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Biological detoxification of furfural and 5-hydroxyl methyl furfural in hydrolysate of oil palm empty fruit bunch by *Enterobacter* sp. FDS8

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ABSTRACT

Biological detoxification of furfural and 5-hydroxyl methyl furfural (HMF) from lignocellulose hydrolysate is commercially attractive, but the lower process efficiency limits its practical applications. Highly efficient detoxification of furfural and HMF was developed by simply adding the whole cells of a newly isolated *Enterobacter* sp. FDS8 without the requirement of adding anything else, giving furfural and HMF degradation rates of up to 0.54 g L^{-1} h^{-1} and 0.12 g L^{-1} h^{-1}, respectively, which are the highest biodetoxification rates ever reported, with a total sugar loss of below 5%. The whole cells were able to be recycled and reused for at least 5 times without losing their detoxification capability. When used for fermentation to produce lactic acid by *Lactobacillus pentosus*, the biologically detoxified oil palm empty fruit bunch (EFB) hydrolysate gave higher lactic acid productivity (1.7-fold), titer (1.5-fold) and yield (1.8-fold) compared to the un-detoxified EFB hydrolysate.

1. Introduction

Pretreatment of lignocellulose to get fermentable sugars is an essential step for converting lignocellulose to value-added chemicals by microbial fermentation. Acid-catalyzed pretreatment of lignocellulose is one of the most extensively investigated pretreatment methods owing to its high efficiency and low process cost [1]. However, during the acid-catalyzed pretreatment, many degradation products such as furfural and 5-hydroxy methyl furfural (HMF), acetic acid, formic acid, ferulic acid, vanillin, 4-hydroxybenzaldehyde, guaiacol and phenolics are formed due to the harsh conditions [2]. These degradation products are potential inhibitors to microbial growth, leading to low productivity and product titer [3,4]. Among the inhibitors, furfural and HMF are most toxic due to their higher concentrations in lignocellulose hydrolysates [5]. They damage microbes by reducing enzymatic and biological activities, breaking down DNA and inhibiting protein and RNA synthesis [6–8].

A lot of physical and chemical methods have been developed to remove or degrade furfural and HMF from lignocellulose hydrolysates, such as over-liming, adsorption using active charcoal, ion exchange and enzymatic conversions [2]. However, these methods are hardly commercially acceptable due to their high process cost or generation of large amount of wastes. An alternative route is biological detoxification, which has the advantages of simple operation and less generation of wastes [2]. However, the efficiency of biological detoxification is usually low. The highest degradation rates of furfural and HMF were reported to be 0.1 and 0.02 g L^{-1} h^{-1}, respectively, with detoxification processes taking 1–4 days to complete [2,6,9–14]. The low efficiency of biological degradation severely limits its practical applications. Therefore, more efficient biological detoxification processes need to be developed for industrial applications in lignocellulose biorefinery.

Here we report a highly efficient process for biological detoxification furfural and HMF from lignocelluloses hydrolysate using *Enterobacter* sp. FDS8 that was isolated from the nature in Singapore. The strains of the same genus had not yet been reported to be used for biological detoxification of furfural and HMF.

2. Materials and methods

2.1. Processing of lignocellulose

EFB (moisture content 7%, w/w) was kindly provided by Wilmar International Limited, Singapore. It was sun-dried and grinded to small particles by a knife mill with 1 mm screen, followed by

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oven-drying at 80 °C for overnight before use. EFB compositions were analyzed following the standard procedures of NREL [15].

2.2. Acid-catalyzed EFB hydrolysis

A typical procedure for the acid-catalyzed EFB hydrolysis was as follows. Into a 1 L Parr reactor (Fike, Blue Springs, MO, USA) was added 30 g of EFB and 300 mL of acid solution containing 0.5% (w/v) of H_2SO_4 and 0.2% (w/v) of H_3PO_4. The reactor was heated to 160 °C for 30 min followed by immediate cooling down to room temperature by circulated cooling water. The solid fraction was removed from the hydrolysate by filtration. The composition of the liquid phase was analyzed by HPLC. The hydrolysate was adjusted to pH 7.0 by using 10 M NaOH before use for detoxification. Exogenous furfural and HMF were added to get the desired higher concentrations of the inhibitors when needed.

In order to obtain high sugar concentrations in the hydrolysate for fermentation, 150 g of EFB and 450 mL of acid solution consisting of 3% (w/v) of H_2SO_4 and 1.2% (w/v) of H_3PO_4 were added to 1 L Parr reactor. The reactor was heated to 130 °C for 45 min followed by immediate cooling down to room temperature by circulated cooling water. The solid fraction was removed from the liquid hydrolysate by filtration. The composition of the hydrolysate was analyzed by HPLC. The hydrolysate was adjusted to pH 7.0 by adding 10 M NaOH prior to biodetoxification.

2.3. Screening furfural and HMF-degrading microbes

The solid PDA plates used for isolating furfural and HMF degrading microbes contained (1 L): 2 g of furfural, 5 g of potato extract, 20 g of glucose and 20 g of agar, pH 6.0. The liquid medium used for cultivating the isolates had the same compositions with the solid PDA plates but without furfural and agar.

