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Tolerance of the Nanocellulose-Producing Bacterium *Gluconacetobacter xylinus* to Lignocellulose-Derived Acids and Aldehydes

Shuo Zhang,[†,‡,#] Sandra Winestrand,[§] Lin Chen,[†,‡] Dengxin Li,[#] Leif J. Jönsson,[†,§] and Feng Hong*[,†,‡]

[†]China–Sweden Associated Research Laboratory in Industrial Biotechnology and [‡]Group of Microbiological Engineering and Industrial Biotechnology, College of Chemistry, Chemical Engineering and Biotechnology, and [#]School of Environmental Science and Engineering, Donghua University, Shanghai 201620, China

[§]Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden

ABSTRACT: Lignocellulosic biomass serves as a potential alternative feedstock for production of bacterial nanocellulose (BNC), a high-value-added product of bacteria such as *Gluconacetobacter xylinus*. The tolerance of *G. xylinus* to lignocellulose-derived inhibitors (formic acid, acetic acid, levulinic acid, furfural, and 5-hydroxymethylfurfural) was investigated. Whereas 100 mM formic acid completely suppressed the metabolism of *G. xylinus*, 250 mM of either acetic acid or levulinic acid still allowed glucose metabolism and BNC production to occur. Complete suppression of glucose utilization and BNC production was observed after inclusion of 20 and 30 mM furfural and 5-hydroxymethylfurfural, respectively. The bacterium oxidized furfural and 5-hydroxymethylfurfural to furoic acid and 5-hydroxymethyl-2-furoic acid, respectively. The highest yields observed were 88% for furoic acid/furfural and 76% for 5-hydroxymethyl-2-furoic acid/5-hydroxymethylfurfural. These results are the first demonstration of the capability of *G. xylinus* to tolerate lignocellulose-derived inhibitors and to convert furan aldehydes.

KEYWORDS: *Gluconacetobacter xylinus, bacterial nanocellulose, lignocellulose, aliphatic acids, furan aldehydes, biotransformation*

INTRODUCTION

Bacterial nanocellulose (BNC) is a pure variety of cellulose (free of lignin and hemicellulose), which is a high-value-added extracellular product obtained by fermentation of bacteria such as *Gluconacetobacter xylinus* (previously named *Acetobacter xylinus*). It is a nanostructured biomaterial with unique physicochemical features, such as high surface area, high degree of crystallinity, high degree of polymerization, high tensile strength in wet state, and good biocompatibility.[1] There are potential applications for BNC in many areas including food, textile, paper, pharmaceuticals, wound dressings, tissue engineering, wastewater treatment, speakers, fuel cells, and mining.[1−6]

Carbon sources utilized in fermentation processes for BNC production include monosaccharides (such as glucose and fructose), disaccharides (such as sucrose and maltose), and alcohols (such as ethanol, glycerol, and mannitol). The choice of carbon source could affect the quantity and also the quality of the BNC produced. However, these substrates are relatively expensive and sometimes give low yields of BNC, which limits the scale of industrial manufacture of BNC and becomes a bottleneck for extending the applications for BNC into areas where larger quantities are needed.[7] In recent years, researchers have attempted to produce BNC by developing cost-effective feedstocks from agricultural and industrial residues, such as konjak glucomannan,[8] wheat straw,[9,10] cotton-based waste textiles,[7] waste fiber sludge,[11] and spruce chips.[12] The advantage of using agricultural or industrial residual streams as feedstocks for production of BNC is the low cost of the raw material. In the studies of utilization of konjak glucomannan or wheat straw as carbon source, the hydrolysates obtained through acid hydrolysis had to be detoxified by using overliming and activated carbon treatment.[8,10] The reason is that when lignocellulosic feedstocks are pretreated or hydrolyzed at high temperature, high pressure, and low pH, they give rise to inhibitory compounds derived from polysaccharides and lignin.[13−15] Inhibitory compounds include aliphatic carboxylic acids, furan aldehydes, and phenolic and other aromatic compounds. Quantitatively important carboxylic acids include formic acid, **1**, acetic acid, **2**, and levulinic acid (4-oxopentanoic acid), **3**, whereas furfural (furan-2-carbaldehyde), **4**, and 5-hydroxymethylfurfural (5-(hydroxymethyl)-2-furaldehyde), **5** (Figure 1), are two common furan aldehydes. Their effect on yeast, for example, *Saccharomyces cerevisiae*, has been well studied.[13−15] The effects of phenolic and other aromatic compounds have also been studied, but there is a wide variety of such compounds and their inhibitory effects have been found to vary more than those of different aliphatic acids and different furan aldehydes, which make them more difficult to investigate. For the BNC-producing bacterium *G. xylinus*, studies on complex media have been performed before, whereas information on the effect of specific inhibitors is still lacking. Clear conclusions about the effects of specific compounds can be drawn from experiments using a simple model medium containing one potential inhibitory compound. Although effects of aromatic compounds have been investigated before,[16] the current study is focused on the effects of quantitatively

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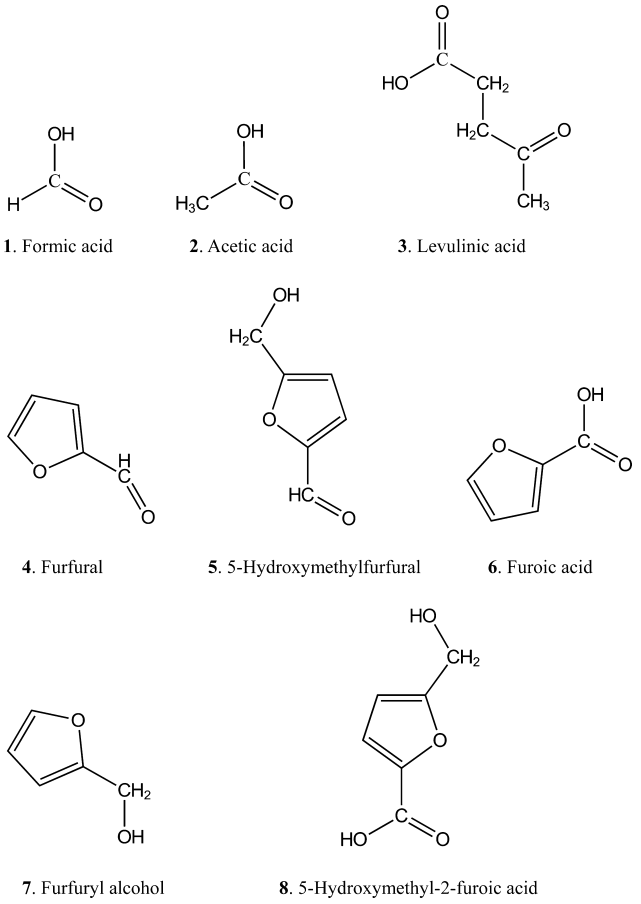

Figure 1. Structures of model inhibitors and related conversion compounds: (**1**) formic acid; (**2**) acetic acid; (**3**) levulinic acid; (**4**) furfural; (**5**) 5-hydroxymethylfurfural; (**6**) furoic acid; (**7**) furfuryl alcohol; (**8**) 5-hydroxymethyl-2-furoic acid.

important aliphatic acids and furan aldehydes on cultures of *G. xylinus*.

