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Isolation of 5-hydroxymethylfurfural biotransforming bacteria to produce 2,5-furan dicarboxylic acid in algal acid hydrolysate

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In dealing with lignocellulosic and algal biomass, thermal acid hydrolysis is an economical and efficient method. In this process, 5-hydroxy-methylfurfural (5-HMF) is formed unavoidably, which inhibits downstream reducing sugar fermentation. Fortunately, 5-HMF can be biotransformed into 2,5-furan-dicarboxylic acid (FDCA), the top 14 biomass platform molecules. Base on the connection between 5-HMF removal and FDCA production, microbes capable of biotransforming 5-HMF into FDCA are beneficial to raise biofuel yield and potential molecule production. In this research, pure strain *Methylobacterium radiotolerans* G-2 capable of transforming 5-HMF into FDCA was enriched and isolated from local campus soil, and its abilities of 5-HMF biotransformation and FDCA production were characterized. Strain *M. radiotolerans* G-2 could completely transform 1000 mg/L 5-HMF into FDCA with maximum concentration of 513.9 mg/L at an initial pH of 7 at 26°C. Algal acid hydrolysate after two-fold dilution was suitable for strain *M. radiotolerans* G-2 to perform 5-HMF biotransformation, and 459.7 mg/L FDCA could be obtained. Interestingly, strain *M. radiotolerans* G-2 did not significantly consume reducing sugar and reducing sugar consuming efficiency was less than 16%.

[**Key words:** Macroalgae biomass; Thermal acid hydrolysis; Biotransformation; 5-Hydroxy-methylfurfural (5-HMF); 2,5-Furan-dicarboxylic acid (FDCA); *Methylobacterium radiotolerans* G-2]

In order to mitigate and solve environmental problems resulted from using fossil fuel, countries around the world focus on developing green and renewable energy. The major advantage of biofuels is their renewable and abundant. The feedstocks used for generating biofuels have been undergone for several generations (1). The chosen biomass used for next generation is always devoid of main disadvantage caused by last generation. Algal biomass is considered as an ideal material for biofuels production in recent years because of its carbon fixation and fast growth rate in various water bodies (2–4). As the feedstock shifts, more procedures must be incorporated to transform complex compounds into simple molecules. Unfortunately, these procedures weaken biofuel competitiveness in the applications.

Thermal acid hydrolysis, one of the economical and efficient method, is extensively used to release monosaccharide from algal biomass as algae-based carbohydrates possess low lignin content (5,6). However, the main drawback of thermal acid hydrolysis is the production of some inhibitory compounds hindering downstream biofuels production process using monosaccharide as substrate (7). Among these inhibitory compounds, 5-hydroxy-methylfurfural (5-HMF) is commonly found in the lignocellulosic acid hydrolysate and mainly formed by hexose dehydration (8). Hydrolysis of macroalgae *Gracilaria verrucosa* with a solid-acid catalyst released not only 61 g/L reducing sugar but also 10.7 g/L 5-HMF (9). Park et al. (10) also indicated that 5-HMF was the main byproduct of acid hydrolysis of red algae *Gelidium amansii*. 5-HMF concentration had a negative effect on bioethanol and biohydrogen ($BioH_2$) production. The cell growth and ethanol production of *Kluyveromyces marxianus* were both obviously reduced after 24 h in the presence of 0.5 g/L 5-HMF (11). The maximum $BioH_2$ production rate was reduced 50% as 5-HMF concentration was 0.48 g/L (12).

Therefore, inhibitors detoxification, especially 5-HMF, is essential to guarantee maximum biofuel yield. Several processes used for hydrolysate detoxification had been provided by Almeida et al. (13). However, these processes mostly imply overall cost increase. Therefore, simultaneous production of valuable byproduct during biofuel producing processes is beneficial to reduce biofuel production cost.

