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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

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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.

Keywords: lignin-degradation, *Bacillus* sp., lactic acid bacteria, cellulase performance, laccase.

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)

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). With organic acids, the quality of the by-product stream improves significantly, as it may be more easily burned in co-firing installations, used as fertilizer or applied in animal feed (Radecki et al., 1988). 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 (Suplementary data 1). Mt. Fuji is the highest mountain in Japan at 3,776 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 9ºC (Mt. Asahi), 13ºC (Mt. Fuji), 25ºC (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 ºC. 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 6,000×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.

2.4 Effects of temperature and pH on alkali lignin degradation

One hundred ml of sterile production medium for bacteria was prepared in different conical flasks at pH 8.0 and inoculated with 2% inoculum. Each flask was incubated at different temperatures (15°C, 25°C, 30°C, 37°C, and 40°C) for 48 h. Total protein concentrations were determined using a Bio-Rad protein assay kit, which contained a bovine γ-globulin standard and a bovine serum albumin standard. Each flask was adjusted to a different pH (4, 5, 6, 8, and 10) using 0.1N NaOH and 0.1N HCl. After sterilization, flasks were inoculated with 2% inoculum. Flasks were then incubated at 37ºC for 48 h.

2.5 Enzyme activity

Lignin peroxidase (LiP, EC1.11.1.14) and manganese peroxidase (EC1.11.1.1) activity were determined as described by Yang et al. (2011).

Laccase activity was determined in both culture fluid and using a crude intracellular enzyme. Laccase activity was measured in 1mL reaction measurements containing 75 mM catechol as the substrate in 50 mM sodium phosphate buffer, pH 5 and 200 μL of culture fluid. The progress of the reaction was monitored at 440 nm for 10 min. One unit of laccase activity was defined as a change in A_{440} of 1 mL in 1 min (Ramesh et al. , 2008).

To determine intracellular laccase activity, bacterial cultures were centrifuged (6,000×g) for 20 min at 4°C to precipitate cellular debris and obtain clear supernatants. Bacterial pellets were then washed with Tris-HCl buffer (0.1 M; pH 7.5) containing 10 mM of phenylmethylsulfonyl fluoride to inhibit protease activity in the supernatant before sonication. Cell extract was obtained by centrifugation (14,000×g) at 4°C for 20 min and used as a crude intracellular enzyme. All spectrophotometric measurements were carried out using a UV–Vis spectrophotometer (UV1800; Shimadzu, Japan). All assays were carried out in triplicate.

2.6 Biodegradation of lignin in rice straw by Bacillus sp. CS-1

Strain CS-1 was pregrown on BSGYP medium for 24 h. Erlenmeyer flasks containing 100 mL of growth medium (pH 8) and 3 g of milled rice straw was inoculated with 2 mL of pregrown pure culture (0.65 mg protein mL^{-1}). Uninoculated (control) and bacterial inoculated flasks were incubated at 37°C on a rotary shaker (120 rpm) in dark

conditions for 3 days.

Rice straw was provided by a farmhouse which cultivates Korean rice in Icheon, Gyeonggi, Korea. Air dried raw material without classification were cut into 3–5 cm lengths and stored in sealed plastic bags at room temperature for pretreatment. Prior to composition analysis, biomass was ground using a Wiley mill and, particles between the sizes of 40 and 80 mesh were collected. Milled rice straws were washed with water to remove dust and dried in an air forced oven at 60°C for 48 h.

As a control, *Thermobifida fusca* (NBRC 14071^{T}), obtained from NITE Biological Resource Center in Japan was used for the removal of lignin in rice straw. Cultivation was performed with nutrient broth medium (pH 6.0) and incubated at 45°C on a rotary shaker (170 rpm) in dark conditions for 10 days.

2.7 Determination of components of rice straw

The components (cellulose, Klason lignin, acid soluble lignin, and ash) of native or pretreated rice straw were determined as described previously (Zhu et al., 2009; Taniguchi et al., 1982). Holocellulose content was determined after solubilization with 72% sulfuric acid according to the phenol-sulfuric acid method using glucose as the standard (Zhu et al., 2009; Taniguchi et al., 1982). Hemicellulose content was calculated by subtracting the weight of cellulose from that of holocellulose.

2.8 Biological pretreatment methods for enhancing cellulase performance

Experiments were planned and divided into four different processing procedures as follows: (1) single pretreatment of rice straw by *Bacillus* sp. CS-1; (2) single pretreatment by two lactic acid bacteria (*Lactobacillus bulgaricus* (NBRC13953) and *Streptococcus thermophiles* (NBRC13957)); (3) two-step processing procedure (sequential pretreatment using lactic acid bacteria followed by *Bacillus* sp. CS-1; (4) two-step processing procedure (sequential pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria). After each pretreatment processing procedure, the content of holocellulose (hemicellulose and cellulose), cellulose, and lignin in rice straw were determined (Zhu et al., 2009; Taniguchi et al., 1982).

For the two-step processing procedure using lactic acid bacteria and *Bacillus* sp. CS-1, the following experiment was conducted. To obtain enough cell volume, pre-cultivation using lactic acid bacteria was performed using each optimum medium as documented by Chang et al. (2012b). Lignin and hemicellose degradation experiments used 300–mL glass–stoppered Erlenmeyer flasks containing 100 mL of growth medium and 3 g of milled rice straw. Growth medium had the following composition in 1000 mL of deionization water: 3 g peptone; 15 g malt extracts; and 40 g glucose. Each pre-cultivated culture (0.25 mg protein mL^{-1}) of the two lactic acid bacteria were inoculated in 100 mL of growth medium with milled rice straw and incubated for 3 days at 120 rpm on a rotary shaker at 30°C for the first processing procedure. Autoclaved growth medium served as the control, with the pH of the growth medium adjusted to 7 with NaOH (1.0 M) before autoclaving. Milled rice straws were then separated by centrifugation (2,180×g for 15 min), rinsed with deionized water twice and reacted for a further 3 days in a BSGYP medium also containing *Bacillus* sp. CS-1 (the second

processing procedure).