The influences of formic acid, acetic acid, levulinic acid, furfural, and 5-hydroxymethylfurfural on static cultures of *G. xylinus* were investigated during a period of 7 days when cell proliferation, sugar consumption, the pH of the cultures, and the concentration of the inhibitor were monitored daily by using modern analytical techniques including fluorescence staining, high-performance anion-exchange chromatography (HPAEC), and high-performance liquid chromatography−UV diode array detection (HPLC-UV-DAD). At the end of the cultivation, the yield of BNC was determined, and bioconversion products derived from furan aldehydes were identified and quantitated. This is the first study of the effects of specific lignocellulose-derived aliphatic acids and furan aldehydes on BNC production by *G. xylinus*, and it is also the first demonstration of the ability of *G. xylinus* to convert furan aldehydes. Studies in this area give a better understanding of the properties of *G. xylinus* and can give important information regarding suitable detoxification methods and directions for efficient production of BNC from lignocellulosic feedstocks.

MATERIALS AND METHODS

Chemicals and Microorganism. Reagent grade chemicals purchased from Sigma-Aldrich (St. Louis, MO, USA) were used in the experiments. The substances added to the bacterial cultures were formic acid, **1**, acetic acid, **2**, levulinic acid, **3**, furfural, **4**, and 5-hydroxymethylfurfural, **5** (Figure 1). *G. xylinus* strain ATCC 23770 was obtained from the American Type Culture Collection (Manassas, VA, USA).

Cultivation of *G. xylinus*. The liquid seed medium, which was prepared using ultrapure water, contained 3.0 g/L yeast extract, 5.0 g/L tryptone, and 25 g/L glucose. The pH of all media was adjusted to 5.0. The fermentation medium was prepared by dissolving 0.75 g of glucose, 0.15 g of tryptone, and 0.09 g of yeast extract in 23.2 mL of ultrapure water.

Pre-inoculum for all experiments was prepared by transferring a bacterial colony grown on agar seed medium into 100 mL of liquid seed medium. After 24 h of agitated cultivation at 30 °C, 1.8 mL of cell suspension was introduced into a 100 mL Erlenmeyer flask containing 23.2 mL of the fermentation medium. The culture was then incubated at 30 °C with agitation for another 24 h to ensure that the bacterial cells were in the exponential growth phase before the addition of inhibitory substances.

Before the addition of the inhibitors, aqueous stock solutions were prepared and the pH of the stock solutions was adjusted to 5 with H_2SO_4 and NaOH. The concentration of the stock solutions of the aliphatic acids was 6 times higher than the concentration in the cultivation medium. For each of the aliphatic acids, 5 mL of the stock solution was added to obtain 25, 100, 175, and 250 mM in the resulting media. Triplicates were performed for each aliphatic acid. Five milliliters of autoclaved ultrapure water was added to reference cultures without inhibitors. The 100 mL Erlenmeyer flasks containing 30 mL of bacterial culture were incubated statically at 30 °C for 7 days. Samples (2 mL) from each flask were taken aseptically every 2 days during this time period. The samples were stored at −20 °C until analyzed.

Distilled furfural was added directly to the culture medium to reach final concentrations of 10, 20, 30, and 40 mM. A stock solution of 5-hydroxymethylfurfural (0.15 g/mL) was prepared in ultrapure water and was then added into the medium to reach the same final concentrations as for furfural. Triplicates were performed for each aliphatic acid.

Determination of BNC Yield. Production of BNC was quantitated gravimetrically on the basis of the dry weight of the insoluble BNC obtained at the end of cultivation. The BNC was collected after the incubation and dried to constant weight at 105 °C. After that, the BNC was weighed for calculation of the volumetric yield (g/L) and the yield on consumed sugar (g/g). The yield of BNC on consumed sugar (g/g) was calculated by using the following equation:

$$\text{BNC yield on consumed sugar (g/g)} = \frac{\text{BNC (g)}}{\text{glucose on first day (g)} - \text{residual glucose (g)}}$$

Analysis of Glucose. The concentration of glucose during the cultivations was monitored by using an Accu-Chek Aviva glucometer (Roche Diagnostics GmbH, Mannheim, Germany). The consumption rate of the glucose during cultivation was calculated by using the following equation:

$$\text{glucose consumption rate (g/[L·day])} = \frac{\text{glucose on first day (g/L)} - \text{residual glucose (g/L)}}{\text{cultivation time (days)}}$$

Analysis of Aliphatic Acids. Quantitation of formic acid, acetic acid, and levulinic acid was done using HPAEC. Before analysis, all samples were filtered through a 0.20 μm Millex-GN syringe-driven filter unit (Millipore, County Cork, Ireland) and diluted with ultrapure water. The HPAEC analysis was performed with a Dionex ICS-3000 system with a conductivity detector. The analytical column used was a 250 mm × 4 mm i.d., 9 μm, IonPac AS15, with a 50 mm × 4 mm i.d., 9 μm, IonPac AG15 guard column (Dionex, Sunnyvale, CA, USA). Isocratic elution with 35 mM sodium hydroxide at a flow rate of 1.2 mL/min was used for separation of the anions. The total run time of the analysis was 16 min. For quantitation of aliphatic acids, standard

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curves covering the range from 0.5 to 50 ppm were prepared using reference standards.