2,5-Furan-dicarboxylic acid (FDCA) is in the list of top 14 biomass platform molecules for the sustainable future announced by the US Department of Energy in 2004 (14). Since FDCA can be used for polyethylene terephthalate and other aromatics containing polymer production, it has come into notice in catalyst field and polyester industry (15). The advantages of biologically producing FDCA include mild operation condition, high selectivity and specificity (16). On the basis of the connection between 5-HMF detoxification in the lignocellulosic and algal hydrolysates and FDCA production, isolation of microbes capable of biotransforming 5-HMF to FDCA is beneficial to high biofuel yield and potential molecule production. In this research, potential bacterial strain capable of biotransforming 5-HMF into FDCA was enriched and isolated. Moreover, potential microbes' ability of 5-HMF biotransformation was investigated under various initial pHs, temperatures and 5-HMF concentrations. The isolate was also used

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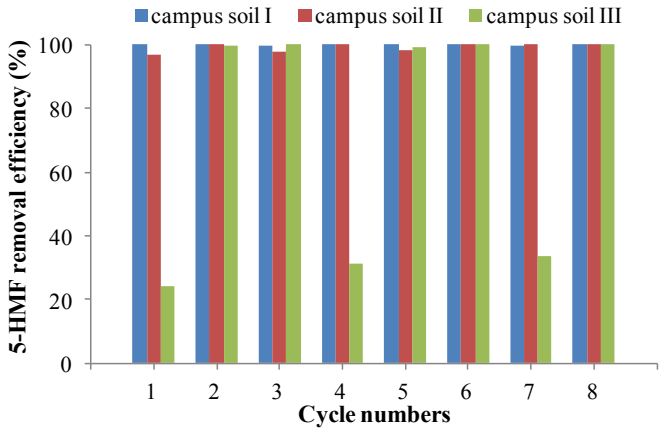

FIG. 1. 5-HMF removal efficiencies in each cycle under various enrichment conditions. Each cycle referred to 24 h.

to biotransform 5-HMF into FDCA in the algal acid hydrolysate. In this research, we provide useful information to optimize biofuel manufacture and produce economical molecule materials.

MATERIALS AND METHODS

Algal acid hydrolysate preparation Pretreatment and thermal acid hydrolysis of macroalgae *Chaetomorpha linum* were conducted according to the previous research (17). The algal acid hydrolysate was obtained by treating 3% algal powder (<100 mesh) with 0.5 M HCl under 121°C for 15 min.

5-HMF biotransforming bacteria enrichment, isolation, and identification For 5-HMF biotransforming bacteria enrichment, 20 g surface soil from the National Yunlin University of Science and Technology campus (23°41′41.0″N, 120°32′12.0″E) was collected. After mixing with mineral salt media (MSM) (17), the soil solution was allowed to stand for 30 min to precipitate large soil granules. Approximately 20–30 mL supernatant was added into a 500-mL flask containing 100 mL MSM. Three different enrichment conditions were designed to simulate the presence of other inhibitors (acetic acid and furfural) in the algal hydrolysate. In all enrichment conditions, 200 mg/L 5-HMF was added as the main carbon source. In addition, 200 mg/L furfural and 100 and 1000 mg/L acetic acids were supplied individually into three enrichments, assigned as campus soil I, II, and III, respectively. Three enrichments were incubated at 120 rpm under 28°C in the dark. When 5-HMF was almost consumed, 200 mg/L 5-HMF was added repeatedly until stable 5-HMF utilization rate was obtained. Bacterial isolation was performed in accordance with our previous research (17). Bacterial 16S rDNA sequencing was carried out by the Mission Biotech Company (Taipei, Taiwan), and the sequence was analyzed using NCBI nucleotide BLAST software to obtain bacterial identification result.

Batch experiments Batch experiments were conducted at a series of 125-mL batch reactors containing 40 mL MSM or the autoclaved algal acid hydrolysate. As to MSM based batch experiments, optimum 5-HMF concentrations (500, 1000, 1500, 2000, and 3000 mg/L), initial pHs (pH 5–8) and incubated temperatures (26°C, 28°C, 30°C, and 32°C) were investigated. The initial amount of biomass started at a cell concentration yielding 0.1 ± 0.02 O.D. units. As to algal hydrolysate media, the initial pH was adjusted in accordance with the batch results of the MSM based media before autoclaving. To avoid the lack of important elements in the algal acid hydrolysate, 5 mL MSM at cell concentration yielding 0.25 ± 0.02 O.D. units was mixed with 35 mL algal acid hydrolysate to the total volume of 40 mL. All reactors were shaken at 120 rpm at the designed temperature and sampled periodically to determine 5-HMF and FDCA concentrations, biomass and pH variations. When the hydrolysate was used as the media, reducing sugar was supplementally measured.