2.9 Enzymatic hydrolysis

After removing microorganisms growing on the rice straw as completely as possible, pretreated rice straw was enzymatically solubilized in a 50 mM sodium citrate buffer (pH 5.0). Hydrolysis experiments were conducted in a shaking water bath at 120 rpm and 40°C for 48 h. Cellulase (Cellulase Y-NC; Yakult Pharmaceutical Industry, Tokyo, Japan) was used at a protein concentration of 100 mg L^{-1}. The specific activity of cellulase was 30,000 U g^{-1}, according to the manufacturers' data. Carboxymethyl cellulose was used as a substrate to measure cellulase activity. The concentration of pretreated rice straw was 10 g L^{-1}. After an appropriate incubation time, the reaction mixture was centrifuged (8,000×g for 5 min), and the supernatant was filtrated with a glass filter (G-100; Advantec Toyo, Tokyo, Japan) to remove the residual substrate. Total soluble sugar and glucose in the resulting filtrate were determined using the phenol-sulfuric acid method (Masai et al., 2007) and high performance liquid chromatography using a Bio-Rad HPX-87H column., respectively.

The net yields of total soluble sugar (TS) and glucose (G) were determined on the basis of the amounts of holocellulose (H: cellulose and hemicellulose) and cellulose (C) in untreated rice straw, respectively, as follows:

Net yield of TS (%) ={ (amount of TS produced from residual straw after pretreatment) / (amount of H in untreated straw) } × 162/180 × 100

Net yield of G (%) ={ (amount of G produced from residual straw after pretreatment) /

(amount of C in untreated straw) } × 162/180 × 100

2.10 Data analysis

All results are shown as mean values with standard deviations (±95% confidence interval) from triplicate experiments, except for the pH and temperature effects on alkali-lignin degradation experiments. Statisticallys significant differences were determined using Student's *t* tests with a *p* value of <0.05.

2.11 Nucleotide sequence accession number

The 16S rRNA gene sequences of the isolates (strain CS-1 and strain CS-2) determined in this study have been deposited at the DNA Data Bank of Japan under Accession No. AB795826 and AB795827, respectively.

3. Results and Discussion

3.1 Isolation of bacterial strains

Of the sixty-eight soil samples, only four samples (Mt. Asahi in Hokkaido (one sample), Mt. Fuji in Shizuoka (one sample), and Mt. Yonahadake on Okinawa Island (two samples)) showed RBBR-decolorizing ability. Of these, the Mt. Asahi soil sample showed the highest decolorizing activity. The soil sample from Mt. Fuji showed

relatively low RBBR-decolorizing activity (data not shown).

Although RBBR-decolorizing activity varied, several RBBR-decolorizing bacteria were isolated from soil throughout Japan (from Hokkaido to Okinawa), clarifying the wide distribution of RBBR-decolorizing bacteria in Japanese forest soil (Table. 1). The RBBR-decolorizing rate of each isolate was determined. Identification of isolates was conducted using commercially available API identification systems (Table 1). As a results, several strains were isolated from Mt. Asahi and Mt. Yonahadake. When soil samples from Mt. Fuji was used in the isolation process, only a few genera of strains could be isolated. Among the isolates, two strains of *Bacillus* sp. from Mt. Asahi and Mt. Yonahadake showed the highest RBBR-decolorizing rates (Table 1). Until a few years ago, strains of *Bacillus* have not been well known as lignin-degrading bacteria. Lately, several lignin-degrading strains of *Bacillus* sp. have been isolated from around the world and their lignin-degrading and/or decolorizing abilities have been investigated (Bandounas et al., 2011). In current study, *Bacillus* sp. strains were found in all RBBR-decolorizing soil samples. These results indicate that *Bacillus* sp. may be an important microorganism and play a key role in degradation of lignin.

On the other hand, bacteria of several genera, including *Pseudomonas, Alcaligenes, Arthrobacter, Nocardia* and *Streptomyces*, can readily degrade the single-ring aromatic compounds that build up the lignin macromolecule (Bugg et al., 2011). There are also a number of literature reports on bacteria (*Streptomyces viridosporus* T7A, *Nocardia*, and *Rhodococcus*) that are able to break down lignin (Yang et al., 2012). Strains of *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA1 were found to show lignin-degrading activity that is comparable with *S. viridosporus* T7A (Yang et al.,

2012). There was also a report of a lignin degradation bacterial consortium named LDC that was screened from the sludge of a reed pond. It could break down 60.9% of the lignin in the reeds at 30 °C under static culture conditions (Wang et al., 2013). Interestingly, strains isolated which are capable of decolorizing RBBR from the soil of Okinawa origin involved several of the same genus that have already been reported as lignin- and aromatic compound-degrading bacteria, such as *Pseudonomas*, *Arthrobacter*, *Nocardia*, and *Rhodococcus* (Bugg et al., 2011; Li et al., 2009). These results indicate that more various lignin-degrading bacteria could be distributed in Mt. Yonahadake compared with those of Mt. Asahi and Mt. Fuji.