Analysis of Furan Derivatives. The concentrations of the furan aldehydes **4** and **5** were analyzed using high-performance liquid chromatography (HPLC) with an Agilent series 1200 instrument equipped with a G1315D diode array multiple wavelength detector (Agilent Technologies, Santa Clara, CA, USA). Samples (10 μL) were diluted 20-fold with ultrapure water and filtered through 0.2 μm Millex-GN syringe-driven filter units (Millipore). A volume of 2 μL of each diluted sample was injected into a column. The column used was a 50 mm × 3.0 mm i.d., 1.8 μm, Zorbax RRHT SB-C18 (Agilent Technologies), and the flow rate was 0.5 mL/min. Elution was performed with a gradient made of a mixture of ultrapure water and acetonitrile, both of which contained 0.1% formic acid. The gradient started with 3% acetonitrile for 3 min, after which the acetonitrile content increased linearly to 10% after 5 min. The column temperature was maintained at 40 °C. For quantitation of furan aldehydes **4** and **5**, standard curves covering the range from 0.5 to 50 ppm were prepared using reference standards. The wavelength used for quantitation of **4** and **5** was 280 nm.

The possible bioconversion of furfural and 5-hydroxymethylfurfural to corresponding acids and alcohols was investigated using HPLC-UV-DAD, and retention times and spectra of reference standards for 2-furoic acid (furan-2-carboxylic acid), **6**, furfuryl alcohol (2-furanmethanol), **7**, and 5-hydroxymethyl-2-furoic acid, **8** (Figure 1). The wavelength for quantitation of **6** and **8** was 254 nm, and the wavelength for quantitation of **7** was 210 nm.

Analysis of Bacterial Viability. The bacterial cells were collected by filtration through Durapore membrane filters in a 1225 sampling manifold (Millipore) and were then resuspended in saline. The viability of the bacterial cells was determined by using a BacLight live/dead bacterial viability fluorescence staining kit (Invitrogen, Grand Island, NY, USA) and a Synergy H4 hybrid microplate reader (BioTek Instruments, Winooski, VT, USA). The protocol for microplate assays supplied by the manufacturer was followed. A standard curve for the relative fluorescence value (RFV) and the number of bacterial cells was made before analysis of samples from the bacterial cultures from experimental series with inhibitors.

RESULTS AND DISCUSSION

Effects of Aliphatic Acids. The effects of the three aliphatic acids **1**, **2**, and **3** (Figure 1) were evaluated in the concentration range of 25−250 mM. Figure 2 shows the effects of acid **1** on sugar consumption, cell viability, and the pH of bacterial cultures. When the concentration of acid **1** was 25 mM, *G. xylinus* could consume glucose at a rate of 3.1 g/(L·day), which was similar to the rate observed for cultures grown in the reference medium (Table 1; Figure 2). During the 7 days of cultivation after the addition of acid **1**, the pH of cultures with 25 mM acid **1** decreased from 4.4 to 3.1, which was slightly higher than the 2.7 in the reference medium. The concentration of live bacteria in the culture with 25 mM acid **1** reached the maximal value, 16.8 × 10^6 cells/mL, at the fourth day, when acid **1** was exhausted. After that, there was a slight decrease in the concentration of live bacteria (Figure 2C). The volumetric yield of BNC in the medium containing 25 mM acid **1** was 3.2 g/L, whereas it was 3.5 g/L in the reference medium (Table 1). The BNC yield on consumed glucose was 0.15 g/g, whereas it was 0.16 g/g in the reference medium (Table 1).

When the initial dosage of acid **1** was increased to 100 mM or higher, the consumption rate of glucose decreased to ≤0.18 g/(L·day) (Table 1) and the pH value did not change much (Figure 2B). Low concentrations of live bacterial cells were observed in the media, and the concentration of acid **1** was constant (Figure 2). There was no synthesis of BNC in the media with 100 mM or higher concentrations of acid **1** (Table 1). Thus, *G. xylinus* could tolerate 25 mM formic acid, but at 100 mM or higher concentrations there was a complete inhibition of both the production of BNC and the growth of the bacterial cells.

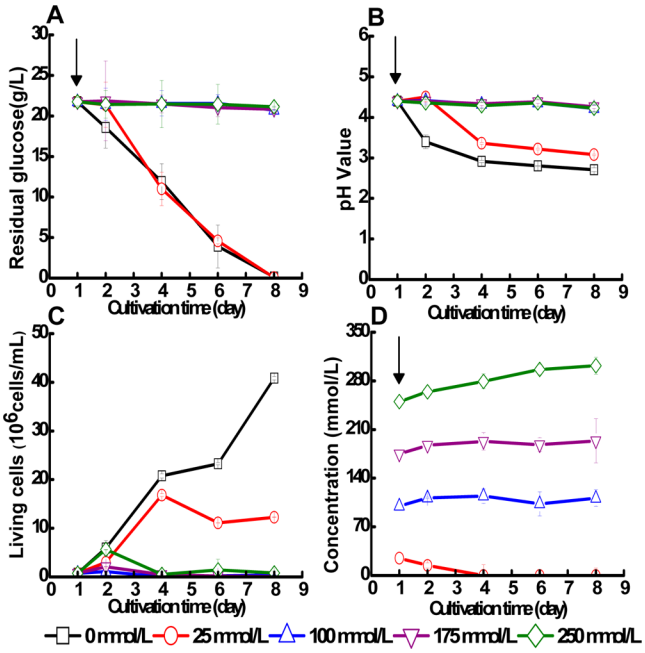

Figure 2. Changes in (A) glucose concentration, (B) pH value, (C) cell growth, and (D) formic acid concentration during cultivation of *G. xylinus* in media supplemented with formic acid. The arrows indicate the time for addition of formic acid. Error bars show standard errors of means of three replicates.

Concentrations of acid **2** up to 250 mM did not stop the growth of *G. xylinus* or the production of BNC (Figure 3; Table 1). The glucose was consumed gradually during the cultivation (Figure 3). In the first 3 days after the addition of acid **2**, the consumption rates of glucose for cultures with acid additions of 25, 100, and 175 mM were 4.8, 5.1, and 4.9 g/(L·day), respectively. These were higher than that for the cultures grown in reference medium, which had a glucose consumption rate of 3.3 g/(L·day) after 3 days. At the end of the cultivation, there was little glucose left in the media except for cultures with an initial addition of 250 mM acetic acid, which contained 3.2 g/L (Figure 3A). The average glucose consumption rate during the 7 day cultivation in media with 25−175 mM acetic acid was 3.1 g/(L·day), which was the same as for cultures grown in reference medium (Table 1). For cultures in medium with 250 mM acid **2**, the average glucose consumption rate was 2.6 g/(L·day), which shows a decrease ($p < 0.05$, t test) compared to cultures grown in reference medium. The volumetric yield of BNC in the media with acetic acid did not differ significantly ($p < 0.05$) from the yield of the cultures in reference medium except for cultures with an initial addition of 250 mM acid **2**, for which it decreased to 3.1 g/L. Even though the glucose consumption rate and the volumetric yield of BNC were lower for medium with 250 mM, the BNC yield on consumed glucose (0.17 g/g) was slightly higher than that of the cultures in reference medium (0.16 g/g). It can be concluded that high concentrations of acetic acid tend to inhibit the proliferation of *G. xylinus*, but not its ability to produce BNC. The pH decreased gradually, but the pH in the medium with high concentration of acetic acid did not drop as much as that in the

