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Investigation on hydrogen production from paper sludge without inoculation and its enhancement by *Clostridium thermocellum*

Qian An[a,b], Ji-Lian Wang[c,d], Yu-Tao Wang[c,d], Zhang-Lin Lin[a,b,*], Ming-Jun Zhu[a,b,c,d,*]

[a] State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China
[b] School of Biology and Biological Engineering, South China University of Technology, Guangzhou Higher Education Mega Center, Panyu, Guangzhou 510006, People's Republic of China
[c] College of Life and Geographic Sciences, Kashgar University, Kashgar 844000, People's Republic of China
[d] The Key Laboratory of Ecology and Biological Resources in Yarkand Oasis at Colleges & Universities under the Department of Education of Xinjiang Uygur Autonomous Region, Kashgar University, Kashgar 844000, People's Republic of China

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ABSTRACT

The feasibility and performance of hydrogen production from paper sludge without inoculation was investigated under thermophilic conditions. The maximum hydrogen production reached 64.32 mM with 7.4% PS. The dynamic changes in bacterial community structures during hydrogen production were investigated by analyzing 16S rDNA gene sequences using high throughput sequencing technology. The results showed that microbial community was dominated by order *Clostridiales* and *Thermoanaerobacterales*. Genus *Thermoanaerobacterium* and *Ruminiclostridium* played a leading role in the fermentation process, which was responsible for the hydrolysis of PS and hydrogen production. Effect of inoculation with *Clostridium thermocellum* on hydrogen production from PS was also studied. The results showed that *C. thermocellum* supplement significantly increased hydrogen yield and holocellulose degradation rate by 96.80% and 32.95%, respectively. In addition, inoculation of *C. thermocellum* enhanced VFA generation and shortened the lag phase of hydrogen production. The present study lays the foundation on the valorization of waste lignocellulose.

1. Introduction

With the rapid economic development, fossil fuels have been increasingly consumed in a large amount leading to a series of ecological and environmental problems. To alleviate the oil crisis and environmental pollution, alternative energy sources have been developed. Biohydrogen production technology is a promising solution to fossil fuel depletion, global warming and air pollution for its mild reaction conditions and environmental benefits. Hydrogen has a high heat value (122 kJ/g), which is 2.75 times that of conventional gasoline (Elsamadony and Tawfik, 2015). There is no greenhouse gas and toxic gases left but only water after hydrogen was combusted. Hydrogen will be a potential contributor to future energy demands due to its environment-friendly character and abundant availability as well as renewability.

Bio-hydrogen production can be achieved by bio-photolysis, photo fermentation and dark fermentation. Bio-photolysis, which is carried out by microalgae, split water molecule into oxygen and hydrogen. However, because of the low photochemical efficiency, it has not yet been practiced in the industry even if the process requires only solar energy and water at standard temperature and pressure (Nikolaidis and Poullikkas, 2017). Dark fermentation (DF) is a process in which microbes convert organic matter to hydrogen, CO_2, organic acids and solvents in the absence of light. DF has been extensively studied during the last decades because hydrogen can be produced from biomass waste in low operation cost (Khanna and Das, 2013).

Paper sludge (PS) is a kind of waste produced in pulping and papermaking process. By 2012, the amount of dry PS production is 0.26 Gt/a in China (Fang et al., 2015) and 0.2–0.6 ton of sludge is generated for every ton of pulp produced (Meyer and Edwards, 2014). Some disposal methods have been used for PS, such as landfill, agricultural application and incineration (Fang et al., 2015). Secondary pollution, such as atmosphere and water contamination, might be caused if the disposal is improperly managed. Incineration has been widely applied for the sludge treatment for the volume reduction and electricity or heat generation. However, high moisture content, high ash and low calorific value restricted its stability (Xie and Ma, 2013). PS consists of paper fillers (such as calcium carbonate, kaolin and soluble

* Corresponding authors at: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China; School of Biology and Biological Engineering, South China University of Technology, Guangzhou Higher Education Mega Center, Panyu, Guangzhou 510006, People's Republic of China.
E-mail addresses: zhanglinlin@scut.edu.cn (Z.-L. Lin), mjzhu@scut.edu.cn (M.-J. Zhu).

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silicates, etc.), fibers (cellulose, hemicellulose and lignin) and lignin-byproducts (Bayr and Rintala, 2012). The composition of the PS is highly relevant to the raw materials used and pulping and papermaking process. Primary sludge (dry weight) from recycled paper mills consists of 54.18%, 7.17%, 7.31%, 2.75% of cellulose, hemicellulose, lignin and ash, respectively (Moreau et al., 2015). Secondary sludge, which is generated in the clarifier of the biological units of the wastewater treatment, has lower wood fiber content compared to primary sludge which is generated from primary clarifier where the big fiber was sieved (Bayr and Rintala, 2012). Therefore, microbes and cell-decay products derived from effluent disposal exist in secondary sludge. PS can be used to produce ethanol through enzymatic hydrolysis and yeast fermentation (Marques et al., 2008). The hydrogen production from PS by anaerobic fermentation has been attempted (Kádár et al., 2004; Lin et al., 2013). Kádár et al. (2004) studied hydrogen production from PS hydrolysate by extreme thermophilic anaerobic fermentation but attained low hydrogen yield. The high price and amount of cellulase undoubtedly increase production costs. Mesophilic anaerobic hydrogen production from co-digestion of pulp & paper sludge and food waste had been explored by Lin et al. (2013), and hydrogen yield of 64.48 mL/g VS_{fed} was achieved. In recent years, the hydrogen production by using indigenous anaerobic microorganisms within the waste material has been studied (Kim et al., 2009; Yokoyama et al., 2007). Thus, it was hypothesized that PS could serve as substrate and hydrogen-producing microbes in DF. In this case, it would simplify the operation for hydrogen production in practical application and be conductive to develop decentralized hydrogen production & biological waste disposal system, which is beneficial for energy and environment security. Biochemical pathways involved in the DF are based on rather complex and diverse microorganisms. Therefore, it is important to understand the diversity and abundance of the microbial communities during the hydrogen production from PS.

