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Interaction among multiple microorganisms and effects of nitrogen and carbon supplementations on lignin degradation

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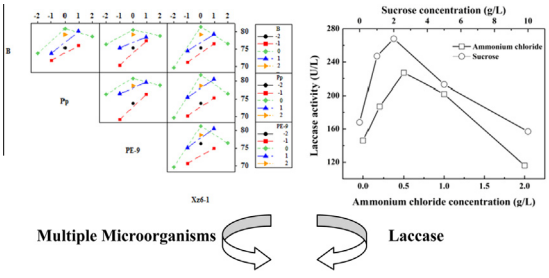
HIGHLIGHTS

- Plackett–Burman and central composite experimental design were performed.
- Mutual interactions among the nine strains and four screened strains were discussed.
- The compositions of the multiple microorganisms were optimized.
- The effects of nitrogen and carbon supplementations were investigated.

GRAPHICAL ABSTRACT

A stable and effective microbial consortium including two indigenous bacteria (*Bacillus* sp. (B) and *Pseudomonas putida* (Pp)) and two inter-kingdom fusants (PE-9 and Xz6-1) was constructed. The multiple microorganisms could produce laccase and exhibited immense environmental adaptability and excellent capability of lignin degradation in alkalescent conditions.



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ABSTRACT

The mutual interactions among the consortium constructed by four indigenous bacteria and five inter-kingdom fusants and the effects of nitrogen and carbon supplementations on lignin degradation and laccase activity were investigated. Analyzed by Plackett–Burman and central composite design, the microbial consortium were optimized, *Bacillus* sp. (B) and PE-9 and *Pseudomonas putida* (Pp) and PE-9 had significant interactions on lignin degradation based on a 5% level of significance. The nitrogen and carbon supplementations played an important role in lignin degradation and laccase production. The ultimate lignin degradation efficiency of 96.0% and laccase activity of 268 U/L were obtained with 0.5 g/L of ammonium chloride and 2 g/L of sucrose. Results suggested that a stable and effective microbial consortium in alkalescent conditions was successfully achieved through the introduction of fusants, which was significant for its industrial application.

1. Introduction

Lignin is the most abundant aromatic polymer on Earth. Due to its complex aromatic heteropolymer and high molecular weight, lignin is extremely recalcitrant to biodegradation, resulting in great prevention of effective treatment of pulping wastewater. In the field of lignin biodegradation, numerous studies have verified that white-rot (Arora and Sharma, 2009; Fackler

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et al., 2006) and brown-rot (Martinez et al., 2005; Wong, 2009) fungi are able to mineralize lignin and produce a range of functional extracellular ligninolytic enzymes such as laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP). Furthermore, it has been reported that the lignin degrading ability greatly depends on the interactions of the ligninolytic enzymes with lignin (Chen et al., 2011a,b). Although fungi exhibit excellent capability of lignin degradation, they are always unstable in practical treatment, especially under the extreme environmental and cultural conditions (Hatakka, 1994).

Recently, a number of literatures report that the bacteria also have an ability to break down lignin. *Streptomyces viridosporus* T7A can degrade lignin with an extracellular lignin peroxidase enzyme (Ramachandra et al., 1988). *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA1 are also found to depolymerize lignin. Meanwhile, some soil bacteria such as *Nocardia* and *Rhodococcus* are identified to break down lignin, using an assay involving ^{14}C-labelled lignin (Zimmermann, 1990). *Aneurinibacillus aneurinilyticus* exhibit high lignin-degrading capacity. They can depolymerize lignin to low molecular weight aromatic compounds such as guaiacol, acetoguaiacone, gallic acid and ferulic acid (Raj et al., 2007). Furthermore, several bacteria isolated from termite guts are confirmed to have the capability of degrading lignin (Geib et al., 2008). These previous researches indicate that bacteria, which have immense environmental adaptability and biochemical versatility, also contribute to lignin degradation. However, their degradation efficiency of lignin is not satisfied compared with fungi.

The microbial degradation of lignin has been well studied in single fungi or bacterium, but seldom investigated in multiple microorganisms, which has a huge potential for solving the problem of environmental pollution due to its low cost, easy use and economic security. In the field of wastewater treatment, the multiple microorganisms have more industrial applications than single microorganism. Hisashi et al. (2000) established a specific consortium using immobilized photosynthetic bacteria, *Rhodobacter sphaeroides* S, *Rb. sphaeroides* NR-3 and *Rhodopseudomonas palustris*, fixed on ceramic media for wastewater treatment. They found that mixture of the three bacteria was more effective for the removal of COD, nitrate, phosphate and odor compared with using a single strain. It is important for the introduced strains to establish stable microbial communities with the indigenous bacterial consortium for treatment improvement (Briones and Raskin, 2003; Smith et al., 2003). Recently, bioaugmentation of group of microorganisms is regarded as an efficient way to treat the pulp and paper wastewater which contains abundant of lignin. Chandra (2001) used active microbial consortia, composed of *P. putida*, *Citrobacter* sp. and *Enterobacter* sp., to treat the pulp and paper mill effluent by activated sludge process. He found the organisms not only decolorized effluent up to 97% but reduced BOD, COD, phenolics and sulfide up to 96.63%, 96.80%, 96.92% and 96.67%, respectively within 24 h. Also, our group (Chen et al., 2010, 2011a,b) constructed two groups of multiple microorganisms from six strains, i.e., *Agrobacterium* sp., *Bacillus* sp., *Enterobacter cloacae*, *Gordonia*, *Pseudomonas stutzeri*, *P. putida*, which both have effective removal of COD and TOC of pulping effluent.