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Table 1. Yield of Bacterial Nanocellulose and Glucose Consumption Rate in 7 Day Cultures Supplemented with Aliphatic Acids

	concentration				
acid	0 mM	25 mM	100 mM	175 mM	250 mM
			Volumetric Yield of Bacterial Nanocellulose (g/L)		
1	3.5 ± 0.3	3.2 ± 0.2	ND[a]	ND	ND
2	3.5 ± 0.3	3.4 ± 0.7	3.5 ± 0.9	3.6 ± 0.5	3.1 ± 0.3
3	3.5 ± 0.3	3.5 ± 0.5	5.0 ± 0.9	4.2 ± 0.9	3.8 ± 0.5
			Bacterial Nanocellulose Yield on Consumed Glucose (g/g)		
1	0.16 ± 0.01	0.15 ± 0.01	ND	ND	ND
2	0.16 ± 0.01	0.16 ± 0.03	0.16 ± 0.04	0.17 ± 0.02	0.17 ± 0.01
3	0.16 ± 0.01	0.16 ± 0.02	0.23 ± 0.04	0.20 ± 0.04	0.18 ± 0.02
			Glucose Consumption Rate (g/(L·day))		
1	3.1 ± 0.1	3.1 ± 0.1	0.18 ± 0.30	0.13 ± 0.10	0.09 ± 0.01
2	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	2.6 ± 0.1
3	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1

[a]None detected.

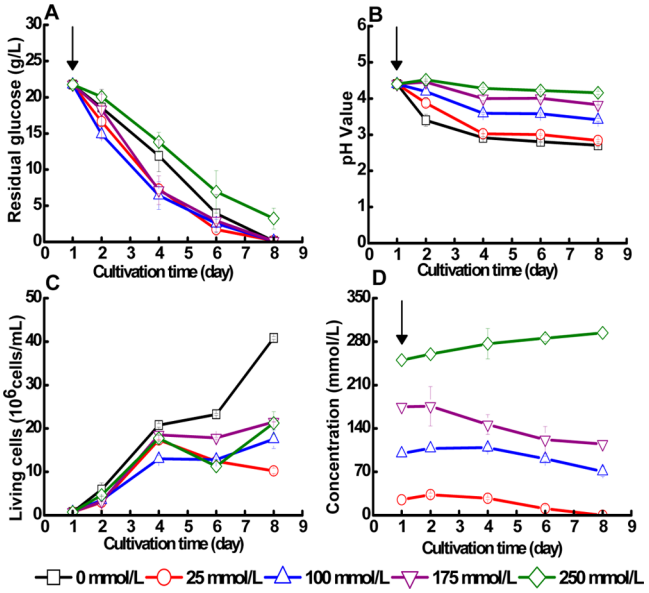

Figure 3. Changes in (A) glucose concentration, (B) pH value, (C) cell growth, and (D) acetic acid concentration during cultivation of *G. xylinus* in media supplemented with acetic acid. The arrows indicate the time for addition of acetic acid. Error bars show standard errors of means of three replicates.

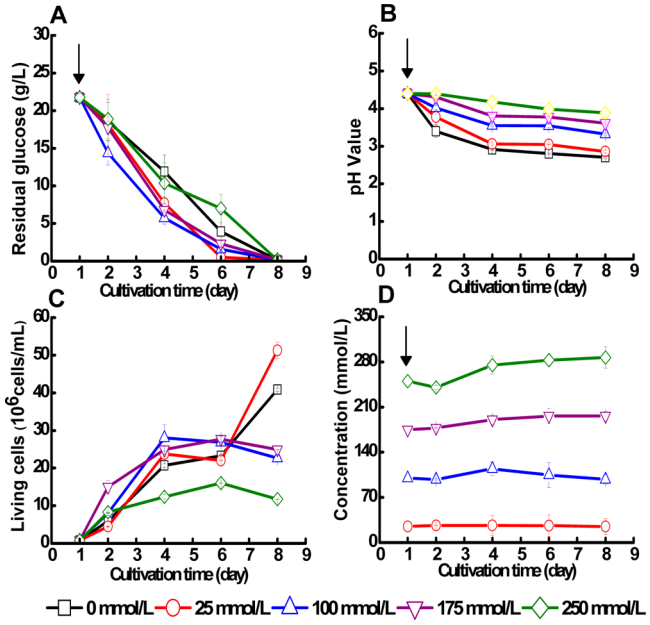

Figure 4. Changes in (A) glucose concentration, (B) pH value, (C) cell growth, and (D) levulinic acid concentration during cultivation of *G. xylinus* in media supplemented with levulinic acid. The arrows indicate the time for addition of levulinic acid. Error bars show standard errors of means of three replicates.

medium with low concentration of acetic acid. This can be attributed to the buffering capacity of acetic acid, which has a pK_a of 4.76, close to the initial pH of the cultures. *G. xylinus* consumed acetic acid when the initial concentration was 175 mM or lower (Figure 3D). The concentration of acetic acid increased slightly in cultures with an initial concentration of 250 mM (Figure 3). This may be due to evaporation of water during the cultivation and to the bacterium not being able to convert as much acetic acid as in the other media.