Two RBBR-decolorizing bacteria (strains of *Bacillus* sp.) were isolated and named CS-1 and CS-2. 16S rDNA sequence (1,455 bp) analysis indicated that CS-1 from Mt. Asahi and CS-2 from Mt. Yonahadake were *Bacillus* sp. (100% identity with *Bacillus subtilis* (1514 bp); GenBank Accession No. HQ727971.1 and 100% identity with *Bacillus* sp. B37 (1508 bp); GenBank Accession No. JN656409.1), respevtively (data not shown)

The isolated strains were Gram-positive and rod-shaped bacterium. CS-1 and CS-2 were able to produce catalase, oxidase, and urease but not indole (Supplementary data 2). Utilization of citrate was positive but not propionate. The strains were positive for nitrate reduction and hydrolysis of casein tests but not H_2S production. CS-1 and CS-2 could grow using the following carbon sources: D-glucose, fructose, sucrose, glycerol, D-xylose, maltose, lactose, and D-mannitol. These data indicated that CS-1 and CS-2 resembled a member of the *Bacillus* genus.

3.2 Biodegradation of alkali lignin

As a result of isolation and screening, two strains of *Bacillus* sp. (CS-1 and CS-2) were selected for alkali lignin degradation experiments on the basis of their RBBR-decolorizing efficiency (Table 1). With initial concentrations of 0.05–2.0 g L^{-1}, at least 61% of alkali lignin could be degraded within 48 h (data not shown). There was a significant biodegradation of alkali lignin (0.5 g L^{-1}) in the culture after 24 h; the removal ratio of lignin was up to 40%, reaching 80% on the second day of incubation (Fig. 1). The maximum lignin degradation rate of CS-1 was estimated to be 99.5% at a concentration of 0.05 g L^{-1} (data not shown). This result is much better than that previously reported (Tuomela et al., 2000). The removal percentages of lignin by CS-1 were higher than several novel actinomycete strains, including *Streptomyces* spp. strains F-6 and F-7 (Yang et al., 2012).

Cell growth was in accordance with the lignin degradation ratio (Fig. 1). On the other hand, CS-1 and CS-2 were not able to use lignin as the sole carbon source (data not shown).

Environmental parameters showed great influence on the growth of organisms and the degradation of lignin. The optimum temperature for the best degradation of bacterial isolates was determined using various temperatures (Supplementray data 3). Experiments indicated that this was 37ºC. Temperature influence on the growth of CS-1 and the degradation of lignin was in accordance with cell growth (Supplementray data 3). Results also indicated that the optimum pH for the degradation of lignin was around 8. Like temperature, degradation activity relied on cell growth of CS-1.

A number of bacteria capable of degrading lignin have been reported (Bugg et al., 2011; Tuomela et al., 2000). Of those bacteria, lately, several *Bacillus* sp. strains have been reported as lignin-degrading bacteria. *Bacillus* sp. (CSA105) was isolated from sediment core from the pulp and paper mill industries and purified ligninolytic enzyme from the cell extract. In addition, several kraft lignin-degrading *Bacillus* sp. have been isolated (Bandounas et al., 2011, Chandra et al., 2007; Raj et al., 2007). These results indicate that *Bacillus* sp. may be an important microorganism and may play a key role in lignin biodegradation..

3.3 Enzyme activity

The activities of three enzymes were determined. Both *Bacillus* sp. strain CS-1 and CS-2 showed very low manganese peroxidase activity (data not shown). Laccase activity of both CS-1 and CS-2 were at high levels (Fig. 2). LiP activity was not observed (data not shown). Laccase activity was associated with growth in CS-1 and CS-2 (Fig. 2). Intracellular laccase activity was 3.4 times higher than the extracellular laccase activity (data not shown). This result suggests that more local activity was cell-associated.

Alkali lignin degradation rate of CS-1 was slightly higher than that of CS-2 (data not shown). This result seems to be resulted in the difference of laccase activity (Fig. 2). The effect of temperature on laccase activity using a crude intracellular enzyme was investigated. The optimum temperature for ABTS oxidation was determined to be between 55–75°C. For ABTS oxidation, a steady increase of activity up to 70°C was

monitored, demonstrating the high temperature tolerance of laccase (data not shown). Due to this unusual property, laccase from *Bacillus* sp. CS-1 and CS-2 may be of significant importance in industrial applications. Generally, the rate of biological pretreatment is too slow for industrial purposes. However, the two isolated *Bcillus* sp. strains, CS-1 and CS-2, possessed high lignolytic enzyme activities (laccase activities), and lignin-degrading time is very fast.

Laccase and laccase-producing microorganisms play an important role in bioremediation of aromatic compounds from contaminated soils, industrial pollutants and xenobiotics. Laccases are generally found in plants and fungi, but they have also been reported in a few bacteria, including *Azospirillum lipoferum*, *Bacillus sphaericus*, *Marinomonas mediterranea*, *Streptomyces griseus*, and *Serratia marcescens* (Sheikhi et al., 2012). Bacterial laccases are more amenable to genetic manipulation than fungal laccases. Therefore, research and study of bacterial laccases is very interesting.

Bacterial laccase of *Bacillus* genus was first reported by Claus and Filip (1997). Since, then, more bacterial laccases have been found. *Bacillus subtilis* WPI showed laccase-like activity towards the oxidizing substrates ABTS and guaiacol (Sheikhi et al., 2012). However, *Bacillus megaterium* and *Bacillus* sp. (CSA105) strain showed no correlation with laccase activity on bioalteration of kraft lignin (Kharayat and Thakur, 2012). These results indicate that the type of ligninolytic enzymes involved in lignin degradation might be differ from the biochemical characteristics, even if the strain is of the same genus.

