic sludge	55	5.2	[39]
Glucose	*Enterobacter aerogenes*	37	32.5	This study

534 [a] Mixed sugars include glucose, xylose, mannose, arabinose and galactose. [a] IC50 = inhibitor concentration, at
535 which 50% inhibition occurs.