Results obtained with acid **3** are shown in Figure 4 and Table 1. After the addition of acid **3** to the medium, there was a sharp decrease in the concentration of glucose during the first 3 days of cultivation (Figure 4A). The average glucose consumption rate in cultures with 25, 100, 175, and 250 mM levulinic acid during the first 3 days were 4.7, 5.3, 5.0, and 3.8 g/(L·day), respectively. They were higher than that of the reference medium (3.3 g/(L·day)). At the end of the cultivation, there was little glucose left in the media (Figure 4A). The average glucose consumption rate during the 7 days of cultivation with levulinic acid was 3.1 g/(L·day) for all media (Table 1). The pH value in all media decreased gradually, although the decrease of pH in media with higher concentrations of levulinic acid was smaller than that in media with lower concentrations or no levulinic acid. The buffering effect of levulinic acid (Figure 4B) may explain the slightly more rapid initial glucose consumption rates of cultures containing levulinic acid (Figure 4A). The bacterium grew well in media with levulinic acid (Figure 4C), and the maximum concentration of live bacteria (51.3×10^6 cells/mL) was observed in the medium with 25 mM levulinic acid. This concentration was even higher than that in the reference medium, which was 40.8×10^6 cells/mL. The lowest concentration of live cells, 11.7×10^6 cells/mL, was obtained in the medium with 250 mM levulinic acid. The concentration of levulinic acid did not change during the cultivation (Figure 4D), which indicates that *G. xylinus* was not

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able to utilize levulinic acid. The concentration of levulinic acid in cultures with an initial concentration of 250 mM increased slightly at the end of the cultivation, which can be attributed to evaporation of water in combination with the inability of *G. xylinus* to utilize levulinic acid. The volumetric yield of BNC increased significantly ($p < 0.05$) as the concentration of levulinic acid got higher. The maximum yield of BNC, 5.0 g/L, was obtained with an initial concentration of levulinic acid of 100 mM, whereas cultures with reference medium reached only 3.5 g/L (Table 1). Even cultures with an initial concentration of 250 mM gave higher yields than cultures with reference medium (Table 1). There was a similar trend for the BNC yield on consumed glucose (Table 1). The BNC yield on consumed glucose in medium with 100 mM, 0.23 g/g, was the highest value for media with or without aliphatic acids (Table 1).

The results demonstrate that formic acid is more inhibitory to *G. xylinus* than acetic acid, whereas inhibitory concentrations of levulinic acid were not reached in the interval studied. The result is in one way similar to results obtained in experiments with a baker's yeast strain, where formic acid was found to be more inhibitory than levulinic acid or acetic acid.[17] Larsson et al.[17] found that concentrations exceeding around 100 mM tended to decrease the ethanol yield, whereas lower concentrations instead stimulated ethanol production at the expense of biomass synthesis. There are also differences, as levulinic acid was found to be more inhibitory to yeast than acetic acid[18] and the effects of acetic acid and levulinic acid on *G. xylinus* were positive at least up to 175 mM (this work).

Acids may be inhibitory for several reasons. One possibility is that the protonated form of the acid passes across the plasma membrane and releases the proton in the cytoplasm, where the pH is higher than in the surrounding medium.[19,20] To maintain a constant intracellular pH value, protons are pumped out of the cell by a plasma membrane ATPase. This may lead to excessive consumption of ATP and, in the long run, to depletion of the pool of ATP. Another possibility is that they would act as uncouplers, amphiphilic molecules that dissolve in the inner mitochondrial membrane of eukaryotes or in the plasma membrane of prokaryotes and transfer protons across the membrane, which would uncouple the respiratory chain and the oxidative phosphorylation and disturb the regeneration of ATP. However, the anions of formic acid, acetic acid, and levulinic acid are hydrophilic and could not readily traverse the cell membrane.[21] A third possibility could be intracellular anion accumulation. After dissociation, the anionic form of the acid would be captured in the cell.[19] It has been speculated that formic acid would have higher anion toxicity to yeast.[17] It has also been suggested that formic acid would increase the membrane leakage of ethanologenic *Escherichia coli* LY01.[22]

There are several studies of the effect of weak acids on *Acetobacter*, another genus of acetic acid bacteria, which is characterized by a strong ability to oxidize alcohols and sugars to acetic acid and by high resistance to the acid.[23] An efflux pump of *Acetobacter aceti* IFO 3283 has been reported to enable the bacterium to grow in the presence of high concentrations of acetic acid.[24] Addition of acetic acid to cultures of *Acetobacter xylinum* strain DA has been shown to enhance the cellulose yield from glucose.[25] Glucose is catabolized to generate energy for bacterial anabolism and can be used as a building block in the synthesis of bacterial cellulose. If glucose is partly substituted by another carbon source (such as acetic acid), the conversion of glucose to cellulose may be improved.[25] This is a possible explanation for some of our results with *G. xylinus*, where relatively low concentrations of bacteria in media with high concentrations of acetic acid gave similar yields of BNC as obtained with the reference medium (Table 1; Figure 3C).

Effects of Aldehydes. As shown in Figure 5, furfural (**4**, Figure 1) had a very clear negative effect on *G. xylinus*. Only 5.6

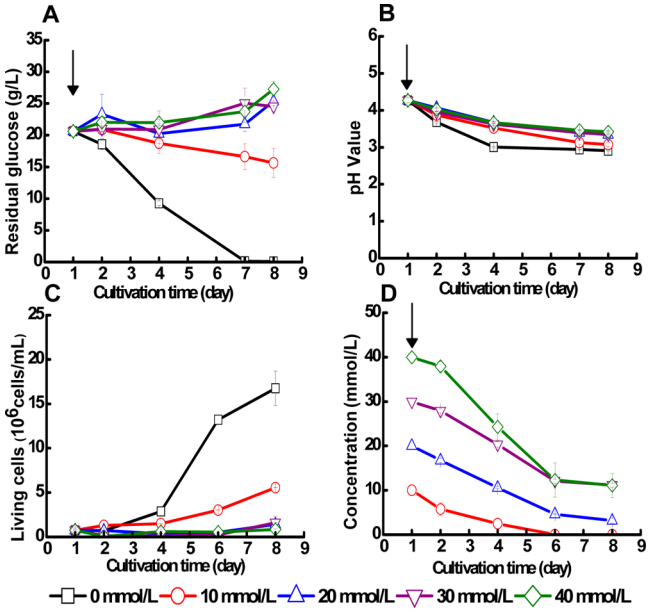

Figure 5. Changes in (A) glucose concentration, (B) pH value, (C) cell growth, and (D) furfural concentration during cultivation of *G. xylinus* in media supplemented with furfural. The arrows indicate the time for addition of furfural. Error bars show standard errors of means of three replicates.

