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Characterization of anaerobic consortia coupled lignin depolymerization with biomethane generation

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HIGHLIGHTS

- Two anaerobic microbial consortia were established to depolymerize lignin.
- The consortia could produce biomethane during lignin depolymerization.
- The consortia could increase the hydrolytic efficiency of lignocellulosic biomass (e.g. OPEFB).

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ABSTRACT

Two sediment-free microbial consortia (LI3 and LP3) were established to depolymerize lignin under anaerobic conditions. During depolymerizing high molecular weight lignin to low molecular weight molecules, the two cultures produced biomethane up to 151.7 and 113.0 mL g^{-1} total lignin. Furthermore, LI3 and LP3 could also utilize the biomass – oil palm empty fruit bunch fiber (OPEFB) to produce 190.6 and 195.6 mL methane g^{-1} total lignin in OPEFB, and at the same time improve the bioavailability of lignocellulosic matters for further enzymatic hydrolysis. The microbial community analysis by denature gradient gel electrophoresis (DGGE) and the high-density 16S rDNA gene microarray (PhyloChip) exhibited that *Methanomethylovorans* sp. (LI3) and *Methanoculleus* sp. (LP3) were the main methanogens present, and phylum *Firmicutes* and *Bacteroidetes* were mainly involved in the lignin depolymerization. The established microbial consortia with both lignin depolymerization and biomethane production provide profound application on the environmental friendly pretreatment of lignocellulosic materials.

1. Introduction

Utilization of lignocellulosic materials to generate bioenergy is attracting much attention because of their abundance and sustainability in nature. Plant biomass derived from crop waste or dedicated feedstock (e.g., perennial plants) has potential for biofuel production if a robust, efficient and economc system is established to utilize these substrates. Moreover, conversion of biomass to bioenergy is a sustainable approach in terms of reducing environmental pollutants, especially greenhouse gas through traditional combustion process (Charles, 2009; Feng et al., 2011). However, one of the major barriers to the lignocellulosic biofuel engineering is the presence of lignin, which blocks the enzymatic hydrolysis on the internal cellulose and hemicellulose so as to limit their bioavailability.

Lignin constitutes one of the main components in the lignocellulosic materials and can comprise ~10–30% of plant biomass, which is usually located on the surface of xylem cells of the plants (DeAngelis et al., 2011). As the integral consistency of plant cell wall, lignin functions as the connection with hemicellulose and cellulose structure and also provides the strength as well as resistance (DeAngelis et al., 2011). Lignin represents a complex and non-repeating three-dimensioned polymer connected by both ether and carbon–carbon linkage with the basic repeating units of phenolic monomer, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Bugg et al., 2010; Vanholme et al., 2008). Traditional pretreatment of the lignocellulosic materials is through chemical or physical process to destroy lignin structure in order to release polysaccharides. These processes are expensive, and most importantly they usually generate a variety of toxic contaminants that will affect the efficiency of following on hydrolysis and fermentation processes (Mishra and Thakur, 2010). Therefore, conversion of raw lignocellulosic materials via biological methods could become a more direct, economic, and favorable process.

Currently, lignin has been reported to be degraded by fungal species as their powerful lignin-degrading enzymatic systems (Vanholme et al., 2008). However, the stability of fungi is not attractive in practical treatment when they are exposed to certain environmental conditions, such as higher pH (>7) or anaerobic

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conditions. Bacteria may be a potential candidate possessing ligninolytic activity because of their immense environmental adaptation and biochemical versatility (Bugg et al., 2010; Chandra et al., 2007). Bacteria with phenolic compounds degrading capability may degrade lignin as well (Peng et al., 2008). Actually, in natural environment, lignocellulosic substances are found to be aerobically or anaerobically transformed and degraded by different bacteria in syntrophic association (DeAngelis et al., 2011). However, reports on lignin degradation by anaerobic bacteria are still limited, thus investigations on anaerobic bacteria capable of lignin-degradation would be beneficial to the industrial production of the next-generation biofuels, with merits of environmental friendly treatment of substrates as well as the economical and compatible application to downstream hydrolysis and fermentation at anaerobic conditions (Bugg et al., 2010; DeAngelis et al., 2010).

In this study, anaerobic microbial consortia were discovered to be capable of utilizing lignin as a sole carbon source and producing biogas – methane (CH_4). Further investigation was conducted on applying the microbial consortia to depolymerize the raw lignocellulosic material – oil palm empty fruit bunch fiber. The 16S rRNA gene based tools – denaturing gradient gel electrophoresis (DGGE) and microarray (PhyloChip) – were employed to characterize the diversity and richness of these lignin-depolymerizing microbial communities.