3.4 Biodegradation of lignin in rice straw by Bacillus sp. CS-1

Native straw was composed of cellulose (38%), hemicellulose (25%), Klason lignin (21%), acid-soluble lignin (4.8%) and other materials, mainly ash (11.2%). The growth of CS-1 resulted in weight loss of dry rice straw (data not shown). All the main components (cellulose, hemicellulose, and Klason lignin) were partially degraded (Table 2). In bacterial pretreatment, 3.2% cellulose and 20% Klason lignin were degraded with CS-1. The ratio of hemicellulose removed was only 19.2% (Table 2).

Thermobifida fusca (NBRC 14071^{T}), is considered as one of the most effective fungi for the selective removal of lignin on rice straw (McCarthy and Broda, 1984). In this study, *Thermobifida fusca* was able to remove 18% of Klason lignin in rice straw during incubation (Fig. 3). This degradation activity was lower than that of other fungi, for example, *P. ostreatus* (30% Klason lignin), but comparable to that of *P. simplicissimum* (15.1%) (Yang et al., 2012).

CS-1 tested in this study might be promising because the removal of Klason lignin on rice straw was comparable with that of fungi (*Thermobifida fusca* and *P. ostreatus*). Otherwise, the lignin-degrading activity was lower than that of other fungi, for example, *Fusarium moniliforme* (34.7%) and *Penicillium* sp. strain apw-tt2 (66.3%), which isolated from rice straw (Wulandari et al., 2013; Chang et al., 2012a).

3.5 Biological pretreatment methods for enhancing cellulase performance

Figure 4 shows the changes in the components of rice straw after pretreatment. The composition ratio of cellulose in the two-step process procedure was higher than that of

the single pretreatment. The maximum composition ratio of cellulose was observed after the two-step processing procedure (sequential pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria) at 53.1% (Fig. 4). The composition ratio of Klason lignin was the lowest at 11.4% in this condition. The composition ratio of hemicellulose after the single pretreatment using lactic acid bacteria was lower than that of CS-1.

With the single pretreatment by *Bacillus* sp. CS-1, 16% hemicellulose and 3.2% cellulose were degraded and the ratio of Klason lignin removed was 20%. With the two-step lactic acid bacteria pretreatment, 40% hemicellulose and 7.9% cellulose were degraded and the ratio of Klason lignin removed was 14.3%. The maximum degradation ratio of Klason lignin was observed after the two-step processing procedure (sequential pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria) at 61.9% (Table 2). This lignin-degrading ratio is comparable to that of several fungi which have shown a high capacity of lignin degradation (Corredor, 2008).

To evaluate the effect of pretreatment of rice straw, pretreated rice straw was hydrolyzed with a commercial crude cellulase. The amount of total soluble sugar obtained from rice straw pretreated with each pretreatment process procedure was approximately 1.5 or 4.3 times higher than that of untreated rice straw (Table 3).

The amount of glucose in all enzymatic hydrolysates of pretreated rice straw was less than one-half of the amount of total soluble sugar (Table 3). These findings show the presence of some oligosaccharides and/or pentose (mainly xylose), which were detected using high performance liquid chromatography (data not shown).

In this study, lactic acid bacteria producing lactic acid and pyruvic acid were examined to investigate the removal of hemicellulose in rice straw. The amounts of pyruvic and

lactic acids in the reactive liquid were measured after cultivation. The maximum concentration of pyruvic acid and lactic acid produced was 1.65 and 1.54 (g L^{-1}), respectively; and consequentially, the pH of the cultures decreased below 3.7 (Supplementray data 4).

Hemicellulose is a physical barrier which surrounds cellulose fibers and can protect the cellulose from enzymatic attack. Cellulose and hemicellulose are cemented together by lignin, preventing swelling of lignocelluloses and decreasing the yield of bioconversion (Mohammad and Keikhosro, 2008). The results indicate that lactic acid and pyruvic acid could be used for removing hemicellulose because about 40% of the initial hemicellulose was degraded in the reaction liquid (Table 3). A single pretreatment using lactic acid bacteria was more effective compared with *Bacillus* sp. CS-1 and untreated (control) samples on the basis of net yields of sugars, even though the lignin degradation ratio was slightly lower than that of *Bacillus* sp. CS-1 (Table 3). This result suggest that hemicellulose elimination is also an important factor with the lignin degradation on rice straw for saccharification. The removing of hemicellulose might result in improving surface area accessible to cellulose. These results are directly in line with previously reported literatures (Rollin et al. 2011; Corredor, 2008).

Both of the two-step processing procedures were more effective compared with the single pretreatment method in net yields of sugars, probably because the extent of lignin and hemicellulose degradation increased cellulase accessibility to the cellulose of rice straw. Judging from the net yields of sugars after enzymatic hydrolysis, the most effective method was the two-step processing procedure (pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria) at 68.6% (Table 3). The second most effective

method was the two-step processing procedure (pretreatment using lactic acid bacteria followed by *Bacillus* sp. CS-1) at 57.1%. Nearly 30% of the rice straw was decomposed. A similar ability was observed with a fungus; *C. stercoreus* TY-2 showed an enzymatic saccharification yield of 57% for 25 days (Yamagishi et al., 2011).