$\times 10^6$ live cells/mL were found in cultures with an initial furfural concentration of 10 mM, which can be compared to 16.8×10^6 live cells/mL in cultures with reference medium. With an addition of 10 mM furfural, the average consumption rate of glucose was 0.71 g/(L·day), only 24% of the rate observed for cultures in reference medium (Table 2). With 10 mM furfural, a BNC yield of 1.1 g/L was achieved, which was 20% of the yield obtained with reference medium. The decreased volumetric yield of BNC in the presence of furfural was mainly due to the reduction of the concentration of live bacteria, although the BNC yield on consumed glucose was also lower (0.22 g/g) than in cultures with reference medium (0.27 g/g). When the concentration of furfural was increased to 20 mM, there was no glucose consumption in the cultures (Figure 5A) and there were hardly any live bacteria in the culture medium (Figure 5C). The slight increase in glucose concentration in cultures with an initial furfural concentration between 20 and 40 mM can be explained by evaporation of water during the incubation (Figure 5A).

Figure 5D indicates that the concentration of furfural decreased as the cultivation continued. This could be due not only to bioconversion of furfural by *G. xylinus* but also to evaporation of furfural. In cultures with furfural, furoic acid was identified as a main product (Table 3). Furoic acid (**6**, Figure 1) was formed in all cultures to which furfural was added, but the highest yield, 88%, was observed in cultures with an initial furfural concentration of 10 mM. No furfuryl alcohol was detected in the culture media. Control experiments with medium with furfural but without bacterial inoculum were

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Table 2. Yield of Bacterial Nanocellulose and Glucose Consumption Rate in 7 Day Cultures Supplemented with Furan Aldehydes

	concentration				
aldehyde	0 mM	10 mM	20 mM	30 mM	40 mM
	Volumetric Yield of Bacterial Nanocellulose (g/L)				
4	5.6 ± 0.8	1.1 ± 0.5	ND[a]	ND	ND
5	5.6 ± 0.8	4.3 ± 0.1	0.81 ± 0.01	ND	ND
	Bacterial Nanocellulose Yield on Consumed Glucose (g/g)				
4	0.27 ± 0.04	0.22 ± 0.01	ND	ND	ND
5	0.27 ± 0.04	0.31 ± 0.01	0.22 ± 0.02	ND	ND
	Glucose Consumption Rate (g/(L·day))				
4	2.9 ± 0.1	0.71 ± 0.33	ND	ND	ND
5	2.9 ± 0.1	2.0 ± 0.2	0.34 ± 0.20	ND	ND

[a]None detected.

Table 3. Conversion of Furan Aldehydes in Bacterial Cultures

initial concentration of **4** (mM)	10	20	30	40
yield of **6** (%)	88	62	42	34
initial concentration of **5** (mM)	10	20	30	40
yield of **8** (%)	76	32	15	12

performed. No conversion products from furfural were detected in the medium, which indicates that the furoic acid formed in media with bacteria was a bioconversion product and not a product caused by oxidation by air or by other media components. Apart from bioconversion, the decrease of the concentration of furfural in the culture media (Table 3) can be attributed to evaporation of furfural. Experiments with acid wood hydrolysates have shown that furfural easily evaporates. After evaporation of 10% of the volume, the concentration of furfural was reduced with 37%, whereas the concentration of 5-hydroxymethylfurfural, which is much less volatile than furfural, was unchanged.[18]

The effects of 5-hydroxymethylfurfural (**5**, Figure 1) on the bacterial cultures are shown in Figure 6 and Table 2. When the initial concentration of 5-hydroxymethylfurfural was 10 mM, the growth rate increased rapidly from the fourth day, and the maximum concentration of viable cells, 13.3×10^6 cells/mL, was obtained on day 7. The average consumption rate of glucose was 2.0 g/(L·day), which was lower than for the culture in control medium (2.9 g/(L·day)). When the initial concentration of 5-hydroxymethylfurfural was increased to 20 mM, the consumption rate of glucose decreased sharply to 0.34 g/(L·day). As the initial concentration of 5-hydroxymethylfurfural was increased further, the growth of the bacteria diminished and the glucose consumption rate was negligible. The pH value decreased gradually during the cultivation. When the initial 5-hydroxymethylfurfural concentrations were 10 and 20 mM, the yields of BNC were 0.31 and 0.22 g/g, respectively (Table 2). As the yield of BNC in the reference medium was 0.27 g/g, low concentrations of 5-hydroxymethylfurfural may have a positive effect on the production of BNC. The data in Table 2 and Figure 6 indicate that 5-hydroxymethylfurfural was less toxic than furfural to *G. xylinus*.

When the initial concentration of 5-hydroxymethylfurfural was 10 mM, all of the 5-hydroxymethylfurfural could be converted by the bacteria (Figure 6D). When the initial concentration of 5-hydroxymethylfurfural was higher, the conversion rate seemed to decline. Analyses of the culture

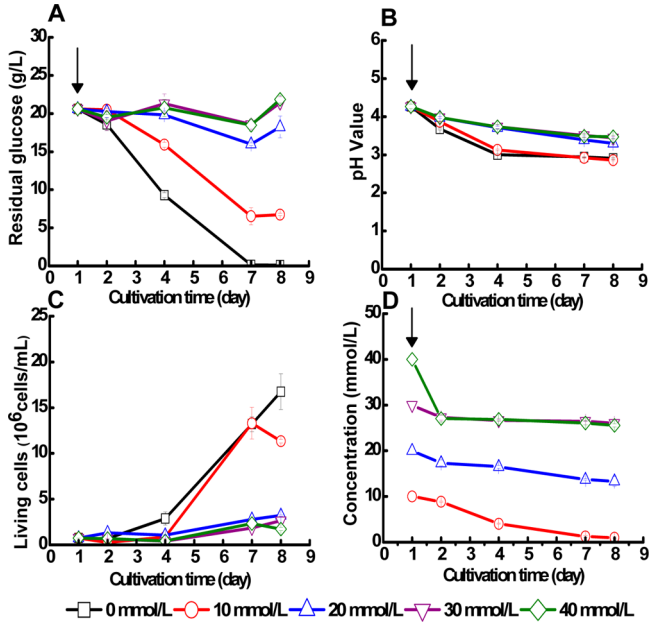

Figure 6. Changes in (A) glucose concentration, (B) pH value, (C) cell growth, and (D) 5-hydroxymethylfurfural concentration during cultivation of *G. xylinus* in media supplemented with 5-hydroxymethylfurfural. The arrows indicate the time for addition of 5-hydroxymethylfurfural. Error bars show standard errors of means of three replicates.

fluid indicated formation of 5-hydroxymethyl-2-furoic acid (**8**, Figure 1). The yield of 5-hydroxymethyl-2-furoic acid was highest (76%) in cultures with an initial concentration of 5-hydroxymethylfurfural of 10 mM (Table 3), which clearly indicates that it was the predominant bioconversion product. At higher initial concentrations of 5-hydroxymethylfurfural, the yield of 5-hydroxymethyl-2-furoic acid dropped. It is noteworthy that there was formation of 5-hydroxymethyl-2-furoic acid (12% yield) even when the initial concentration of 5-hydroxymethylfurfural was as high as 40 mM, and there were no clear signs of glucose consumption, the concentration of viable cells was low, and there were only minor changes in pH indicating low metabolic activity (Figure 6). Control experiments (without inoculation but with addition of 5-hydroxymethylfurfural) showed no conversion products from 5-hydroxymethylfurfural, which supports the conclusion that 5-hydroxymethyl-2-furoic acid was formed by biocatalytic conversion.

