Characterization of a Novel Laccase-producing *Bacillus* sp. A4 and its Application in *Miscanthus* Degradation

Haipeng Guo,[a,b] Chaoyang Lin,[a,c] Shihui Wang,[a,d] Dean Jiang,[b] Bingsong Zheng,[e] Yun Liu,[d] and Wensheng Qin [a,*]

Bacillus sp. A4 exhibiting laccase production was isolated from forest soil. Its laccase secreted into a LB medium exhibited a maximum activity of 3.9 U mg^{-1} protein at the optimal temperature (37 °C) and pH (6.0). The purified laccase of *Bacillus* sp. A4 demonstrated a low molecular mass of 33 kDa, and its optimal temperature and pH were 40 °C and 4.6, respectively, when using ABTS as a substrate. The activity of the purified laccase was significantly increased in the presence of Cu^{2+}, methanol, and ethanol, but it was totally inhibited by L-cysteine. The laccase production of this strain was markedly stimulated when the strain was incubated with 0.5% different lignocellulosic biomasses. The highest activity of laccase (22.6 U mg^{-1} protein) was obtained in using algal biomass. This new strain efficiently decreased the lignin content of lignocellulose biomasses after 9 d of incubation at 37 °C, especially lignin from grasses. Further analysis showed that, compared to that of all tested biomasses, the new strain was a more efficient decomposer of the lignin of *Miscanthus*, which exhibited much more lignin loss and cell wall structure destruction in a short span of time. Therefore, the potential use of this strain could be advantageous for using lignin in *Miscanthus* for industrial processes.

Keywords: Laccase; Bacillus sp.; Miscanthus; Lignin; Scanning electron microscope

Contact information: a: Department of Biology, Lakehead University, 955 Oliver Road, Thunder Bay, ON P7B 5E1 Canada; b: College of Life Sciences, Zhejiang University, Hangzhou, ZJ 310058 China; c: State Key Laboratory of Rice Biology and Institute of Insect Science, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, ZJ 310058 China; d: Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029 China; e: State Key Laboratory of Subtropical Silviculture, Zhejiang A & F University, Linan, ZJ 311300 China;
** Corresponding author: wqin@lakeheadu.ca*

INTRODUCTION

Laccases (EC 1.10.3.2) are a group of copper-containing oxidases that play an important role in oxidizing a range of aromatic compounds in nature, such as phenolic units and amine compounds in lignin (Leonowicz *et al.* 2001; Majeau *et al.* 2010). The characteristics of laccase have been widely studied in fungi, especially white rot fungi, which have been regarded as the most efficient laccase producers and are used in industrial processes (Senthivelan *et al.* 2016). However, fungi often grow slowly and require a long time to produce laccase; also, most of their laccases have the drawbacks of low thermostability and a limited pH-activity range (pH 4.0 to 6.0) (Majeau *et al.* 2010; Mukhopadhyay *et al.* 2013). The majority of fungal laccases usually rapidly drop their activity at temperatures above 60 °C and the half-life range of their activities at 60 °C was reported as 0.3 to 2 h (Hildén *et al*. 2009). To overcome the obstacles inherent in fungal laccases, more and more researchers have focused on bacterial laccases due to their rapid production, superior stability at high temperatures and pH values, and easy

genetic manipulation, although the laccase production yields of bacteria are lower than those of fungi (Martins *et al.* 2002; Muthukumarasamy and Murugan 2014).

To date, bacterial laccases have been widely reported and applied to lignocellulosic degradation and bioremediation. For example, Saritha and Lata (2012) isolated a *Streptomyces griseus* from leaf litter and found that it selectively decreased the Klason lignin content of hardwood and softwood and produced high levels of cellulose complex when growing on wood substrates. A recombinant protein CotA from *Bacillus licheniformis* showed spectroscopic properties and effectively oxidized syringic acid to 2,6-demethoxy-1,4-benzoquinone (Koschorreck *et al.* 2008). A newly discovered *Bacillus licheniformis* LS04, exhibiting high laccase activity, was isolated from forest soil and was shown to be able to decolorize various chemical dyes (Lu *et al.* 2012). Lončar *et al.* (2013) screened a laccase production strain and identified it as *Bacillus amyloliquefaciens,* which showed wastewater decolorization ability. Furthermore, it was reported that bacterial laccases showed superior tolerance to various harsh environments. Fang *et al.* (2011) isolated a bacterial laccase from a marine microbial metagenome that exhibited chloride tolerance and dye decolorization ability. The spore laccase of *Bacillus licheniformis* LS04 was found to be quite stable at high temperatures, showed no loss of laccase activity after 10 days of incubation at pH 9.0, and demonstrated organic solvent tolerance (Lu *et al.* 2012).

Lignocellulosic biomass consisting of carbohydrate polymers (cellulose and hemicellulose) and an aromatic polymer (lignin) is thought to be the most abundantly sustainable raw material on Earth that can be used for the production of biofuels and chemicals. Among various biomass sources, the *Miscanthus* species are promising crops for bioethanol production due to their high biomass yield and remarkable adaptability to different environments (Brosse *et al.* 2012). However, biomass recalcitrance has posed a significant challenge in biofuel production. This is due to the fact that lignin-carbohydrate complexes formed by lignin are intimately interlaced with carbohydrates through ester and ether bonds, preventing the enzymatic hydrolysis of fermentable sugars (Zhao *et al.* 2012; De Souza *et al.* 2015). Among the various techniques developed for the removal of biomass recalcitrance from lignocellulosic biomass, the engineering of microbial fermentation has been proposed as a cost-efficient, eco-friendly, and sustainable development strategy to reduce the risk to the environment and improve the efficiency of biofuel production (Maki *et al.* 2009; Weber *et al.* 2010). In this study, the authors isolated a novel laccase-producing *Bacillus* sp. A4 strain that showed excellent growth and laccase production characteristics. Furthermore, the strain was used to evaluate its degradative capability using various biomasses as carbon sources.

