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Isolation of *Bacillus* sp. strains capable of decomposing alkali lignin and their application in combination with lactic acid bacteria for enhancing cellulase performance

Young-Cheol Chang [a,*,1], DuBok Choi [b,*,1], Kazuhiro Takamizawa [c], Shintaro Kikuchi [a]

[a] Division of Applied Sciences, College of Environmental Technology, Graduate School of Engineering, Muroran Institute of Technology, 27-1 Mizumoto, Muroran 050-8585, Hokkaido, Japan
[b] Department of Pharmacy, College of Pharmacy, Chungbuk National University, Cheongju 361-763, Republic of Korea
[c] Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan

HIGHLIGHTS

- Two alkali lignin-degrading bacteria (CS-1 and CS-2) were isolated from forest soils in Japan.
- CS-1 and CS-2 displayed alkali lignin degradation capability.
- High laccase activities were observed in crude enzyme extracts.
- Improving surface area accessible to cellulose is an important.
- A two-step procedure is effective at accelerating cellulase performance.

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ABSTRACT

Effective biological pretreatment method for enhancing cellulase performance was investigated. Two alkali lignin-degrading bacteria were isolated from forest soils in Japan and named CS-1 and CS-2. 16S rDNA sequence analysis indicated that CS-1 and CS-2 were *Bacillus* sp. Strains CS-1 and CS-2 displayed alkali lignin degradation capability. With initial concentrations of 0.05–2.0 g L^{-1}, at least 61% alkali lignin could be degraded within 48 h. High laccase activities were observed in crude enzyme extracts from the isolated strains. This result indicated that alkali lignin degradation was correlated with laccase activities. Judging from the net yields of sugars after enzymatic hydrolysis, the most effective pretreatment method for enhancing cellulase performance was a two-step processing procedure (pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria) at 68.6%. These results suggest that the two-step pretreatment procedure is effective at accelerating cellulase performance.

1. Introduction

In contrast to fungal lignin degradation, the enzymology of bacterial lignin breakdown is currently not well understood, but extracellular peroxidase and laccase enzymes appear to be involved (Bugg et al., 2011). The advantage of taking a lignin-degrading enzyme from bacteria rather than fungi is that bacteria are much more amenable to genetic modification. This means allows scientists to transfer genes that codes for enzymes into different species of bacteria, such as the industrial workhorse *Escherichia coli*, and also modify the metabolic pathways to enhance the enzyme's lignin-degrading activity (Bugg et al., 2011). In addition, harnessing the biosynthetic ability of microorganisms is becoming an increasingly important platform for producing value-added chemical products (Du et al., 2011).

To date, extensive research and developmental studies on the effective utilization of lignocellulosic materials has been conducted. However, the largest obstacle to the economic production of cellulosic biofuels is cost-effectively releasing sugars from recalcitrant lignocellulose (Zhang, 2008). One of the key problems hindering the effective utilization of this renewable resource as a raw material for chemical reactions and feeds is the low susceptibility of lignocellulose to hydrolysis, which is attributable to the crystalline structure of cellulose fibrils surrounded by hemicellulose and the presence of the lignin seal which prevents penetration by degrading enzymes (Gong et al., 1999).

* Corresponding authors. Tel./fax: +81 143 46 5757 (Y.-C. Chang), tel./fax: +82 63 466 2984 (D. Choi).
E-mail addresses: ychang@mmm.muroran-it.ac.jp (Y.-C. Chang), choidubok@naver.com, choidubok@empal.com (D. Choi).
[1] These authors contributed equally to this work.

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Therefore, an ideal pretreatment is needed to reduce the lignin content and crystallinity of cellulose, and increase the surface area of these materials (Wang et al., 1998). Removal of lignin from biomass before biological processing improves cellulose digestibility, reduces downstream agitation power requirements, provides less sites for nonproductive cellulase adsorption, reduces dissolved lignin compounds that are toxic to fermentations, facilitates cell and enzyme recovery and recycling, and simplifies the distillation steps (Wyman et al., 2004).

Dilute acid treatment is one of the most effective pretreatment methods for lignocellulosic biomass. A common pretreatment uses dilute sulfuric acid (50–300 mM) at 100–200 °C. During hot acid pretreatment, some polysaccharides are hydrolyzed, mostly hemicellulose (Zhu et al., 2009; Lloyd and Wyman, 2005; Mosier et al., 2005). The resulting free sugars can degrade to furfural (from pentoses) or to 5-hydroxymethylfurfural (HMF; from hexoses) (Agbor et al., 2011). These compounds inhibit yeast cells and lead to decreased specific growth rates, specific ethanol production rates and ethanol yields. To resolve this inhibitor problem, organic acids (maleic acid and fumaric acid) have been suggested as alternatives to sulfuric acid during pretreatment. Both organic acids promote the hydrolysis of polysaccharides but, unlike sulfuric acid, neither promotes the degradation of free sugars to furfural and HMF (Kootstra et al., 2009). Recently, Rollin et al. (2011) reported that improving the surface area accessible to cellulose is a more important factor for achieving a high sugar yield rather than attempting to improve the enzymatic digestibility of biomass by removing lignin. Organic acids that do not result in inhibition, such as furfural and HMF, may increase porosity and improves enzymatic digestibility, resulting in hemicellulose removal (Kootstra et al., 2009). However, this sophisticated method requires a heating process of 130–170 °C. Thus, although hemicellulose can be eventually removed from substrates, the high energy requirements will remain problematic.