Although, chemical pretreatment using sulfuric acid and a heating procedure at 120–200°C could achieve greater than 90% conversion of cellulose and hemicellulose to fermentable sugars (Wang et al., 1998), the net sugar yields of total soluble sugar obtained in this study are higher than fungal pretreatements with *P. ostreatus* (33%) or chemical pretreatments with NaOH, peracetic acid and sodium chlorite (32–42%), as described previously (Taniguchi et al., 1982).

4. Conclusions

Effective biological pretreatment method for enhancing cellulase performance was investigated. The pretreated substrate (rice straw) was found to have greatly increased cellulase performance in accordance with the decreased amount of lignin and hemicellulose when the two-step pretreatment procedure using CS-1 and lactic acid bacteria was implemented. These results suggest that the two-step pretreatment procedure is effective at accelerating cellulase performance. However, to meet the requirements of large-scale pretreatment procedures, further studies including configuration of a suitable process and optimization of culture conditions for efficient recovery of sugars are needed.

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Figure legends

Fig. 1. Time course of biodegradation of alkali lignin by *Bacillus* sp. CS-1 with 500 mg L^{-1} of alkali lignin. Data represent means of triplicate experiments. Error bars indicate 95% confidence intervals.

Fig. 2. Time courses of alkali lignin degradation and laccase activity in the culture of strain CS-1 and strain CS-2. Initial concentration of alkali lignin was 500 mg L^{-1}. Data represent means of duplicate experiments.

Fig. 3. Time course of Klason lignin degradation in rice straw. Comparison of Klason lignin-degrading activity was performed with *Thermobifida fusca* and strain CS-1. Cultivation of *Thermobifida fusca* and strain CS-1 was performed at 45°C and 37°C on a rotary shaker. Data represent means of triplicate experiments. Error bars indicate 95% confidence intervals.

Fig. 4. Changes in percentage composition of components of rice straw after single and two-step procedure pretreatment. Components: HC, hemicellulose; C, cellulose; AL, acid soluble lignin; KL, Klason lignin; O, others (mainly ash).

Fig. 1

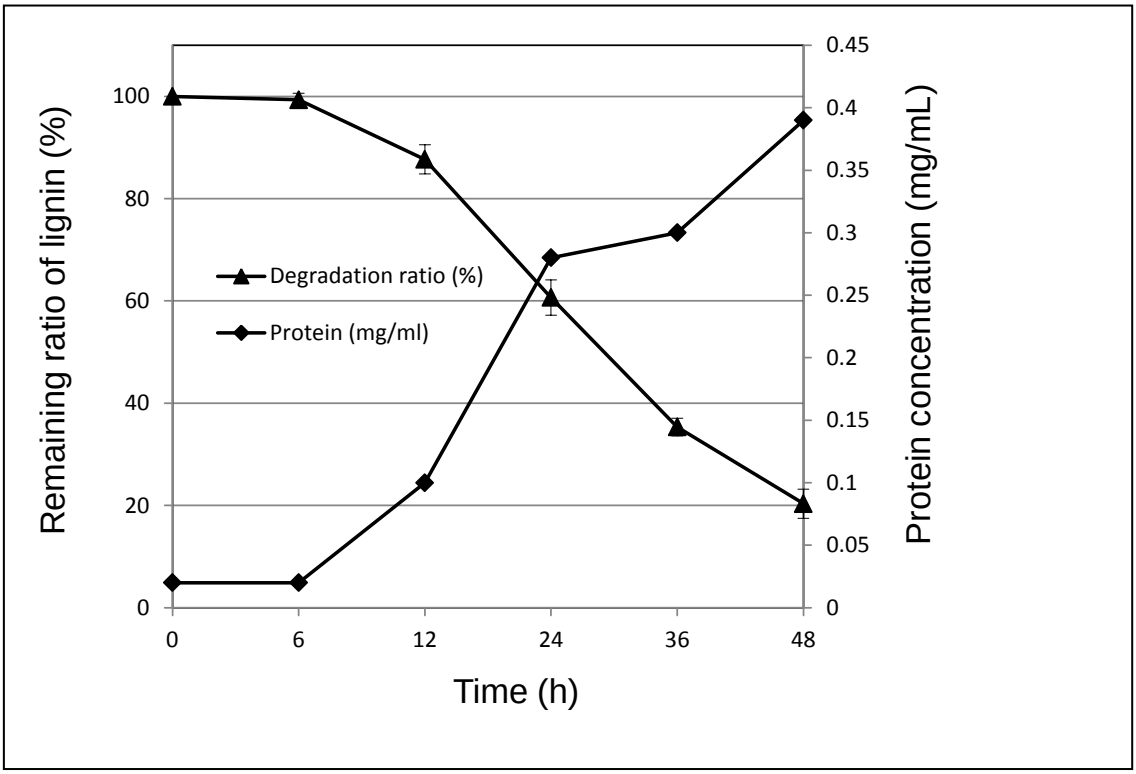

Remaining ratio of lignin (%)
Protein concentration (mg/mL)
Time (h)
Degradation ratio (%)
Protein (mg/ml)
0
20
40
60
80
100
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0
6
12
24
36
48