EXPERIMENTAL

Materials

Isolation and identification of laccase-producing strain Bacillus sp. A4

Bacillus sp. A4 was isolated from forest soil (Thunder Bay, Ontario, Canada). A soil sample (5.0 g) were suspended in 50 mL of sterile distilled water; the mixture was shaken at 200 rpm and 37 °C for 30 min. Then, the isolate was spread *via* the standard serial dilution plate method using a Luria-Bertani (LB) medium that contained 0.5 mM guaiacol. The plates were incubated at 37 °C for 3 to 5 days. The laccase-producing strains were visualized *via* the formation of reddish brown zones in the medium (Coll *et*

al. 1993). All the above experiments were performed in a super clean bench under sterile conditions. The A4 strain was picked up and purified for further study through single-colony isolation because it showed the largest reddish brown zone and the highest laccase activity. To identify the A4 strain, its genomic DNA was extracted with a Bacteria DNA kit (Bio Basic, Markham, Ontario, Canada). The internal transcribed spacer (ITS) region was amplified using the universal primers HAD-1 (5'-GACTCCTACGGGAGGCAGC AT-3') and E1115R (5'-AGGGTTGCGCTCGTTGCGGG-3'). The polymerase chain reaction (PCR) products were cloned into the pUCM-T vector and sequenced. Then, the sequence was retrieved by basic local alignment search tool (BLAST) searches on the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) website, aligned using ClustalX 1.83 (Thompson *et al*. 1997), and subjected to phylogenetic analysis using the neighbor-joining method with MEGA6 (MEGA Inc., Englewood, NJ) using 1,000 bootstraps (Lin *et al.* 2015).

Growth characterization of Bacillus sp. A4

In all experimental conditions, the A4 strain was incubated in a 250-mL Erlenmeyer flask containing 50 mL of the LB medium with agitation at 200 rpm. To determine the point of maximal strain growth and laccase production, the optical density at 600 nm (OD_{600}) and laccase activities in the supernatant and cells were determined on each day over a 9-day period, starting from the first day of inoculation. The OD_{600} cells were measured as a growth parameter. To investigate laccase activity, 1 mL of bacterial culture was harvested and centrifuged for 3 min at 12,000 g. The activities of the supernatant were immediately measured. The cells were re-suspended in 0.1 M potassium phosphate buffer (pH 7.4) that contained 50 mM KCl and were sonicated at a resonance of 40 kHz and 4 °C for 2 min using the "duty cycle" control, which consisted of 10 s of working time and 10 s of stop time. The cell extract was obtained *via* centrifugation at 12,000 g for 3 min as crude intracellular enzyme and kept at 4 °C until activity measurement. To investigate laccase production under different temperature and pH conditions, laccase activity was measured after culturing the strain at various temperatures (20 °C to 42 °C) and various pH values (4.4 to 8.7). The effect of the initial inoculum concentration on laccase production was investigated by inoculating the strain at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (v/v) concentrations.

Laccase activity and protein content assay

The laccase activity was measured according to the method of Guo *et al.* (2017), with some modifications. Briefly, a total of 200 µL of reaction mixture containing 20 µL of diluted crude enzyme and 20 µL of 20 mM 2 2'-Azino-bis [3-ethylbenzothiazoline-6-sulfonate] (ABTS; Sigma, St. Louis, MO, USA) in a 0.1 M citrate buffer (pH 4.6) was incubated at 40 °C for 3 min. The absorbance was then determined at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) using a Microplate Spectrophotometer (Epoch, Bio Tek Instruments, Inc., Winooski, VT, USA). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per min. The protein content was measured by the Bradford Protein Assay Kit (Bio Basic Canada Inc., Markhan, Ontario, Canada) according to the manufacturer's instructions.

Biological Characterization of Laccase

On the third day, the crude intracellular enzyme was obtained *via* the above-described method and purified according to the method of Wang *et al.* (2016). Then, the purified laccase was used in the following experiments.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis

To determine the molecular weight of the laccase from the A4 strain, the purified enzyme was run, along with standard protein marker, using a 12% (W/V) polyacrylamide gel with an 8% stacking gel according to the method of Lin *et al.* (2015). After electrophoresis, the gel was divided into two parts. One part of the gel was stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Oakville, Canada) to visualize the protein. The other part of gel was immersed in 1% Triton-X 100 for 30 min to remove the SDS. Following this, the protein bands with laccase activity were visualized by soaking the gel in 0.1 M citrate buffer (pH 4.6) containing 1.0 mM ABTS.

The effects of pH and temperature on laccase activity

The activity of purified laccase was assayed in a pH range of 3.8 to 8.2 at 40 °C. The buffer solutions used were as follows: pH 3.8 to 6.6 in 0.1 M citrate buffer and pH 7.0 to 8.2 in 0.1 M Tris-HCl buffer. The optimum temperature for the purified laccase was determined over the temperature range of 30 °C to 80 °C, using ABTS as the substrate, in 0.1 M citrate buffer (pH 4.6).

The effects of metal ions, organic solvents, and inhibitors on laccase activity

The effects of various metal ions on laccase activity were determined in the presence of 1.0 mM Li^{+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Al^{3+}, and Co^{2+} and 5.0 mM of the same metals. To determine the optimum Cu^{2+} concentration, laccase activity was measured at different Cu^{2+} concentrations from 0 mM to 10 mM. The effects of organic solvents, methanol, ethanol, acetone, and dimethyl sulphoxide on laccase activity were measured by adding 5%, 10%, and 20% (v/v) of these into the reaction mixture. The influences of SDS, L-cysteine, and ethylenediaminetetraacetic acid (EDTA) on laccase were examined after 30 min of incubation with the enzymes at 40 °C. To determine which concentration of L-cysteine could completely inhibit laccase activity, the activity of laccase was measured in the presence of 0.1 mM to 1.0 mM L-cysteine.

Lignin degradation of various biomasses using Bacillus sp. A4

To measure the degradation of lignin in various biomasses using *Bacillus* sp. A4, the strain was grown overnight in a LB broth medium. Then, 1 mL of the above-described bacterial suspension was transferred to 50 mL of a minimal salt medium containing 1.0 g L^{-1} sodium nitrate ($NaNO_3$), 1.0 g L^{-1} dipotassium phosphate (K_2HPO_4), 1.0 g L^{-1} potassium chloride (KCl), 0.5 g L^{-1} magnesium sulfate ($MgSO_4$), 0.5 g L^{-1} yeast extract, and 5% algae, agave, wild rice husk, 2% NaOH-pretreated *Miscanthus sacchariflorus* (*M. sacchariflorus*) [incubated *M. sacchariflorus* with 2% sodium hydroxide (NaOH) for 3 h at 50 °C], wheat straw, non-pretreatment *M. sacchariflorus*, wood dust, or pine in 250-mL Erlenmeyer flasks. The flasks were placed in an incubator shaker at 37 °C with agitation at 200 rpm for 9 d. The fermentation broth was harvested every day and centrifuged at 12,000 g for 3 min. The supernatants were used to complete the laccase activity measurements.