In the current study, numerous forest soil samples from throughout Japan (from Hokkaido to Okinawa) were collected to better understand the diversity of lignin-decomposing bacteria. After the isolation process, two isolated strains (*Bacillus* sp. strains) were further studied to evaluate their alkali lignin-degrading ability. In addition, their application in lignin degradation was examined using rice straw. A biological pretreatment method was also optimized, which focused on the development of an environmentally-friendly and low energy method for the removal of lignin and to enhance cellulase performance. Two lactic acid bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) were also examined in an attempt to increase the surface area accessible to cellulose resulting in hemicellulose elimination. Application of *Bacillus* sp. strains in combination with lactic acid bacteria for lignin degradation and enhancing cellulase performance were also studied.

2. Methods

2.1. Soil samples and isolation procedure

Soil samples were taken randomly from different forests (Mt. Asahi, Mt. Fuji, and Mt. Yonahadake) located in Hokkaido, Shizuoka, and Okinawa Island (Supplementary data 1). Mt. Fuji is the highest mountain in Japan at 3776 m and an active stratovolcano. In addition, recently it was registrated as a World Heritage site. Mt. Asahi is also an active stratovolcano and the tallest peak in Hokkaido (2290 m). Mt. Yonahadake is the highest mountain on Okinawa Island at 503 m. The temperature of sampling sites was 9 °C (Mt. Asahi), 13 °C (Mt. Fuji), 25 °C (Mt. Yonahadake), respectively. Soil samples were taken at 0–15 cm depth.

Sixty eight soil samples (36 samples (Mt. Asahi); 20 samples (Mt. Fuji); 12 samples (Mt. Yonahadake)) from the above mentioned sites were used as the source of inoculum. As a rapid screening method for detection of ligninolytic ability decolorization of Remazol Brilliant Blue R (RBBR) has been used. RBBR decolorization experiments were set up in 20 mL test tube containing 10 mL of a basal salt medium. The basal salt medium used in this study contained 0.05 g of K_2HPO_4, 0.05 g of KH_2PO_4, 0.1 g of NaCl, 0.3 g of $MgSO_4{\cdot}7H_2O$, 0.2 g of $CaCl_2{\cdot}2H_2O$, 0.6 mg of H_3BO_3, 0.169 mg of $CoCl_2{\cdot}6H_2O$, 0.085 mg of $CuCl_2{\cdot}2H_2O$, 0.099 mg of $MnCl_2{\cdot}4H_2O$, and 0.22 mg of $ZnCl_2$, and was supplemented with 0.01% (w/v) RBBR, 1.0% (w/v) glucose, 0.018% (w/v) yeast extract, and 0.5% (w/v) peptone (BSGYP) in 1000 mL of deionized water (pH 6.0). Cultures were performed under aerobic conditions by inoculating 1 g of each soil sample.

Isolation procedures were performed using cultures from the forest soil samples which represented RBBR-decolorizing activity. The cultures in which RBBR decolorization was observed were subsequently transferred to fresh medium. To isolate colonies, 10-fold dilution of log-phase cells of cultures were spread on petri plates containing BSGYP medium with 1.5% agar. Plates were then incubated under aerobic conditions at 30 °C. The ability of RBBR-decolorization was determined by inoculating colonies into liquid BSGYP medium supplemented with 0.01% (w/v) RBBR and decolorization of RBBR was monitored using a UV–Vis spectrophotometer (UV1800; Shimadzu, Japan) at 592 nm for 10 min. Using this isolation procedure, some representative RBBR-decolorizing bacteria were successfully isolated. The purity of isolated cultures were confirmed using an inverted microscope (Diaphot TMD300; Nikon, Tokyo, Japan).

2.2. 16S rDNA sequence determination and physiological characteristics

For phylogenetic identification of two representative isolates (strains of *Bacillus* sp.), the 16S rRNA gene fragment was amplified by polymerase chain reaction with a pair of universal primers, 27f and 1392r, and DNA sequencing was determined as described by Chang et al. (2011). Phylogenetic analysis was determined as described by Okeke and Lu (2011). Physiological characteristics of the isolates were also determined using commercially available identification systems (API 20E, API 10S, API 50 CHE, API 20 NE; API Staph, API Coryne, API 20A®, API 20C AUX, API® 50 CH, API® 50 CHB, rapid ID 32A API, API Coryne; bioMérieux, Kobe, Japan).

2.3. Biodegradation of alkali lignin

For biodegradation of alkali lignin, two most effective RBBR-decolorizing strains (*Bacillus* sp. CS-1 and CS-2) were selected. Biodegradation experiments were carried out in BSGYP (as mentioned above) containing 0.05 g L^{-1} of alkali lignin. Two isolates were pregrown on BSGYP medium for 24 h. Erlenmeyer flasks (250 mL) containing 100 mL of autoclaved (20 min, 121 °C) BSGYP (pH 6.0) were inoculated with 2 mL of pregrown pure culture (0.65 mg protein mL^{-1}) in log phase. The uninoculated (control) and bacterial inoculated flasks were incubated at 30 °C on a rotary shaker (120 rpm) in dark conditions for 3 days. The time course of lignin degradation was followed while shaking the flasks for 3 days. Disappearance of alkali was monitored by aseptically removing 1 mL samples for measurement of ultraviolet absorption spectra at 280 nm after centrifugation at 6000×*g* for 5 min. All assays were performed at least in duplicate with their corresponding controls. Both non-inoculated media (blanks) and inoculated autoclaved samples (controls) were used.