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A novel lignin degradation bacterial consortium for efficient pulping

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HIGHLIGHTS

- A lignin degradation bacterial consortium was screened from sludge of a reeds pond.
- Approximate 60.9% lignin and 43.0% hemicelluloses, but 2.0% cellulose in reeds was degraded.
- The consortium was consisted of cultured six isolates and uncultured bacteria.
- It is feasible for the consortium to pulping by measuring physical characteristics of paper hand-sheets.

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ABSTRACT

A lignin degradation bacterial consortium named LDC was screened from the sludge of a reeds pond by a restricted subculture. It could break down 60.9% lignin in reeds at 30 °C under conditions of static culture within 15 days. In order to analyze the diversity of LDC, plate isolation, 16S rDNA clone library and ARDRA (Amplified Ribosomal DNA Restriction Analysis) were performed. Six bacterial strains were isolated from LDC and eighteen DNA phylotypes were identified from 230 bacterial analyzed clones. They were classified into *Clostridiales* (9.1%), *Geovibrio thiophilus* (5.1%), *Desulfomicrobium* (10.9%), *Pseudomonas* sp. (25.2%), *Azoarcus* sp. (5.1%), *Thauera* (5.1%), *Paenibacillus* sp. (5.1%), *Cohnella* sp. (2.2%), *Acinetobacter* sp. (3.1%), *Microbacterium* (7.8%), and uncultured bacterium (21.3%). In addition, physical characteristics of paper hand-sheets between biological pretreatment and chemical pretreatment were compared. The results showed that LDC had the capability of lignin degradation and was efficient for pulping, which would provide a new choice for biopulping.

1. Introduction

Lignin is the second abundant biomass that is associated with cellulose and hemicellulose in plant cell. Its complicated aromatic polymer and high molecular weight make it resistant to microorganisms, resulting in the prevention of effective cellulose utilization (Wang et al., 2011). In the field of lignin biodegradation, white-rot and brown-rot fungi are well known for obvious degradation and functional extracellular oxidative enzymes. Ligninolytic enzymes mainly include laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP). It has been demonstrated that ligninolytic enzymes mediate effective degradation of lignin, and the degrading ability greatly depends on the interactions of ligninolytic enzymes with lignin. Furthermore, it was detected that residues GLU460, PRO346 and SER113 in Lac, residues ARG43, ALA180 and ASP183 in LiP and residues ARG42, HIS173 and ARG177 in MnP were most crucial in binding of lignin (Chen et al., 2011). Therefore, lignin degradation is a complex process with synergism among more enzymes.

It has been shown that fungi have high capability of lignin degradation, but they are sometimes not stable in practical treatment under extreme environmental and substrate conditions (Hatakka, 1994). Bacteria, in particular, deserve to be studied for ligninolytic potential because of their immense environmental adaptability and biochemical versatility. Lignin can be degraded and assimilated by some bacteria. *Streptomyces viridosporus* T7A can depolymerize lignin using an extracellular lignin-inducible peroxidase (Ramachandra et al., 1988). *Pseudomonas paucimobilis* SYK-6 can break down various dimeric lignin compounds like DDVA, vanillate, and syringic acid by secreting protocatechuate- 4, 5- dioxygenase (Katayama et al., 1988). Bacteria isolated from compost soil like *Azotobacter*, *Bacillus megatarium* and *Serratia marcescens* are capable of decolorizing or solubilizing lignin. *Azotobacter* sp. HM121 produces laccase and its activity correlates positively with lignin mineralization and solubilization (Morii et al., 1995). *Aneurinibacillus aneurinilyticus* can depolymerize lignin to low molecular weight aromatic compounds such as guaiacol, acetoguaiacone, gallic acid and ferulic acid (Raj et al., 2007). Several known aromatic degraders were also found to be lignin degraders, such as *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA1 (Ahmad et al., 2010). There are also several reports of bacteria isolated from termite guts that have aromatic degradation capability (Geib et al.,

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Table 1
the condition used in chemical pretreatment reeds for ordinary pressure and high pressure.

No.	Sample	Concentration of NaOH (%)	Time (mins)	Solid–liquid ratio	Temperature (°C)
1	Ordinary pressure	6	150	1:4	100
2	High pressure	6	10	1:4	130

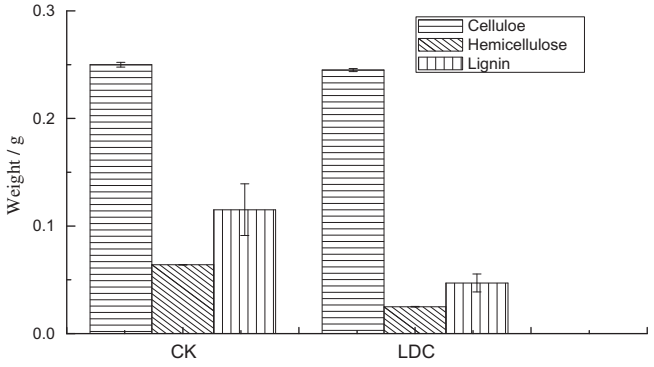

Fig. 1. The residual weight of cellulose, hemicelluloses and lignin in 1 g reeds after degradation by LDC.