In present work, four native bacteria, *Bacillus* sp. (B), *E. Cloacae*, (E) *Gordonia* sp. (G) and *P. putida* (Pp), which were kept in our laboratory and five inter-kingdom fusants of Eukarya and Prokaryote were selected for study. Here this work aims to construct a stable microbial community in which the fusants and indigenous bacteria can survive together. Meanwhile, the microbial community also has excellent capacity of depolymerizing lignin in alkalescent environmental conditions. There were several studies on the construction of multiple microorganisms, but the information about mutual interactions among multiple microorganisms was still sparse. Therefore, the interplays among the multiple microorganisms was analyzed by using Plackett–Burman (PB) and central composite experimental design (CCD). Then microbial community structure was optimized. Furthermore, the effects of nitrogen and carbon sources on lignin degradation by the multiple microorganisms were also investigated.

2. Methods

2.1. Microorganisms, nutrient medium, and solutions

Nine strains of bacterium were used in this study. *Bacillus* sp. (B), *E. cloacae*, (E) *Gordonia* sp. (G) and *P. putida* (Pp) were selected based on an established mixed culture in a portable wastewater treatment system and kept in our laboratory. Xz6-1 (Chen et al., 2013), PB-3, PE-9 (Sun et al., 2012), PG-1 and Pps-3 were screened from the inter-kingdom fusants of Eukarya and Prokaryote in our laboratory. The lignin (typical Mn 5000; typical Mw 28,000) was purchased from Sigma.

The nutrient medium contained 3 g/L of beef extract, 5 g/L of peptone, and mineral salt medium (MSM) at pH 7. The composition of MSM was KH_2PO_4·(0.42 g/L), K_2HPO_4·(0.375 g/L), $(NH_4)_2SO_4$·(0.244 g/L), NaCl (0.015 g/L), $CaCl_2·2H_2O$ (0.015 g/L), $MgSO_4·7H_2O$ (0.05 g/L), and $FeCl_3·6H_2O$ (0.054 g/L). A phosphate buffer (pH 7) was prepared by dissolving NaCl (8 g/L), KCl (0.2 g/L), K_2HPO_4 (1.15 g/L), and KH_2PO_4 (0.2 g/L) in deionized water (Millipore, Milli-Q), and was used for diluting concentrated cells. All chemicals were analytical reagent grade. The lignin substrate medium was prepared by dissolving lignin in MSM to the required concentrations and the pH was adjusted into 9.0.

2.2. Bacteria cultivation and biodegradation experiments

One loop of each of the nine bacteria was separately transferred to 200 mL of the nutrient medium in a glass flask. Each bacterium was activated at 30 °C. These activated cells were harvested as inocula after 48 h, respectively. The cells were collected by centrifugation (10,000 rpm for 5 min) and cleaned with PBS. Nine inocula were separately prepared by inoculating the nine activated strains into the lignin substrate medium (200 mg/L), to give an initial optical density at 600 nm (OD_{600}) of 0.30 ± 0.01. Then each 50 mL inoculums containing 0 or 0.3 OD_{600} of bacteria (according to the Plackett–Burman design detailed in Table S2) was added aseptically to Erlenmeyer flasks (500 mL) yielding a final volume of 300 mL. Samples were withdrawn every day, and the concentrations of lignin were analyzed at 280 nm by an UV/visible spectrophotometer (Jasco UV-550, Japan). Laccase activity was determined by UV/visible spectrophotometer (Knežević et al., 2013).

2.3. Plackett–Burman design

As an efficient method to screen the important factors among numbers of variables, Plackett–Burman design was used in our study to screen several high-efficiency strains that had a significant influence on lignin degradation from nine strains. In this study, a 12-run Plackett–Burman design was applied to evaluate the nine strains. For each factor, two levels were designated: −1 for the low level and +1 for the high level. The lignin degradation efficiency (LDE) was used as the response value in the experiment. Table S1 illustrated the factors as well as the levels of each factor used in the experimental design, whereas Table S2 represents design matrix and the results.