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The inhibition by furan aldehydes of ethanologenic microorganisms, such as *S. cerevisiae*, has been studied extensively.[14,15] *S. cerevisiae* would typically be expected to reduce furfural to furfuryl alcohol[26] and 5-hydroxymethylfurfural to 5-hydroxymethylfurfuryl alcohol (2,5-bis-hydroxymethylfuran).[27] However, during respiratory growth *S. cerevisiae* exclusively converted furfural to furoic acid.[28] Under both aerobic and anaerobic conditions, 5-hydroxymethylfurfural was mainly reduced to 5-hydroxymethyl furfuryl alcohol by yeast and only small amounts of 5-hydroxymethyl furoic acid were found.[27] Methanobacteria have been found to convert furfural to furfuryl alcohol,[29] whereas *Clostridium acetobutylicum* was found to reduce both furfural and 5-hydroxymethylfurfural to the corresponding alcohols.[30] Wahlbom and Hahn-Hägerdal[31] studied *S. cerevisiae* TMB 3001 and found that the reduction of furfural was associated with consumption of NADH, whereas the reduction of 5-hydroxymethylfurfural was associated with the consumption of NADPH. NADH plays an important part in many cellular reactions, including respiratory and fermentative catabolism, whereas NADPH is mainly utilized in biosynthetic reactions. NADPH may also function as an essential cofactor for enzymes involved in protection against oxidative stress. Besides intracellular redox balance, stress also affects energy metabolism, as many stress-responsive processes are dependent on ATP availability. The ability of *G. xylinus* to oxidize furan aldehydes has not been reported before. Biotransformation by oxidation is not self-evident, as *G. xylinus* has been found to reduce phenolic aldehydes to the corresponding alcohols.[16]

NAD(P)H- and $NAD(P)^+$-dependent reactions have also been studied in *G. xylinus*.[1] *A. aceti* MIM2000/28 has been reported to have the ability to convert polyconjugated aldehydes to the corresponding acids, an ability that has been used for a number of selective bio-oxidations.[32] The probable reaction behind conversion of furfural by *G. xylinus* would be

$$\text{furfural} + H_2O + NAD(P)^+ \rightarrow \text{furoic acid} + NAD(P)H + H^+$$

Similarly, 5-hydroxymethylfurfural would probably be oxidized to 5-hydroxymethyl-2-furoic acid in a reaction involving the reduction of $NAD(P)^+$. Assuming that these reactions have relatively high priority, as indicated by furan aldehyde bioconversion taking place (Table 3) without concurrent glucose catabolism or BNC production at the higher furan aldehyde concentrations studied (Table 2), the furan aldehydes would be expected to interfere with critical cellular reactions involving oxidized nicotinamide adenine dinucleotides. It is a possibility that the furoic acids are less toxic than the corresponding aldehydes, and in that case the reactions indicated above would represent detoxification reactions, but that possibility needs to be addressed in future investigations.

Kubiak et al.[33] identified a megaplasmid of *G. xylinus* E25 and found some genes connected to oxidoreductases. Megaplasmids may be important for the survival of bacteria under stress. Oxidoreductases would probably be involved in the catalysis of oxidation of furan aldehydes to the corresponding carboxylic acids. The potential effects of *G. xylinus* oxidoreductases on furan aldehydes warrant attention in future studies.

In summary, the effects of presumed inhibitors, three aliphatic carboxylic acids and two furan aldehydes, on cultures of *G. xylinus* have been elucidated. Whereas formic acid, furfural, and 5-hydroxymethylfurfural had an inhibitory effect, appropriately high concentrations of levulinic acid and acetic acid were found to stimulate BNC production. The sensitivity of *G. xylinus* to furan aldehydes differed from that observed for other microorganisms, such as *S. cerevisiae*, which indicates that results obtained with other microorganisms cannot be extrapolated to *G. xylinus*. The main bioconversion products of furfural and 5-hydroxymethylfurfural were found to be the corresponding carboxylic acids. The effects of carboxylic acids need to be further investigated using pH-controlled cultures. Information provided by the current study paves the way for future studies on potential synergistic effects of different inhibitory compounds. A better understanding of inhibitory and stimulatory effects of lignocellulose-derived compounds will facilitate the development of robust processes for the production of BNC from lignocellulosic resources.

AUTHOR INFORMATION

Corresponding Author

*(F.H.) E-mail: fhong@dhu.edu.cn. Phone: +86-21-67792649. Fax: +86-21-67792649.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BNC, bacterial nanocellulose; HPAEC, high-performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; HPLC-UV-DAD, high-performance liquid chromatography−UV diode array detection; RFV, relative fluorescence value

REFERENCES

(1) Bielecki, S.; Krystynowicz, A.; Turkiewicz, M.; Kalinowska, H. Bacterial cellulose. In *Biopolymers (Polysaccharides I: Polysaccharides from Prokaryotes)*; Vandamme, J., Baets, S. D., Steinbüchel, A., Eds.; Wiley-VCH Verlag: Weinheim, Germany, 2002; Vol. 5, pp 37−90.

(2) Chawla, P. R.; Bajaj, I. B.; Survase, S. A.; Singhal, R. S. Microbial cellulose: fermentative production and applications. *Food Technol. Biotechnol.* **2009**, *47*, 107−124.

(3) Fu, L.; Zhang, J.; Yang, G. Present status and applications of bacterial cellulose-based materials for skin tissue repair. *Carbohydr. Polym.* **2013**, *92*, 1432−1442.