Fig. 2

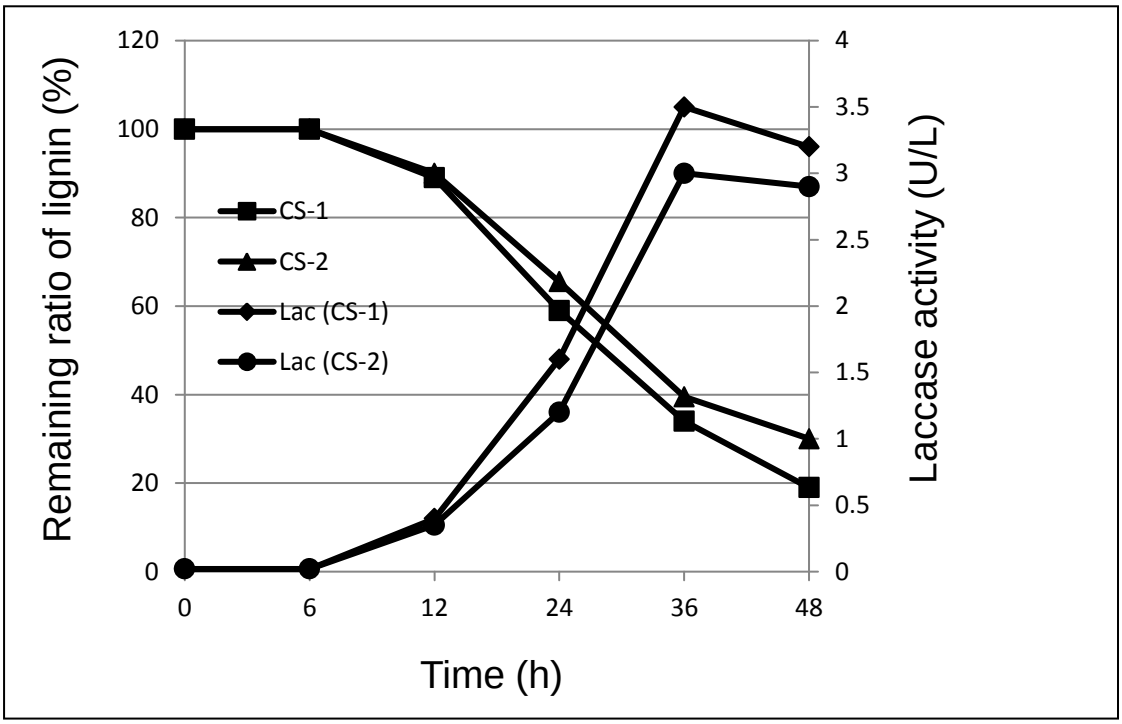

Remaining ratio of lignin (%)
Laccase activity (U/L)
Time (h)
CS-1
CS-2
Lac (CS-1)
Lac (CS-2)
120
100
80
60
40
20
0
4
3.5
3
2.5
2
1.5
1
0.5
0
0
6
12
24
36
48

Fig. 3

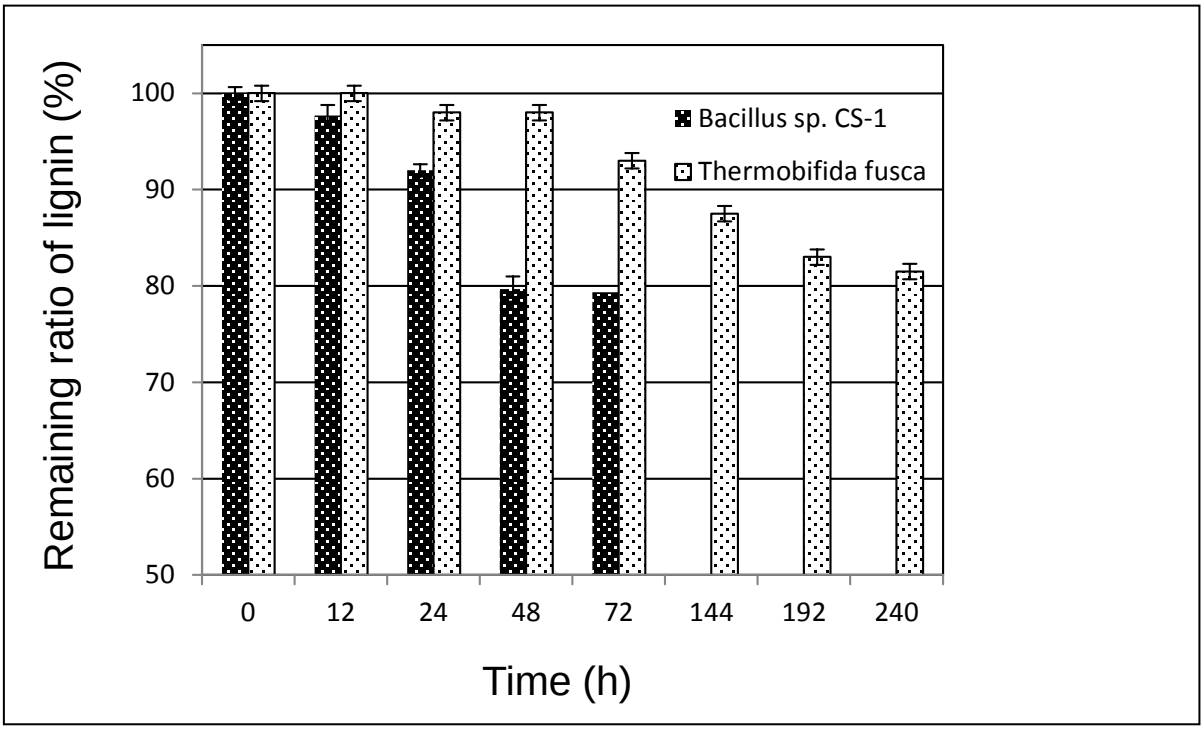

Remaining ratio of lignin (%)
100
90
80
70
60
50
0
12
24
48
72
144
192
240
Time (h)
Bacillus sp. CS-1
Thermobifida fusca