Analytical methods The OD_{600} and pH were measured using UV/Vis spectrophotometer (Genesys 10 UV–Vis; ThermoSpectronic, Rochester, NY, USA) and pH meter (EL20, Mettler Toledo, Switzerland), respectively. Cell biomass was determined according to a standard curve relating optical density (OD) to dry weight to translate the OD value into biomass concentration. OD_{600} value of 0.2 represented 0.10 g (dry weight) per liter for bacterial cells. This relationship was linearly up to at least an OD value of 1.2. Reducing sugar was determined using 3,5-dinitrosalicylic acid assay (18). 5-HMF, FDCA, and other inhibitor compounds (furfural, formic, acetic and levulinic acids) were analyzed by using high performance liquid chromatography (HPLC) (17).

Nucleotide sequence accession numbers The 16S rDNA sequence of strains G-2 isolated in this research has been deposited in GenBank under accession no. KT281919.

RESULTS AND DISCUSSION

Enrichment and isolation of 5-HMF biotransforming bacteria Furfural and acetic acid are also common inhibiters in the lignocellulosic and algal acid hydrolysate. The former one is mainly produced because of pentose dehydration and 5-HMF pyrolysis and the latter one was mainly formed from acetylated sugars in the hemicelluloses (19). Therefore, during enrichment, two inhibitors were chosen for additional supply. Fig. 1 presents 5-HMF removal efficiency in each cycle under various enrichment conditions. 5-HMF was re-added after complete 5-HMF consumption. The results indicated that 5-HMF removal efficiency was not significantly influenced in the campus soil I and II enrichments when containing second carbon source of 200 mg/L furfural or 100 mg/L acetic acid. 5-HMF removal efficiencies were maintained higher than 98% in each cycle. However, with the increase of acetic acid concentration upto 1000 mg/L in the campus soil III enrichment, at least 2 cycle numbers (48 h) were needed to complete 5-HMF utilization compared to the other two enrichments. This implied that high acetic acid hindered 5-HMF utilization but this phenomenon was improved during enrichment. 5-HMF removal rate was gradually increased in the campus soil III enrichment, from 2.0 mg/L/h to 3.2 mg/L/h (data not shown).

Because 5-HMF consumption was observed in three campus soil enrichments, 5-HMF biotransforming bacteria were then isolated from three enrichments. Bacterial strains F-1, F-2, and F-3 were isolated from campus soil I. Two pure cultures assigned as strains G-1, and G-2 and 4 pure cultures of strains H-1, H-2, H-3, and H-4 were isolated from campus soil II and III enrichments, respectively. After preliminary screening, 6 strains (F-1, G-1, G-2, H-1, H-2, and H-3) could grow in the medium containing 200 mg/L 5-HMF as the sole carbon source. Among 6 strains, 4 strains (G-2, H-1, H-2, and H-3) were capable of biotransforming 5-HMF into FDCA. Strain G-2 was chosen for further study in this research since its significant 5-HMF biotransformation efficiency. Strain G-2 was then identified as *Methylobacterium radiotolerans* using the 16S rDNA gene sequencing method with the similarity of 100%. Cells of strain G-2 are Gram-stain-negative rods, and 2.83–3.29 μm long. Colonies are light pink with smooth and circular in form with an entire margin on MSM agar plate containing 200 mg/L 5-HMF, 1.25 g/L yeast extract and 1.25 g/L glucose.

Effect of 5-HMF concentrations on 5-HMF biotransformation Fig. 2 presents the biotransformation of various 5-HMF concentrations into FDCA using strain *M. radiotolerans* G-2. When the initial 5-HMF concentrations were 500 and 1000 mg/L, 5-HMF could be completely biotransformed within 12 and 24 h and received 223.1 and 415 mg/L FDCA, respectively. 5-HMF could not be totally removed as the initial 5-HMF concentration was higher than 1500 mg/L. 5-HMF removal efficiencies were 93.2%, 88.3%, and 71%, and 544.7, 837.8, and 1610 mg/L FDCA were obtained at the initial 5-HMF concentrations of 1500, 2000, and 3000 mg/L, respectively. The FDCA producing efficiencies were calculated by actual obtained FDCA mole divided by theoretical FDCA mole. The order of FDCA producing efficiencies from low to high initial 5-HMF concentrations was 36%, 33%, 29%, 34%, and 43%, respectively. The FDCA curve trend at each 5-HMF concentration increased and then slightly decreased, except for 3000 mg/L 5-HMF as the initial concentration. This phenomenon implied that the produced FDCA could be further utilized by strain *M. radiotolerans* G-2. The accumulated FDCA caused a

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