After 9 d of fermentation, the pretreated biomass, after being separated through filtration, was dried at 80 °C for 72 h and used for the lignin content analysis. The lignin content levels of various biomasses were determined *via* an acetyl bromide procedure (Johnson *et al.* 1961; Iiyama and Wallis 1988). Briefly, the samples (1 mg) were placed in 15-mL glass reaction bottles with solutions of 25% (w/w) acetyl bromide in acetic acid (1 mL). The bottles were sealed with polytetrafluoroethylene-coated silicone caps and incubated at 70 °C for 2 h. Then, the mixture was diluted to 10 mL with acetic acid that contained 2 M sodium hydroxide and 7.5 M hydroxylamine hydrochloride. The lignin content was measured by examining the absorbance at 280 nm, using lignin absorptivities of 23.3 g^{-1} L cm^{-1} for algae, agave, wild rice husk, *M. sacchariflorus,* and wheat straw and 23.6 g^{-1} L cm^{-1} for wood dust and pine.

Methods

Scanning electron microscope (SEM) observation

After 16 d of incubation, the remaining residues from the bacteria-treated non-pretreatment *M. sacchariflorus* were collected through filtration and washed with distilled water three times. Then, the samples were dried at 40 °C, sputter-coated with gold (Ion sputter E-1030; Hitachi Ltd., Tokyo, Japan), and observed *via* scanning electron microscopy (SEM Hitachi AU-70, Tokyo, Japan).

Statistical analysis

All of the experiments were performed in triplicate, and the results are shown as means ± SDs. The statistical analysis was performed *via* a one-way analysis of variance using SPSS (SPSS Inc., Chicago, USA, Version 13.0).

RESULTS AND DISCUSSION

Isolation and Identification of A4 Strain

A total of 12 isolates showed a brown color around the margin of the colonies on the LB medium plate that contained 0.5 mM guaiacol.

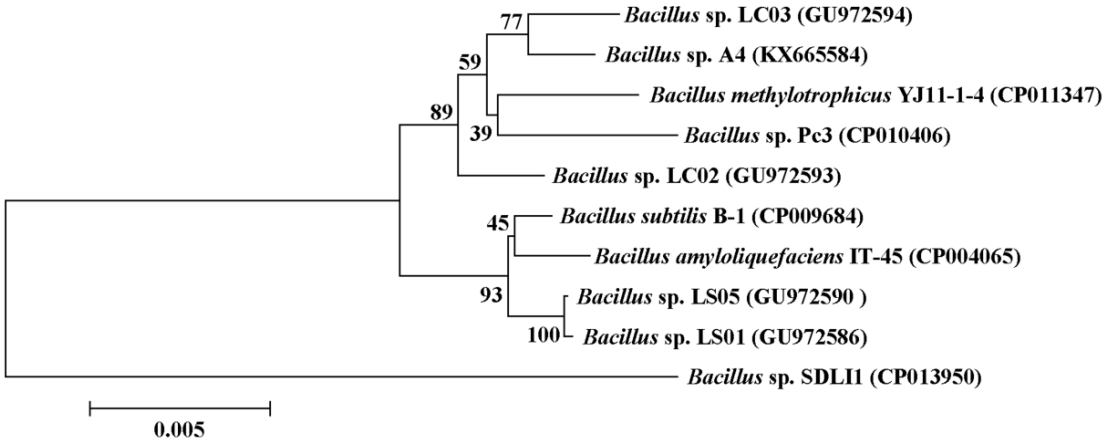

Fig. 1. Phylogenetic tree of 16S rDNA gene sequences between strain A4 and other related *Bacillus* species; 16S rDNA gene sequences were retrieved by BLAST searches in NCBI and subjected to phylogenetic analysis using the neighbor-joining method with MEGA6 using 1,000 bootstraps

One isolate, A4, which could oxidize ABTS in 1 min and thus exhibited the highest level of laccase activity, was selected for further study. The strain showed colonies of approximately 1 mm in diameter that were white in color and Gram-positive on the LB medium plate without guaiacol. Furthermore, the A4 strain also exhibited cellulose activity in the carboxymethyl cellulose agar plate assay (data not shown). To identify the strain, the 16S rDNA gene sequence of A4 was amplified and sequenced. The blast analysis of the sequence in NCBI and the phylogenetic tree showed that this strain belonged to the *Bacillus* genus (Fig. 1). Therefore, the A4 strain was finally identified as *Bacillus* sp. according to the morphological and phylogenetic characteristics of this strain, as well as the comparative analysis of the 16S rDNA sequence.

Strain Growth Characteristic and Laccase Production

The effects of incubation time, temperature, pH, and inoculum concentration on A4 strain growth and laccase production were measured by recording the OD_{600} and relative laccase activity of the fermentation supernatant under various conditions (Figs. 2 and 3). The strain growth and laccase production of the supernatant and cells of *Bacillus* sp. A4 significantly increased initially and then decreased with increased incubation time (Fig. 2). The OD_{600} of the A4 strain reached a maximum of 2.55 after 2 d of incubation, at which time the laccase activity in the supernatant was first detected, and then reached a peak after 5 d with a value of 3.9 U mg^{-1} protein. Thereafter, the laccase activity level decreased and remained relatively constant until Day 8.

The intracellular laccase activity of the A4 strain increased significantly with incubation time up to 3 d, at which point it reached a value of 5.4 U mg^{-1} protein, and then decreased with additional incubation time (Fig. 2). Furthermore, the optimal temperature, pH, and inoculum concentration for laccase production were 37 °C, 6.0, and 2.0%, respectively (Fig. 3).

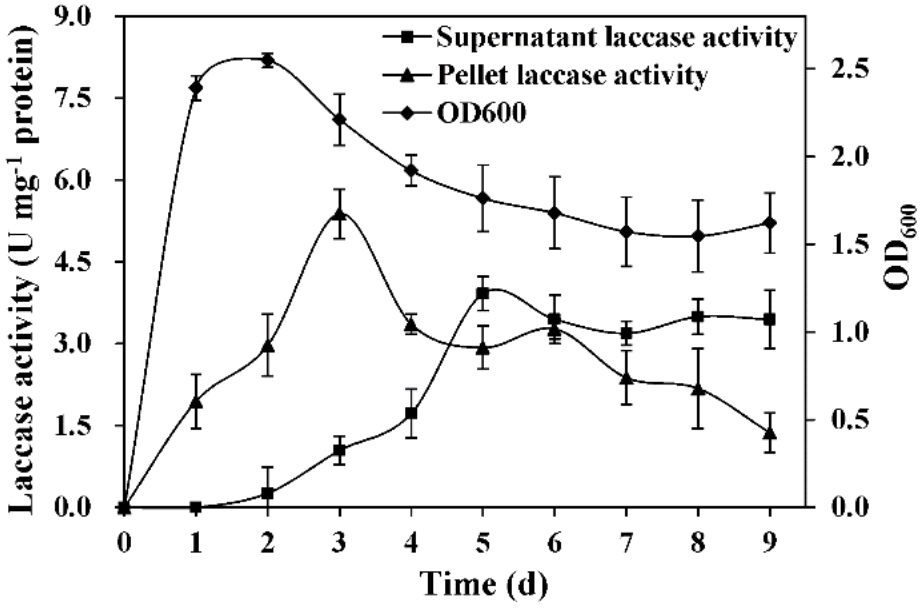

Fig. 2. Time course of OD_{600} and laccase production in supernatant and cells at 37 °C; Data shown are means ± SDs (n = 3)