Fig. 4

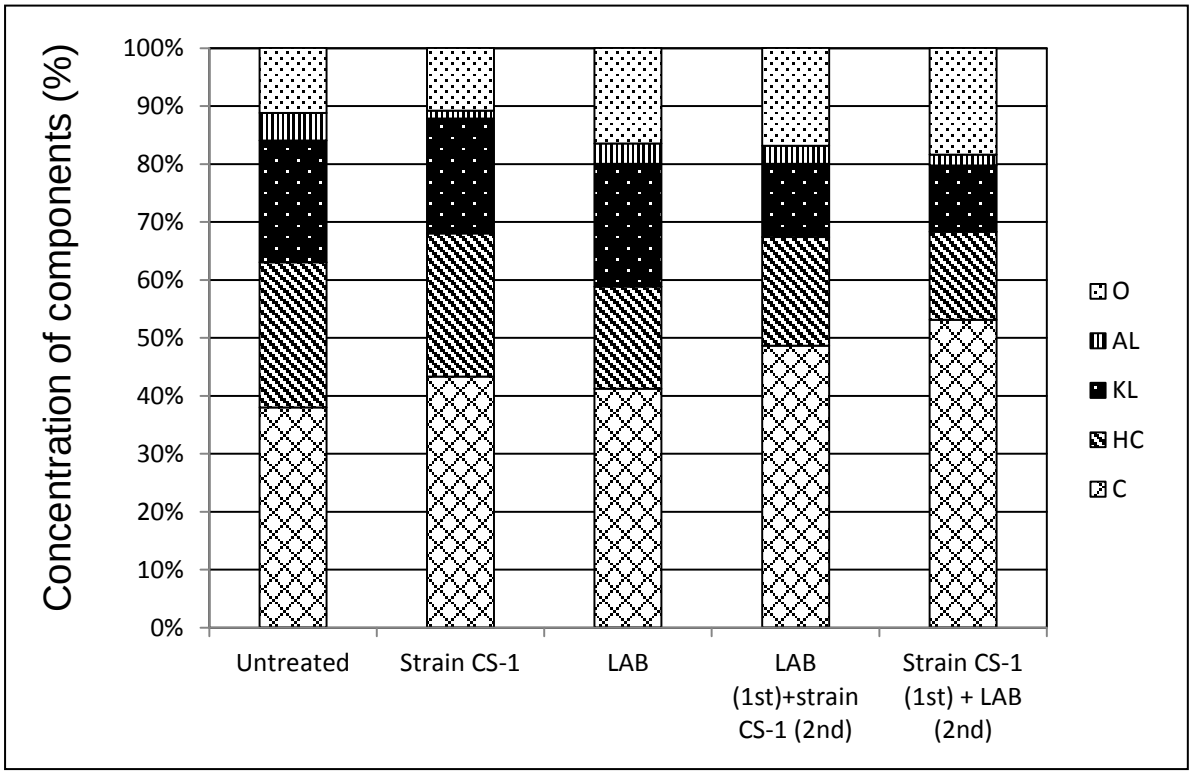

Concentration of components (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Untreated
Strain CS-1
LAB
LAB (1st)+strain CS-1 (2nd)
Strain CS-1 (1st) + LAB (2nd)
O
AL
KL
HC
C

Table 1. Decolorization of Remazol Brilliant Blue R (RBBR) by various soil samples.

Source	Species	Percentage depletion after 3 days[a]
Mt. Fuji, Shizuoka	*Bacillus* sp.	27
	Actinomyces sp.	20
	Pseudonomas sp.	18
Mt. Asahi, Hokkaido	*Bacillus* sp.	100
	Burkholderia sp.	38
	Ralstonia pickettii	37
Mt. Yonahadake, Okinawa Island	*Bacillus* sp.	95
	Pseudonomas fluorescens	38
	Arthrobacter spp.	59
	Nocardia spp.	49
	Sphingomonas sp.	48
	Rhodococcus sp.	25

The initial concentration of RBBR was 0.01 % (v/w). Identification was only performed using isolates capable of decolorizing RBBR more than 15%.

[a] Results are expressed as a percentage of the decolorized RBBR after 3 days of incubation. The initial percentage before incubation was considered to be 0%. Each value represents an average of two analyses (differences of data obtained from two analyses was within 3.8%).

Table 2. Residual amounts of components of rice straw after pretreatment process.

Pretreatment	Residual amounts (%)		
	HC	C	KL
Bacillus sp. CS-1	80.8	96.8	80.0
Lactic acid bacteria	60.0	92.1	85.7
Two-step (lactic acid bacteria (1st) and *Bacillus* sp. CS-1 (2nd))[a]	32.0	73.7	66.7
Two-step (*Bacillus* sp. CS-1 (1st) and lactic acid bacteria (2nd))[b]	48.0	81.6	38.1

[a] Two-step processing procedure (pretreatment using lactic acid bacteria followed by *Bacillus* sp. CS-1).
[b] Two-step processing procedure (pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria).
The initial concentration of rice straw was 10 g/L. Native straw was made up of cellulose (C, 38%), hemicellulose (HC, 25%), and Klason lignin (KL, 21%). The chemical compositions of pretreated rice straw were determined based on the amounts of C, HC, and KL in the untreated rice straw.

Table 3. Net yields of total soluble sugar and glucose from rice straw samples pretreated with each pretreatment method.