Clostridium thermocellum is thermophilic and Gram-positive bacterium, which can convert cellulose into hydrogen, ethanol, carbon dioxide and acetic acid, etc. at the same time. It has attained a wide range of attention in biorefinery for its potential application in Consolidated Bioprocessing (CBP), a process combining cellulase production, saccharification and fermentation (Cheng and Zhu, 2013). The inoculation of cellulose degrading bacteria to the anaerobic fermentation system can improve the fermentation efficiency and the yield of products. Tsapekos et al. (2017) revealed that the *C. thermocellum* addition increased methane yield up to 34% when studying bio-methane production from lignocellulosic biomass.

The valorization of PS for hydrogen production by DF offers a big potential for both clean fuel production and waste disposal. The purpose of this study was to explore the dynamic changes of microbes in the thermophilic anaerobic fermentation process of PS without extra inoculation by analyzing 16S rDNA gene sequences. Then, *C. thermocellum* was inoculated to the process for higher hydrogen yield and substrate degradation rate in batch fermentation, and the PS concentration was also optimized for maximum hydrogen production.

2. Materials and methods

The dynamic changes of microbes in the thermophilic anaerobic fermentation process of PS without extra inoculation were analyzed by 16S rDNA gene sequences using the Illumina HiSeq 2500 PE250 platform and UPARSE software (Uparse v7.0.1001). Then, *C. thermocellum* DSM 1313 was inoculated in 125 mL serum bottles with a working volume of 40 mL to improve the performance of hydrogen yield and substrate degradation rate and the PS concentration was also optimized for maximum hydrogen production in batch fermentation.

2.1. Substrate and inoculum

PS in this study was obtained from a wastewater treatment unit at Donghua paper Co., Ltd. (Foshan, Guangdong province, China) which produce paper towel from waste paper. The PS was stored at −20 °C after dehydration, and thawed at 4 °C before use.

C. thermocellum DSM 1313 was purchased from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *C. thermocellum* inoculum was prepared anaerobically with 10 g/L Avicel pH-105 (FMC, USA) as carbon source in DSMZ 122 medium (Leibniz Inst. DSMZ, 2016). *C. thermocellum* was transferred into the serum bottles with 10% (v/v) inoculum size by injection syringe. All of the hydrogen production as follows was performed at 55 °C with rotary shaking at 150 rpm.

2.2. Hydrogen production from PS without inoculation

125 mL serum bottles were used with a working volume of 40 mL for all the fermentation. Briefly, each bottle contained 7.4% (w/v, dry weight) PS to replace carbon source in the medium and was sealed with a butyl rubber stopper and aluminum seal. The bottles were incubated in a shaking incubator (C24KC refrigerated incubator shaker, Edison, New Jersey, United States) immediately after purged and gassed with 99.99% nitrogen three times. The fermentation was performed in 24 replicates and lasted 8 days, where three of the replicates were sacrificed for hydrogen test each day and liquid samples were taken from it. The liquid samples were centrifuged at 6800 *g* for 10 min, and the supernatants were used for organic acid and ethanol test. The sediments of 1 d, 4 d and 7 d were stored at −80 °C for further DNA extraction.

2.3. DNA extraction and microbial community analysis

Microbial genomic DNA of 1 d, 4 d and 7 d precipitation were extracted by the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer protocol. DNA quantity and specificity were determined by NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, USA) and agarose gel electrophoresis, respectively.

The microbial community of the samples was identified by amplifying and analyzing the V4 region of 16S rRNA from the genome DNA. The amplicons sequencing was conducted on the Illumina HiSeq 2500 PE250 platform by Novogene Bioinformatics Technology (Beijing, China).

After sequencing, paired-end reads from the original DNA fragments were merged using FLASH (Zhang et al., 2014) and assigned to each sample with the unique barcodes. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs), and analysis was performed using UPARSE software (Uparse v7.0.1001) (Edgar, 2013). Representative sequences were picked for each OTU and annotated the taxonomic information using RDP classifier (Version 2.2). The reported OTU data represent the average of two sequencing results.

Sequencing results are available through the NCBI sequence read archive (SRA) database with the accession number SRP134226.

2.4. Hydrogen production from PS enhanced by Clostridium thermocellum

48 fermentation serum bottles were the same as described in 2.2 and was divided into two groups. *C. thermocellum* was transferred into one group which called inoculation group with 10% (v/v) inoculum size by injection syringe, and the other group called non-inoculation group was injected equivalent medium without carbon source. The residues in the endpoint were collected and dried at 60 °C for components analysis. For the Optimization of substrate concentration, a series of substrate concentration (5.4, 7.4 and 9.3%, w/v, dry weight) were considered in batch fermentation experiments for 4 days.