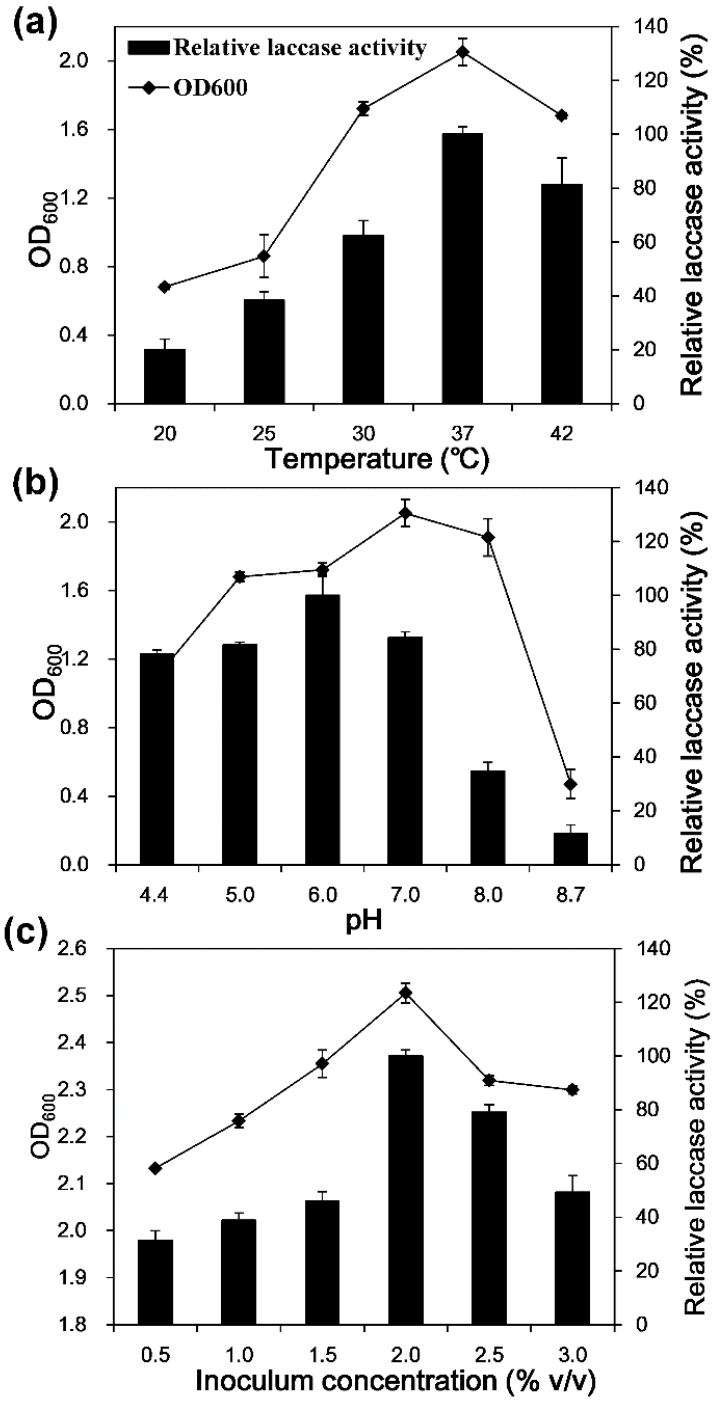

Fig. 3. Effects of culture temperature (a), pH (b), and incubation concentration (c) on OD_{600} and laccase activity in LB medium after 5 d co-cultivation; Data shown are means ± SDs (n = 3)

SDS-PAGE and Zymogram Analysis

The purified laccase was confirmed *via* SDS-PAGE and zymogram analysis, in which appeared a single protein band (Fig. 4). The molecular weight of the enzyme was 33 kDa, as measured by SDS-PAGE (Fig. 4), and the activity at that size was shown *via* a zymogram analysis (Fig. 4).

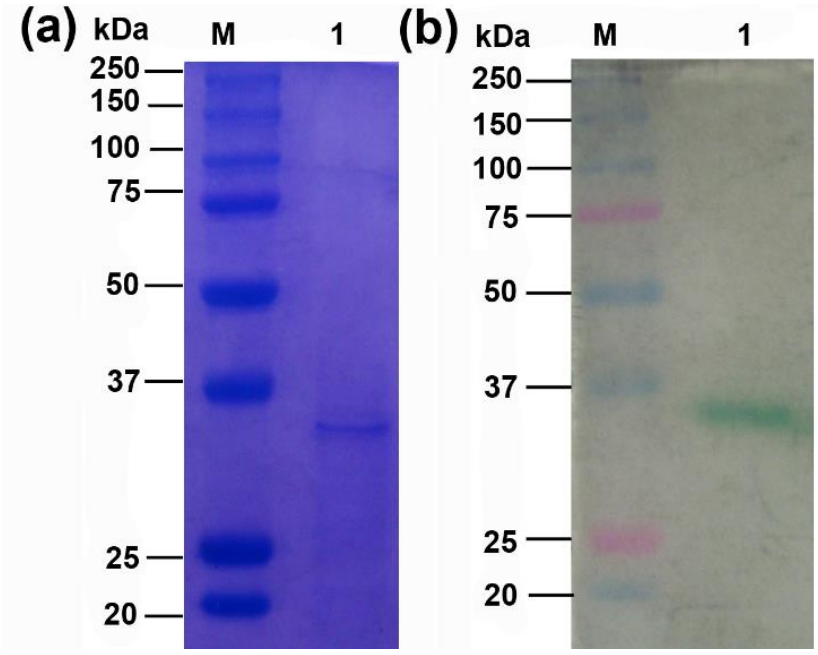

Fig. 4. Electrophoretic analysis of the laccase purified by crude intracellular enzyme, (a) SDS-PAGE was stained by Coomassie Brilliant Blue R-250; (b) Zymogram was strained by 1 mM ABTS; Lane M: protein marker; Lane 1: Purified laccase; the protein size is about 33 kDa