2. Methods

2.1. Enrichment of microbial consortia

Sediments were collected from mangrove area (Singapore, five samples) and paddy fields (Indonesia, three samples) by using sealed falcon tubes and transferred into the laboratory within 24 h, which were used as microbial inocula in the microcosm setup. The microcosms were setup in an anaerobic chamber as described previously (Lee et al., 2011). The mineral salts medium contained: (g L^{-1}) NaCl 1.0, $MgCl_2{\cdot}H_2O$ 0.5, KH_2PO_4 0.2, NH_4Cl 0.3, KCl 0.3, $CaCl_2{\cdot}H_2O$ 0.015; (mg L^{-1}) $FeCl_2{\cdot}4H_2O$ 1.5, $CoCl_2{\cdot}6H_2O$ 0.19, $MnCl_2{\cdot}4H_2O$ 0.1, $ZnCl_2$ 0.07, $Na_2MoO_4{\cdot}2H_2O$ 0.036, $NiCl_2{\cdot}6H_2O$ 0.024, $Na_2WO_4{\cdot}2H_2O$ 0.008, $Na_2SeO_3{\cdot}5H_2O$ 0.006, H_3BO_3 0.006, $CuCl_2{\cdot}2H_2O$ 0.002; 10 mM TES and 30 mM $NaHCO_3$ as buffers; 0.2 mM L-cysteine, 0.2 mM $Na_2S{\cdot}9H_2O$, 0.5 mM DL-dithiothreitol as reductants and 0.1% resazurin as the anaerobic indicator. After dispensing mineral salts medium (30 mL) to serum bottles (60 mL) under N_2 flushing and sealed with butyl rubber stopper and aluminum cap, 1.0 g L^{-1} pure kraft alkali lignin (5% moisture and probably (hemi)cellulose impurities, Sigma–Aldrich) was spiked to the medium as a sole carbon source and pH was adjusted to 7.2 ± 0.1. The medium bottles with lignin were autoclaved before use unless stated otherwise. Microcosms were incubated at 35 °C with a shaking speed of 150 rpm. Sediment-free cultures were obtained after four transfers (4 weeks of intervals) of the liquids into same fresh medium (10% of inocula). Control samples were established with autoclaved inocula or prepared in the absence of microbes.

2.2. Determination on structure changes of lignin by HPLC

Depolymerization of lignin structure by the microbial consortia was measured by high performance liquid chromatography (HPLC) with minor modification of protocol from Chandra et al. (2007, 2011). The supernatant of active cultures and negative controls (with autoclaved inocula) at each sampling point was collected and pH was adjusted to 1–2 with 5 M HCl. The acidified samples were analyzed on a reverse phase Atlantis™ dC_{18} column (4.6 mm × 150 mm, particle size 5 μm) (Waters, Ireland) by using an Agilent 1200 series HPLC, at a wavelength of 280 nm and temperature of 35 °C. 10 μL of each sample was injected to the column running with a mobile phase of HPLC grade acetonitrile/water (80:20) at a flow rate of 0.5 mL min^{-1}.

2.3. Analysis of methane production

Methane collected from the headspace of serum bottles was injected manually with a glass, gas-tight, luer lock syringe (Hamilton Co., USA) into a gas chromatograph (GC) 6890 N equipped with a flame ionization detector (Agilent Technologies, USA) and a GS-GasPro column (30 m × 0.32 mm I.D. × 0.25 μm film thickness) (J&W Scientific, USA). Helium was used as a carrier gas at a flow rate of 1.5 mL min^{-1}. The oven temperature program was set as follows: held at 80°C for 1 min and raised from 80 to 110 °C at a rate of 30 °C min^{-1} and finally held at 110 °C for 1 min. The injector and detector temperature was set at 200 and 250 °C, respectively. The identification and quantification of methane was based on the retention time and the total volume of methane was calculated by analyzing 200 μL of headspace gas by GC-FID. The standard sample contain 0, 1, 2, 5, 10 mL of pure methane in a N_2 filled serum bottle (60 mL, containing 30 mL mineral salts medium to ensure that standards and samples are prepared at same conditions) under the standard atmospheric pressure.

2.4. Application of oil palm empty fruit bunch fiber (OPEFB) as a substrate

The OPEFB used in this study has a typical composition of cellulose (41.2%, dry wt., w/w), hemicellulose (34.4%), lignin (17.1%), ash (3.4%) and ethanol solubles (2.3%) (Abdul Khalil et al., 2006). Prior to adding to the culture bottles as a substrate, the fiber was washed by distilled water for three times, air-dried in oven at 60 °C overnight, and crushed by a fruit grinder. The fiber powder with sizes less than 250 μm was obtained by sieving, and then added to the medium bottles as a sole carbon source with a ratio of 1:200 (dry wt. of OPEFB/volume of medium, equal to 1 g L^{-1} lignin contents). After autoclaving, 1% (v/v) of sediment-free culture was inoculated into the serum bottle, and incubated under 35 °C at a shaking speed of 150 rpm. For every 10 days, the bio-pretreated fiber was filtered, washed by distilled water twice to remove any chemicals from culture medium and impurities, and finally dried at 60 °C overnight. In order to understand whether the polysaccharides are more bioavailable after the bio-depolymerization of lignocellulosic materials, 100 mg of the bio-treated OPEFB was then digested by 40 U cellulase from *Trchoderma reesei* ATCC 26921 (Sigma, USA) at 50 °C in 10 mL of 100 mM citrate buffer (pH 4.8) for 24 h. Sample aliquots were centrifuged at 12,000 rpm for 10 min, and the reducing sugars generated by cellulase in the supernatants were determined using the dinitrosalicylic acid (DNS) method (Miller et al., 1960). For volatile solids (VS) analysis, the substrate was heated in a muffle furnace at 550 °C for 1 h, and the amount of VS was calculated by subtracting the weight of remaining ash from the original sample (Frigon and Guiot, 2010).

2.5. Investigation of microbial community by denaturing gradient gel electrophoresis (DGGE)

The genomic DNA of microbial consortium was extracted and purified by Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer instructions. The 16S rRNA genes of bacteria were amplified using a primer pair of F338GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGACTCCTACGGGAGGCAGCAG-3′) (the GC clamp is underlined) and 518R (5′-ATTACCGCGGCTGCTGG-3′) on an Eppendorf Mastercycler ep Gradient S PCR machine (Eppendorf, Germany), while the 16S rRNA genes of methanogens were amplified by using a primer pair of