2008).These previous studies illustrate that bacteria also contribute to lignin degradation as well as fungus**.** However, it was found that bacterial consortiums were more effective in lignocellulose degradation than other isolates (Wang et al., 2011).

In China, due to the shortage of wood-pulp, non-wood pulp was employed in many areas. Reeds, which are found in the wetlands of China, are used as raw papermaking material. It has been demonstrated that pulping pretreated by fungi reduces energy costs and improves the paper quality by chemical pulping (Zhang et al., 2012; Gulsoy and Eroglu, 2011). However, there is very little understanding of the bacterial impact on pulping. Moreover, whether reeds, the non-wood raw materials, better contribute to pulping need to be investigated.

In this study, a bacterial consortium named LDC with the capability of lignin degradation was screened from the sludge of a reeds pond in Daqing (China). Lignin and hemicellulose in reeds was decomposed efficiently by LDC, however, cellulose was not as efficiently degraded by LDC. The microbial diversity of this community was analyzed to explore the relationships between the strains and their utilized substrates. Moreover, in order to study the feasibility for mechanical pulping by LDC, the physical characteristics of paper hand-sheets pretreated by chemical methods and LDC were compared. The high lignin degradation efficiency of LDC could attract more attention to the lignin degradation by bacteria, and the particular substrate utilization may also be an important clue to reeds pulping through biological pretreatment.

2. Methods

2.1. Lignin materials

Guaiacol and alkali lignin (low sulfonate content; Mn 10,000; MW 60,000) were purchased from SIGMA (Aldrich). All the chemicals used in this work were all analytical or molecular biology grade. Reed straws were all harvested from the wetlands of Daqing, China. The surface layer of the reed straws was removed, soaked in 3% NaOH solution for 24 h, and washed with distilled water until the pH was 7.0. The straws were dried at 60 °C and cut into 3–4 cm in length for use (Yan et al., 2012).

2.2. Sample collection

Daqing, the biggest oil field of China, has a large area of wetland containing reeds. The long-term growth and decay of reeds left mass humus including lignin and aromatic compounds, which provided a natural environment for the growth of lignin degradation microorganisms. The soil samples for function microbial screen were collected randomly beneath the rotten reed straws.

2.3. Bacterial screening and cultivation

The basic medium used in this study was as follow: 2.5 g/L $NaNO_3$, 1.0 g/L KH_2PO_4, 1.0 g/L $MgSO_4$, 1.0 g/L NaCl, 0.5 g/L $CaCl_2$, Trace element solution 1 mL/L (0.16 g/L $FeCl_3{\cdot}6H_2O$, 1.5 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CoCl_2{\cdot}6H_2O$, 0.15 g/L $CuSO_4{\cdot}5H_2O$, 1.5 g/L $MnSO_4{\cdot}H_2O$, 0.3 g/L H_3BO_3, 0.1 g/L $Na_2MoO_4{\cdot}2H_2O$, pH 8.0). Soil samples were treated as follows. 1 g of each soil sample was suspended in sterile water and shaken at 200 rpm for 2 h. The supernatant, which contained microorganisms, was collected for use. The screening procedure was divided into 3 parts. First, the samples treated in the aforementioned steps were treated with basic medium containing 9 mmol/L guaiacol. After being cultured statically at 30 °C for 15 days, the samples that exhibited a color change from transparent to purple were regarded as having positive guaiacol degradation activity. These samples were sorted out for the next step of screening. These soil samples were transferred to a basic medium containing 1 g/L alkali lignin (AL). After being cultured statically at 30 °C for 15 days, we were able to determine which samples could decompose alkali lignin by measuring the amount of weight lost (Raj et al., 2007). For the last screening procedure, the pretreated reed straws were added into basic medium at a concentration of 20 g/L. The positive samples were cultured at 30 °C for 15 days. Measuring the lignocelluloses degradation in reeds was determined by Ankom^{220} Fiber Analyzer in accordance with the procedure used by Goering and Van Soest (1970). The effective samples were subsequently cultured 10 generations until the degradation efficiency of lignin was stable. The most effective enrichment was selected as the ideal lignin degradation community and analyzed.

2.4. Isolation and identification of cultured-bacterial strains from LDC

The samples of LDC for isolation were taken at stable growth phase. In order to explore the bacteria isolated from LDC, we utilized different molecular weights lignin fractions and reeds, took ten-fold dilutions to spread the LDC on solid mediums containing the carbon sources guaiacol, AL, and reeds (The reed straws were pulverized, passed through 1 mm screens). After individual colonies were isolated, they were identified by 16S rDNA sequencing.

2.5. DNA extraction, amplification and cloning of 16S rDNA

Total genomic DNA was extracted from stable growth phase bacteria using the benzyl chloride method as described by (Zhu et al., 1993). The 16S rDNA gene was performed as follow. The 50 μL PCR mixture contained: 1 μL DNA template; 0.5 μL 50 mM primer of 27 F(5′-AGAGTTTGATCCTGGCTCAG-3′); 0.5 μL 50 mM 1492R (5′-GGTTACCTTGTTACGACTT-3′); 5 μL 10× PCR buffer; 0.5 μL rTaq DNA (5 units/μL, TAKARA); and 41.5 mL ddH_2O. PCR was done using the following program: 1 cycle of initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 5 min, annealing at 55 °C for 1 min, and extension at 72 °C for