Effects of Temperature, pH, Metal Ions, Organic Solvents, and Inhibitors on Laccase Activity

The laccase activity significantly differed with changing incubation temperatures and pH values (Fig. 5). The purified laccase showed activity over a broad range of temperatures (30 °C to 80 °C), with the optimal activity at 40 °C. The relative enzyme activity levels at 30 °C, 50 °C, 60 °C, 70 °C, and 80 °C were 89%, 85%, 81%, 65%, and 51% of that seen at 40 °C, respectively (Fig. 5a). The enzyme showed more than 50% relative activity in the 3.8 to 6.2 pH range, with the optimum pH being 4.6. Beyond a pH 4.6, laccase activity declined rapidly, and the relative activity decreased 90% at pH 8.2 (Fig. 5b).

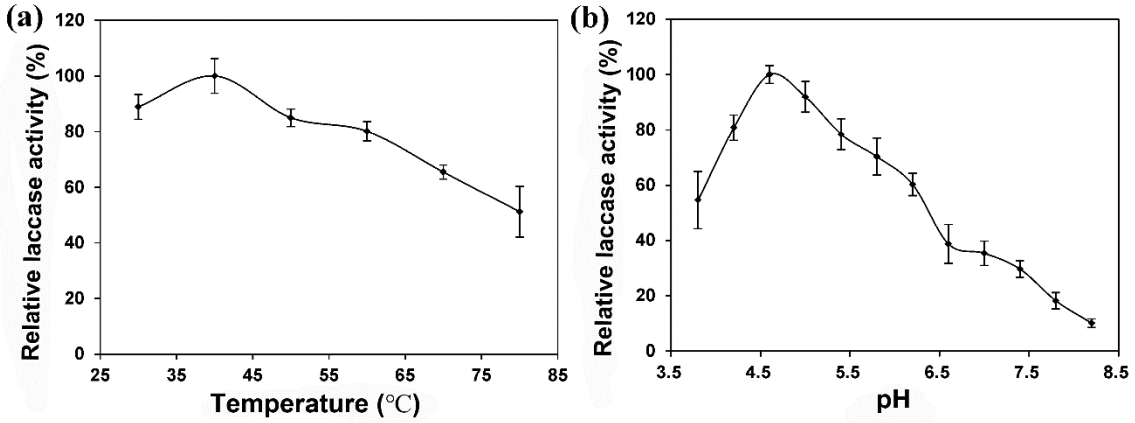

Fig. 5. Effects of temperature (a) and pH (b) on the activity of the laccase enzyme from *Bacillus* sp. A4.; Data shown are means ± SDs (n = 3)

The effects of metal ions, organic solvents, and inhibitors on laccase activity were measured by adding these into the reaction mixture (Fig. 6). The metal ions had almost no effect on laccase activity, but Cu^{2+} significantly increased activity, especially at concentrations of 0.5 mM and 1.0 mM, which increased the activity 45% and 80%, respectively (Figs. 6a and 7a). Moreover, laccase activity was also induced by ≤ 10% (v/v) methanol, ethanol, and acetone. Enzyme activity increased 62%, 30%, and 25% in the presence of 10% methanol, ethanol, and acetone but was inhibited by 20% concentrations of these and all concentrations of dimethyl sulfoxide (Fig. 6b). The laccase was almost totally inhibited with increased L-cysteine concentration, and the reductions were 50%, 78%, and 96% in 0.1 mM, 0.5 mM, and 1.0 mM L-cysteine, respectively (Figs. 6c and 7b). Also, SDS and EDTA strongly restrained the activity of laccase, which decreased 59% and 52% in the presence of these at 1.0 mM, respectively (Fig. 6c).

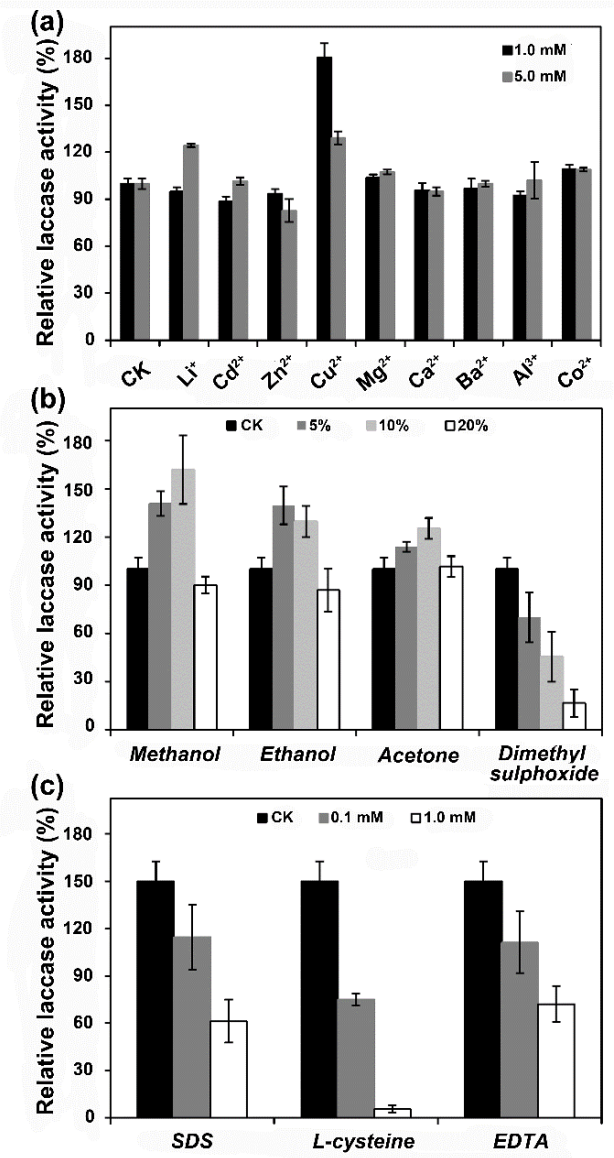

Fig. 6. Effects of metal ions (a), organic solvents (b), and inhibitors (c) on the activity of the laccase enzyme from Bacillus sp. A4; CK means the control without adding any ions in (a); Data shown are means ± SDs (n = 3)