Soil samples (0.5 g) taken from various locations in Singapore were mixed with 5 mL liquid medium and cultivated at 30 °C, 150 rpm for 3 days. Then the upper liquid was collected, diluted and spread onto PDA plates. The plates were kept in an incubator at 30 °C for 2–3 days until the occurrence of clear colonies. The colonies were picked up and cultivated in 40 mL of liquid medium for 24 h. Then 5 mL of this cell culture was inoculated into the EFB hydrolysate containing 2 g L^{-1} of furfural and 0.5 g L^{-1} of HMF. The mixture was incubated at 30 °C for 1 week and liquid samples (1 mL) were regularly taken for HPLC analysis to monitor the degradation of furfural and HMF.

2.4. Identification of isolates

The 16S rDNAs of the isolates were amplified by PCR using the two primers, F27 (5′-agagtttgatcctggctcag-3′) and 1492R (5′-ggttaccttgttacgactt-3′), and blasted with the NCBI nucleotide database to identify the strains.

2.5. Detoxification of furfural and HMF by Enterobacter sp. FDS8

For degradation of furfural and HMF, *Enterobacter* sp. FDS8 was cultivated in 40 mL of the liquid medium in 250 mL flaks for 16 h. The cells from 20, 30 and 40 mL of cultures were harvested by centrifugation at 4000 rpm for 30 min and respectively added to 20 mL of lignocellulose hydrolysate in 250 mL flasks. The mixtures were incubated at 30 °C, 150 rpm for 3 h. The supernatants were collected by centrifugation and analyzed by HPLC.

For recycle and reuse of the cells, the used *Enterobacter* sp. FDS8 cells from the last detoxification experiment were collected by centrifugation, added to 40 mL of fresh EFB hydrolysate and cultivated at 30 °C, 150 rpm for 3 h. Then the cells were collected and reused for next round of detoxification following the same procedures as described above. The cells were stored at 4 °C when not in use.

For detoxification of the EFB hydrolysate with high sugar contents, the *Enterobacter* sp. FDS8 cells prepared in 400 mL of liquid seed culture were collected by centrifugation and added to 100 mL of EFB hydrolysate. The mixture was cultivated at 30 °C, 150 rpm for 24 h. Afterwards the cells were removed by centrifugation and the supernatant was stored at 4 °C prior to use for fermentation.

The inhibitor degradation rate is defined as the amount of the inhibitor consumed per liter per hour (g L^{-1} h^{-1}). Specific degradation rate (h^{-1}) was calculated as the degradation rate divided by cell concentration (g L^{-1}, dry weight). The reduction of inhibitors within the first 1 h of detoxification was measured for the calculations.

2.6. Fermentation of EFB hydrolysates to lactic acid

The fermentation medium was prepared by adding yeast extract (40 g L^{-1}) into 30 mL of detoxified or un-detoxified EFB hydrolysate with high sugar concentrations. The mixture was adjusted to pH 6.0 by adding 2 M HCl and sterilized through membrane (0.22 μm) filtration. Then sterilized powder $CaCO_3$ was added into the mixture to reach 40 g L^{-1}. Seed *Lactobacillus pentosus* (ATCC 8041) cells were prepared by cultivating in 30 mL of MRS medium (pH 6.2) in a 50 mL capped tube at 37 °C for 24 h without shaking. The MRS medium was composed of (1 L): 10 g of peptone, 8 g of meat extract, 4 g of yeast extract, 20 g of glucose, 5 g of sodium acetate trihydrate, 1 g of Tween 80, 2 g of dipotassium hydrogen phosphate, 2 g of triammonium citrate, 0.2 g of magnesium sulfate heptahydrate, and 0.05 g of manganese sulfate tetrahydrate. Then 9 mL of this seed culture was inoculated into the above prepared medium to initiate the fermentation. The broth was incubated at 37 °C for 13 days without shaking. The supernatant was collected by centrifugation and analyzed by HPLC. The precipitate was washed by 2 M HCl to remove $CaCO_3$. Then cells were washed by de-ionized water and placed in oven at 105 °C for 48 h to determine the dry weight of cells.

2.7. Analytical methods

Xylose, glucose, arabinose, acetic acid, furfural, HMF and furfuryl alcohol were analyzed by HPLC (LC-10AT, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87 H column (Bio-Rad, Herculse, CA, USA) at 50 °C. Samples were eluted by 5 mM H_2SO_4 at 0.65 mL min^{-1} and detected by a refractive index detector.

3. Results and discussion

3.1. Isolation and identification of furfural and HMF-degrading microbes

The isolated bacterial strain FDS8 showed the highest furfural degradation rate with its 16S rDNA being sequenced (Table 1). Blasting of the 16S rDNA in NCBI showed that it is most homologous to those of *Enterobacter* sp. ATCC 27981, *Enterobacter* sp. LMG 5337, *Enterobacter* sp. ATCC 27990 and *Enterobacter* sp. ATCC 27982 with a homology of 99.7%, 99.7%, 99.6% and 99.2%, respectively. Therefore, the isolate was identified as belonging to genus *Enterobacter* and named as *Enterobacter* sp. FDS8. Several microorganisms including *Amorphotheca resinae*, *Coniochaeta ligniaria*, *Ureibacillus thermosphaericus*, *Issatchenkia occidentalis*, *Escherichia coli* and *Pichia guilliermondii* have been reported to degrade furfural and HMF in lignocellulose hydrolysate [2,4,6,9–11,14]. However, bacteria of the genus *Enterobacter* had not yet been reported to degrade furfural and HMF in lignocellulose hydrolysate.