Pretreatment	Concentrations (g/L)[a]				Net sugar yield (%)[b]	
	TS		G		TS	G
Untreated	1.12		0.63		16.0	14.9
Bacillus sp. CS-1	1.70		0.84		24.3	19.9
Lactic acid bacteria	2.10		1.02		30.0	24.2
	1st[e]	2nd	1st	2nd		
Lactic acid bacteria (1st) and *Bacillus* sp. CS-1 (2nd)[c]	2.10	4.0	1.0	1.94	57.1	45.9
Bacillus sp. CS-1 (1st) and lactic acid bacteria (2nd)[d]	1.70	4.8	0.82	2.30	68.6	54.5

[a] Concentrations of total soluble sugar or glucose after enzymatic hydrolysis for 48 h.
[b] Net sugar yields were calculated as described in the Materials and Methods section.
[c] Two-step processing procedure (pretreatment using lactic acid bacteria followed by *Bacillus* sp. CS-1).
[d] Two-step processing procedure (pretreatment using *Bacillus* sp. CS-1 followed by lactic acid bacteria). The initial concentration of rice straw was 10 g/L. Native straw was made up of cellulose (38%), hemicellulose (25%), Klason lignin (21%), acid-soluble lignin (3.8–4.0%), and ash (12%).
[e] Net yields of total soluble sugar after 1st pretreatment.
TS: total soluble sugar; G: glucose.

Supplementary data 1

Map showing locations where soil samples were collected.

It ve stratovolcano and the tallest peak in Hokkaido (2,290 m). Mt. Yonahadake is the highest mountain on Okinawa Island at 503 m. The temperatures at sampling sites were 9°C (Mt. Asahi), 13°C (Mt. Fuji), and 25°C (Mt. Yonahadake). Soil samples were taken at 0–15 cm depth.

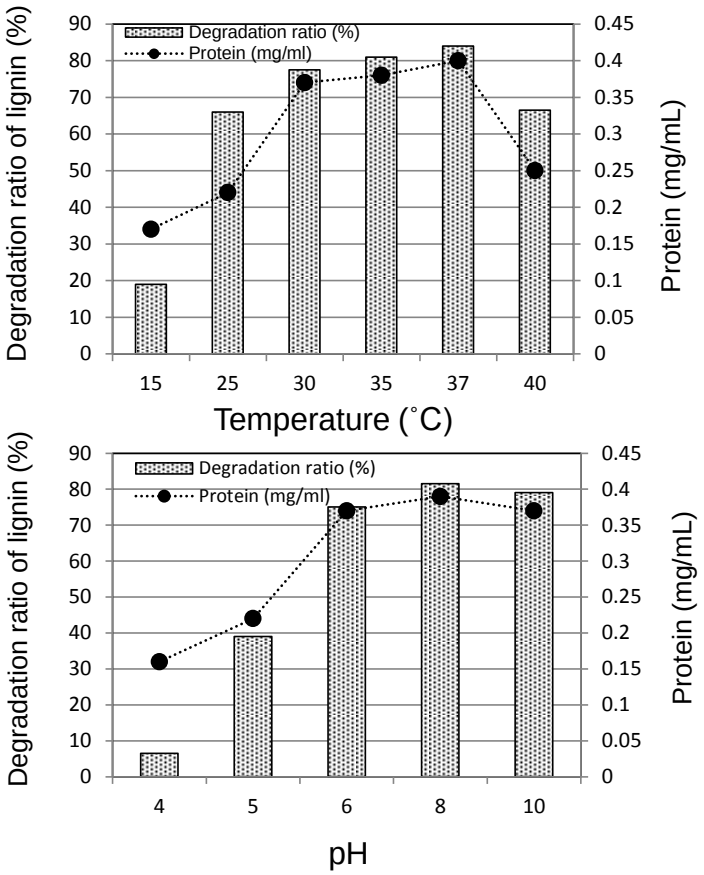
Supplementary data 2

Conventional testes for characterization of bacterium.

Physiological and biochemical testes	Strain CS-1&CS-2	*B. subtilis*
Shape	Rods	Rods
Gram reaction	Gram-positive	Gram-positive
Motility	–	–
Spores	+	+
Growth in air	+	+
Anaerobic growth	–	–
Growth at 50 ºC	+	+
Growth in 10% NaCl	+	+
Catalase	+ (slow)	+ (slow)
Oxidase	+	+
Oxidative/ Fermentative	Non sacchrolytic	Non sacchrolytic
Cellobiose	+ (slow)	+ (slow)
D-glucose	+	+
Sucrose	+	+
Fructose	+	+
Lactose	–	+
Maltose	+	+
Glycerol	+	+
D-xylose	+	+
Casein hydrolysis	+	+
Starch hydrolysis	+	+
Propionate utilization	–	–
Citrate utilization	+	+
H_2S production	+	+
Indole production	–	–
Hydrolysis of tyrosine	–	–
Urease test	+	+
Nitrate reduction	+ (fast)	+ (fast)

+ Positive; - Negative

Supplementary data 3



Effect of temperature (a) and pH (b) on the degradation of alkali lignin. The concentrations of protein were determined after 48 h cultivation. Initial concentration of alkali lignin was 500 mg l^{-1}. Data represent means of duplicate experiments.

Supplementary data 4

Time course of concentrations of pyruvic and lactic acids in reactive liquid.

Reaction time (h)	Pyruvic acid (g l^{-1})	Lactic acid (g l^{-1})
6	0	0
12	0.38	0.32
24	0.87	0.72
48	1.62	1.49
36	1.65	1.54

Experiments were performed in duplicate.