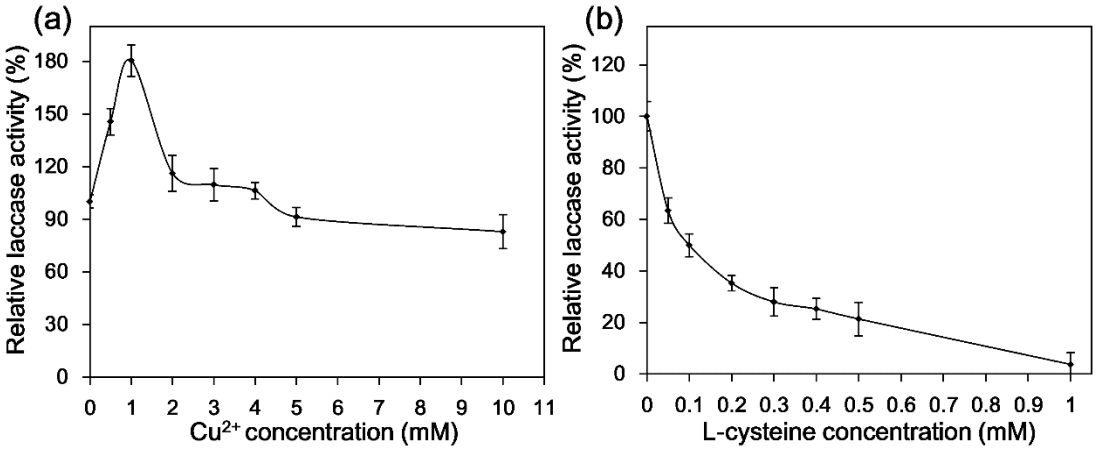

Fig. 7. Effects of Cu^{2+} concentration (a) and L-cysteine concentration (b) on the activity of the laccase enzyme from *Bacillus* sp. A4; Data shown are means ± SDs (n = 3)

Laccase Production and its Effects on Biomass Degradation

The laccase production of the *Bacillus* sp. A4 strain using various biomasses significantly differed with increased incubation time (Fig. 8). Laccase activity was strongly induced by algae, 2% NaOH-pretreated *M. sacchariflorus,* and non-pretreated *M. sacchariflorus* biomass; it was rapidly increased after incubation using algae and 2% NaOH-pretreated *M. sacchariflorus* and reached values of 18.0 U mg^{-1} protein and 14.9 U mg^{-1} protein, respectively, on Day 3. Then, it increased gradually and reached a maximum (22.6 U mg^{-1} protein) after 6 d of incubation using algae, while it decreased with increased incubation time using 2% NaOH-pretreated *M. sacchariflorus*. However, the laccase production of non-pretreated *M. sacchariflorus* was induced gradually and reached a peak (13.5 U mg^{-1} protein) after 7 d of incubation (Fig. 8). Moreover, laccase activity increased significantly when supplemented with agave, wild rice husk, wheat straw, wood dust, and pine biomass, and the corresponded maximum activities were 4.2 U mg^{-1} protein, 4.1 U mg^{-1} protein, 4.1 U mg^{-1} protein, 2.5 U mg^{-1} protein, and 5.3 U mg^{-1} protein, respectively (Fig. 8).

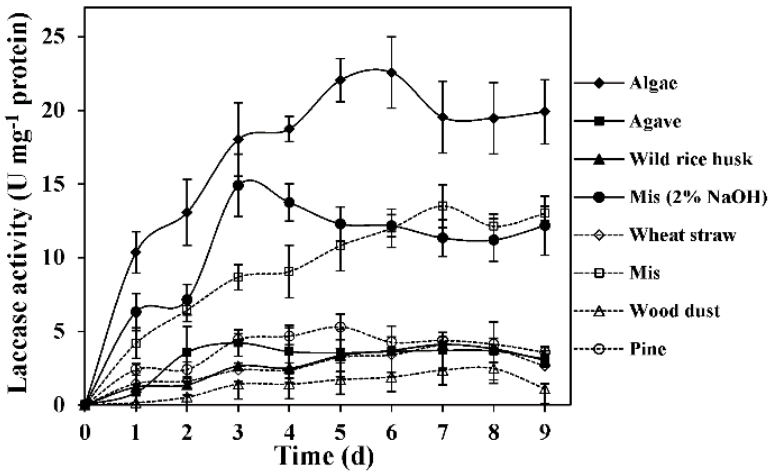

Fig. 8. The effects of various biomasses on the laccase production of *Bacillus* sp. A4. Mis: non-pretreatment *M. sacchariflorus*; Mis (2% NaOH): incubated *M. sacchariflorus* with 2% sodium hydroxide (NaOH) for 3 h at 50 °C; Data shown are means ± SDs (n = 3)

To determine whether the different cell wall components of these biomasses contributed to laccase production, the lignin content of biomasses treated with *Bacillus* sp. A4 was measured, as compared to that of biomasses left untreated. The lignin content levels were significantly different ($p < 0.01$) when the strain was incubated with algae, agave, 2% NaOH-pretreated *M. sacchariflorus,* and non-pretreated *M. sacchariflorus* as compared to those of biomasses untreated with *Bacillus*. The lignin content levels of algae, agave, 2% NaOH-pretreated *M. sacchariflorus,* and non-pretreated *M. sacchariflorus* were 1.9%, 5.6%, 11.7%, and 22.8% of dry weight biomass before incubation with the A4 strain, respectively, and they were 0.5%, 2.7%, 4.4%, and 11.5% of dry weight biomass after incubation, respectively (Fig. 9). Furthermore, the lignin content levels of wild rice husk, wheat straw, wood dust, and pine were also significantly degraded ($p < 0.05$) by the A4 strain; they decreased by 28%, 26%, 11%, and 15%, respectively (Fig. 9).