(4) Jiang, G.; Qiao, J.; Hong, F. Application of phosphoric acid and phytic acid-doped bacterial cellulose as novel proton-conducting membranes to PEMFC. *Int. J. Hydrogen Energy* **2012**, *37*, 9182−9192.

(5) Lu, X.; Tang, S.; Huang, B.; Shen, X.; Hong, F. Preparation and characterization of bacterial cellulose/hydroxypropyl chitosan blend as-spun fibers. *Fiber Polym.* **2013**, *14*, 935−940.

(6) Wei, B.; Yang, G.; Hong, F. Preparation and evaluation of a kind of bacterial cellulose dry films with antibacterial properties. *Carbohydr. Polym.* **2011**, *84*, 533−538.

(7) Hong, F.; Guo, X.; Zhang, S.; Han, S. F.; Yang, G.; Jonsson, L. J. Bacterial cellulose production from cotton-based waste textiles: enzymatic saccharification enhanced by ionic liquid pretreatment. *Bioresour. Technol.* **2012**, *104*, 503−508.

(8) Hong, F.; Qiu, K. An alternative carbon source from konjac powder for enhancing production of bacterial cellulose in static cultures by a model strain *Acetobacter aceti* subsp *xylinus* ATCC 23770. *Carbohydr. Polym.* **2008**, *72*, 545−549.
(9) Chen, L.; Hong, F.; Yang, X.; Han, S. Biotransformation of wheat straw to bacterial cellulose and its mechanism. *Bioresour. Technol.* **2013**, *135*, 464−468.
(10) Hong, F.; Zhu, Y. X.; Yang, G.; Yang, X. X. Wheat straw acid hydrolysate as a potential cost-effective feedstock for production of bacterial cellulose. *J. Chem. Technol. Biotechnol.* **2011**, *86*, 675−680.
(11) Cavka, A.; Guo, X.; Tang, S. J.; Winestrand, S.; Jönsson, L. J.; Hong, F. Production of bacterial cellulose and enzyme from waste fiber sludge. *Biotechnol. Biofuels* **2013**, *6*, 25.
(12) Guo, X.; Cavka, A.; Jönsson, L. J.; Hong, F. Comparison of methods for detoxification of spruce hydrolysate for bacterial cellulose production. *Microb. Cell Fact.* **2013**, *12*, 93.
(13) Jönsson, L. J.; Alriksson, B.; Nilvebrant, N. O. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels* **2013**, *6*, 16.
(14) Nevoigt, E. Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **2008**, *72*, 379−412.
(15) Pienkos, P.; Zhang, M. Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. *Cellulose* **2009**, *16*, 743−762.
(16) Zhang, S.; Winestrand, S.; Guo, X.; Chen, L.; Hong, F.; Jönsson, L. J. Effects of aromatic compounds on the production of bacterial nanocellulose by *Gluconacetobacter xylinus*. *Microb. Cell Fact.* **2014**, *13*, 62.
(17) Larsson, S.; Palmqvist, E.; Hahn-Hägerdal, B.; Tengborg, C.; Stenberg, K.; Zacchi, G.; Nilvebrant, N. O. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb. Technol.* **1999**, *24*, 151−159.
(18) Larsson, S.; Reimann, A.; Nilvebrant, N. O.; Jönsson, L. J. Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. *Appl. Biochem. Biotechnol.* **1999**, *77*−*79*, 91−103.
(19) Piper, P.; Calderon, C. O.; Hatzixanthis, K.; Mollapour, M. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **2001**, *147*, 2635−2642.
(20) Russell, J. B. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J. Appl. Microbiol.* **1992**, *73*, 363−370.
(21) Casal, M.; Cardoso, H.; Leao, C. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. *Microbiology* **1996**, *142*, 1385−1390.
(22) Zaldivar, J.; Ingram, L. O. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. *Biotechnol. Bioeng.* **1999**, *66*, 203−210.
(23) Fukaya, M.; Takemura, H.; Okumura, H.; Kawamura, Y.; Horinouchi, S.; Beppu, T. Cloning of genes responsible for acetic acid resistance in *Acetobacter aceti*. *J. Bacteriol.* **1990**, *172*, 2096−2104.
(24) Matsushita, K.; Inoue, T.; Adachi, O.; Toyama, H. *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. *J. Bacteriol.* **2005**, *187*, 4346−4352.
(25) Toda, K.; Asakura, T.; Fukaya, M.; Entani, E.; Kawamura, Y. Cellulose production by acetic acid-resistant *Acetobacter xylinum*. *J. Ferment. Bioeng.* **1997**, *84*, 228−231.
(26) Palmqvist, E.; Almeida, J. S.; Hahn-Hägerdal, B. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. *Biotechnol. Bioeng.* **1999**, *62*, 447−454.
(27) Taherzadeh, M. J.; Gustafsson, L.; Niklasson, C.; Liden, G. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 701−708.
(28) Sárvári, H. I.; Franzén, C. J.; Taherzadeh, M. J.; Niklasson, C.; Lidén, G. Effects of furfural on the respiratory metabolism of *Saccharomyces cerevisiae* in glucose-limited chemostats. *Appl. Environ. Microbiol.* **2003**, *69*, 4076−4086.
(29) Deppenmeier, U. The unique biochemistry of methanogenesis. *Prog. Nucleic Acid Res.* **2002**, *71*, 223−283.
(30) Zhang, Y.; Han, B.; Ezeji, T. C. Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by *Clostridium acetobutylicum* ATCC 824 during butanol fermentation. *New Biotechnol.* **2012**, *29*, 345−351.
(31) Wahlbom, C. F.; Hahn-Hägerdal, B. Furfural, 5-hydroxymethyl furfural, and acetoin act as external electron acceptors during anaerobic fermentation of xylose in recombinant *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **2002**, *78*, 172−178.
(32) Pini, E.; Bertacche, V.; Molinari, F.; Romano, D.; Gandolfi, R. Direct conversion of polyconjugated compounds into their corresponding carboxylic acids by *Acetobacter aceti*. *Tetrahedron* **2008**, *64*, 8638−8641.
(33) Kubiak, K.; Kurzawa, M.; Jędrzejczak-Krzepkowska, M.; Ludwicka, K.; Krawczyk, M.; Migdalski, A.; Kacprzak, M. M.; Loska, D.; Krystynowicz, A.; Bielecki, S. Complete genome sequence of *Gluconacetobacter xylinus* E25 strain − valuable and effective producer of bacterial nanocellulose. *J. Biotechnol.* **2014**, *176*, 18−19.