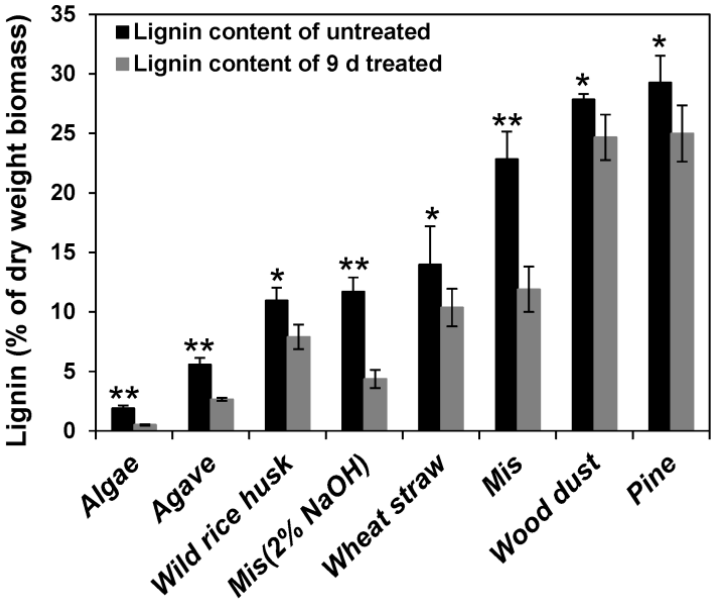

Fig. 9. The lignin content levels of untreated biomass and biomass treated with *Bacillus* sp. A4 for 9 d; Data shown are means ± SDs (n = 3); the * symbol indicates a significant difference at $p < 0.05$, and the ** symbol indicates a very significant difference at $p < 0.01$

To further confirm the degradation of *M. sacchariflorus* by the A4 strain, the cell wall structures of untreated and bacteria-treated non-pretreated *M. sacchariflorus* were observed over an incubation period of 16 d. The bacteria-untreated non-pretreated *M. sacchariflorus's* surface was smooth and tight after 16 d of incubation, which indicated no degradation of the cell wall (Figs. 10 a and b). In contrast, the samples treated with bacteria showed a rough, fragile, and paralyzed surface that indicated degradation after 16 d of incubation (Figs. 10 c and d).

In the present study, a highly laccase-producing strain was isolated on a LB medium plate that contained guaiacol. The laccase produced by this strain showed the ability to oxidize ABTS. Sheikhi *et al.* (2012) reported that the laccase produced by *Bacillus subtilis* WPI was more sensitive to ABTS than guaiacol as a substrate due to the laccase inactivation of guaiacol reaction products (Robles *et al.* 2000). The strain was identified as *Bacillus* sp. A4 according to its morphological and biochemical characteristics (Fig. 1) and the 16S rDNA sequence, which has been widely used to

identify bacteria generally and construct phylogenetic trees due to the slow evolutionary rate of bacteria (Goto *et al.* 2000; Yarza *et al.* 2014). A time course for the intracellular and supernatant laccase activity of *Bacillus* sp. A4 was assayed at the optimal temperature (37 °C) and pH (6.0) with a 2% inoculum concentration. Intracellular laccase could be detected at every measuring time and reached a peak at Day 3, but the laccase activity of the supernatant was first detected at Day 2 of incubation and reached a peak at Day 5 (Fig. 2). This indicated that this enzyme could be slowly released into the medium with increased incubation time. This was also found to be true for *Trametes versicolor,* which produced laccase in medium after 2 d of incubation, reached a peak after 6 d, and then decreased slightly and remained relatively constant up to Day 15 (Collins and Dobson 1997). Lu *et al.* (2013) reported that the laccase activity of the culture supernatant reached a maximum of 227.9 U L^{-1} on Day 7 and then decreased gradually and that this decrease in laccase production might have been caused by the activation of some proteases at a lower pH.

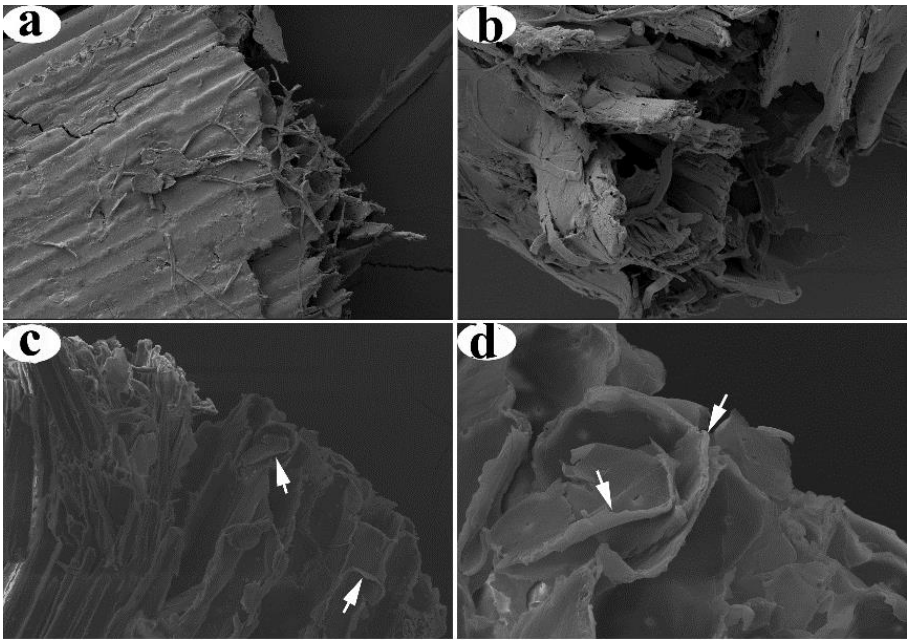

Fig. 10. The scanning electron microscopy (SEM) of non-pretreated *M. sacchariflorus* after co-cultivation with *Bacillus* sp. A4 for 16 d. (a-b) Samples not treated with *Bacillus* sp. A4; (c-d) samples treated with *Bacillus* sp. A4; the arrows indicate the degradation of *M. sacchariflorus* by *Bacillus* sp. A4

The molecular weights of most bacterial and fungal laccase proteins range from 43 kDa to 110 kDa (Yaropolov *et al.* 1994; Madhavi and Lele 2009). In this study, the molecular weight of laccase was approximately 33 kDa (Fig. 4), which was different from most of the reported laccases of bacteria and fungi, though several isoforms of laccases from bacteria have been reported to range from 31 kDa to 40 kDa in size. For example, Sondhi *et al.* (2014) found a novel extracellular thermo-alkali-stable laccase of *Bacillus tequilensis* SN4 that produced a 32 kDa monomeric protein. The molecular weight of laccase from *Streptomyces coelicolor* was around 32 kDa and able to rapidly decolourize dye in the presence of syringaldehyde, which acted as a redox mediator (Dubé *et al.* 2008). The laccase exhibited maximal activity at 40 °C, and retained more than 50% of its activity at 80 °C (Fig. 5a). The optimum temperature for laccase was

lower than those seen in some other bacterial strains, such as 60 °C in *Bacillus licheniformis* LS04 (Lu *et al.* 2012), 65 °C in *Bacillus amyloliquefaciens* 12B (Lončar *et al.* 2013), and 85 °C in *Bacillus vallismortis* fmb-103, and was similar to those of *Bacillus subtilis* (Hullo *et al.* 2001) and *Bacillus* sp. ADR (Telke *et al.* 2011). The optimal pH value for purified laccase in terms of oxidizing ABTS was 4.6 (Fig. 5b), like most bacterial laccase (Hullo *et al.* 2001; Dubé *et al.* 2008; Lu *et al.* 2012).

Furthermore, the effects of metal ions, organic solvents, and inhibitors were measured. Among the metal ions tested, laccase activity was highly induced by < 5.0 mM Cu^{2+}, but it was insensitive to other heavy metals (Figs. 6a and 7a). Murugesan *et al.* (2009) reported that Ca^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} enhanced the laccase activity of *Ganoderma lucidum* up to a 1.0 mM concentration and that chemical dyes were decolorized during 1 h of incubation in the presence of 1.0 mM Cu^{2+}. Lorenzo *et al.* (2005) reported that the addition of Cu^{2+} to the reaction mixture stimulated laccase activity at concentrations lower than 1.0 mM and that Mn^{2+} and Zn^{2+} did not affect enzyme activity at any of the concentrations tested (0.5 mM to 80 mM). The filling of the Type I Copper binding site by Cu^{2+} could be the main cause of this increased laccase activity (Shekher *et al.* 2011; Sondhi *et al.* 2014). Purified laccase activity was induced 5% to 10% methanol and 10% to 20% ethanol (Fig. 6b). Previous studies have shown that bacterial laccases from *Bacillus licheniformis* LS04 (Lu *et al.* 2012) and *Bacillus pumilus* W3 (Guan *et al.* 2014) were highly induced by organic solvents, which allow access to some insoluble substrates in the reaction mixtures and help to detoxify several stable organic pollutants (Torres *et al.* 2003). At a 1.0 mM concentration, the inhibitors SDS and EDTA could partly inactivate the laccase activity of *Bacillus* sp. A4, but laccase activity was completely inhibited by L-cysteine at the same concentration (Fig. 6c and 7b). Partial inactivation by a low concentration of SDS and EDTA was also found in the laccase of *Bacillus licheniformis* (Lu *et al.* 2013) and *Bacillus pumilus* (Guan *et al.* 2014). The total inactivation in the presence of 1.0 mM L-cysteine was similar to that of the recombinant laccase from *Bacillus licheniformis* (Baldrian *et al.* 2004; Lu *et al.* 2013).

Lignin carbohydrate complexes are intimately interlaced with carbohydrates through ester and ether bonds, and this is a primary obstacle to the production of biofuels from lignocellulosic biomass (Studer *et al.* 2011). The lignin content of biomass is usually in the range of 15% to 35% (Azadi *et al.* 2013). The microbial degradation of lignin has been well-studied in fungi, such as white-rot and brown-rot fungi (Leonowicz *et al.* 1999; Guillén *et al.* 2005), but little is known about the degradation of lignin in bacteria. The present study showed that the novel laccase-producing *Bacillus* sp. *A4* produced a great deal of laccase and decreased lignin content after incubation with various biomass (Fig. 8). Laccase activity was maximally induced by algae, which contained only 1.9% lignin content and indicated that the highest level of induction was mostly caused by the algal phytochrome, which could be also used as the oxidation substrate of laccase (Shekher *et al.* 2011). Laccase has been commonly induced by the biomass of grasses (agave, wild rice husk, wheat straw, and *Miscanthus*), but induction was difficult in the presence of wood biomass (wood dusk and pine) (Figs. 8 and 9). The various levels of induction and degradation caused by the lignin from grasses and woods could be attributed to their different structural compositions (Demirbaş 2005). Antai and Crawford (1982) reported that two *Streptomyces* strains substantially degraded both the lignin and the carbohydrate components of softwood, hardwood, and grass lignocelluloses but that they were more efficient decomposers of grass lignin than of

wood lignin. The authors were interested in the fact that *Miscanthus* biomass, both pretreatment and non-pretreatment, significantly stimulated laccase production, which resulted in a significant decrease ($p < 0.01$) in lignin content and the breakage of the cell wall structure in *Miscanthus* (Figs. 8 through 10). The high level of laccase induction could be partially due to the high cellulose/lignin ration and total ester-bound phenolics content compared to other bioenergy crops, such as switchgrass and reed (Lygin *et al.* 2011). The different types of lignin and extractives in the structures of various species of lignocellulosic materials may also lead to different levels of degradation by *Bacillus* sp. A4 (Demirbaş 2001). Furthermore, the efficiency of lignin degradation in *Bacillus* sp. A4 was much higher than that in white rot fungus (Wan and Li 2010; Liu *et al.* 2013). The white rot fungus *Ceriporiopsis subvermispora* degraded 39.2% lignin of corn stover after 42 d of cultivation (Wan and Li 2010). The lignin content of switchgrass decreased by 30% after 36 d of incubation with fungus *Pycnoporus* sp. SYBC-13 (Liu *et al.* 2013), while the *Bacillus* sp. A4 removed 11 to 74% lignin of the tested biomass after only 9 d of cultivation (Fig. 9). Electron microscopic and biochemical studies of lignocellulose degradation by most fungi and bacteria have shown that enzymes, such as lignin peroxidases, laccase, and cellulases, are too large to penetrate undegraded secondary wood cell walls (Evans *et al.* 1994; Blanchette 1995; Tuor *et al.* 1995). Therefore, the high lignin degradation ability of *Bacillus* sp. A4 may be due to the low molecular mass of the laccase, which helped it to penetrate the cell wall tissue and then degrade the lignin of plants (Ten Have and Teunissen 2001).

CONCLUSIONS

1. A novel laccase-producing strain was isolated from forest soil and identified as *Bacillus* sp. A4. The purified laccase retained relatively high levels of activity over a wide temperature range (30 °C to 70 °C). It had a low molecular weight (33 kDa) and was significantly induced by Cu^{2+} and some organic solvents.
2. The laccase production of the strain was markedly stimulated when incubated with various lignocellulosic biomasses. The novel strain could efficiently decrease lignin content of lignocellulosic biomasses after 9 d of incubation, especially the lignin of grasses.
3. The new strain A4 was a more efficient decomposer of lignin in *Miscanthus*, which demonstrated much more lignin loss and cell wall structure destruction in a short span of time.

ACKNOWLEDGEMENTS

The authors wish to thank the support of BioFuelNet Canada and the China Scholarship Council (Grant No. 201506320044).

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Article submitted: February 20, 2017; Peer review completed: April 13, 2017; Revised version received: April 23, 2017; Accepted: May 1, 2017; Published: May 15, 2017.
DOI: 10.15376/biores.12.3.4776-4794