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ORIGINAL PAPER

Characterization of Ligninolytic Bacteria and Analysis of Alkali-Lignin Biodegradation Products

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Abstract

Ligninolytic bacteria degrading lignin were isolates and identified, and their biodegradation mechanism of alkaline-lignin was investigated. Four strains with lignin degradation capability were screened and identified from the soil, straw, and silage based on their decolorizing capacity of aniline blue and colony size on alkaline-lignin medium. The degradation ratio of *Bacillus aryabhattai* BY5, *Acinetobacter johnsonii* LN2, *Acinetobacter lwoffii* LN4, and *Micrococcus yunnanensis* CL32 have been assayed using alkaline-lignin as the unique carbon source. Further, the Lip (lignin peroxidase) and Mnp (manganese peroxidase) activities of strains were investigated. Lip activity of *A. lwoffii* LN4 was highest after 72 h of incubation and reached 7151.7 $U \cdot l^{-1}$. Mnp activity of *M. yunnanensis* CL32 was highest after 48 h and reached 12533 $U \cdot l^{-1}$. The analysis of alkaline-lignin degradation products by GC-MS revealed that the strains screened could utilize aromatic esters compounds such as dibutyl phthalate (DBP), and decomposite monocyclic aromatic compounds through the DBP aerobic metabolic pathway. The results indicate that *B. aryabhattai* BY5, *A. johnsonii* LN2, *A. lwoffii* LN4, and *M. yunnanensis* CL32 have high potential to degrade alkaline-lignin, and might utilize aromatic compounds by DBP aerobic metabolic pathway in the process of lignin degradation.

Key words: isolation, bacteria, alkali-lignin, biodegradation products

Introduction

Lignin is widely distributed in various plants and is the second most abundant natural organic polymer on the Earth (Zakzeski et al. 2010; Ragauskas et al. 2014). It is an amorphous and complex aromatic compound with a substantial molecular weight, which is mainly composed of three main lignin precursors (p-hydroxyphenyl, guaiacyl, and syringyl units) linked via C-C or C-O bonds formed by radical coupling reactions. It contains a variety of oxygen-containing functional groups, such as methoxy, hydroxyl, carboxyl, and other active structures (Zhu et al. 2017; Nishimura et al. 2018). In the plant cells, lignin can be converted via phenylalanine and tyrosine by transamination (Hatfield et al. 2017; Kang et al. 2019). Then, they are joined by chemical bonds such as ester bonds to form highly polymerized macromolecules. The long chains of cellulose are twisted into externally hydrophobic microfibrils, and then the lignin is combined with the allosteric hemicellulose by an electrostatic action. Hemicellulose bridges the hydrophobic regions of cellulose microfibrils, forming complex lignocellulosic composites (Kang et al. 2019). Hence, natural lignin could not be easily degraded in papermaking wastewater, agricultural straw returning, and clean biomass energy development. Because of their complicated structure, a significant obstacle obstructs the development and utilization of natural biomass energy such as straw, forage, and woody feeds.

A growing number of bacteria with lignin degradability have been discovered in recent years. Scientists realized that bacteria play an important role in the lignin industrial utilization process (Bugg et al. 2011a). Ligninolytic bacteria have extensive adaptability in industry and agriculture. Simultaneously, lignin waste can be converted to various value-added products by bacteria (Xu et al. 2018). Besides, both lignin peroxidase and manganese oxide enzyme are lignin-degrading proteases containing ferrous-ions, the former degrading the hydroxyl-containing aromatic ring inside the lignin, and the latter removing the methoxy group on the ring, then, make it easy to enter the next step of degradation

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(Bugg et al. 2011b; Bharagava et al. 2018). Many aerobic bacteria could degrade lignin, which belongs mainly to *Actinobacteria*, *Proteobacteria*, and *Firmicutes* (Bugg et al. 2011a). The litter layer, straw from farmland, has a large number of lignin-degrading bacteria.

The purpose of this study was to characterize and identify bacteria, which degrade lignin in nature, and to compare their degradation capabilities and analyze biodegradation products. Moreover, it could enjoy a broad array of uses in the comprehensive utilization of lignin resources in industry and agriculture.

Experimental

Materials and Methods

Screening and identification of ligninolytic bacteria. The ligninolytic bacteria in this study were mainly isolated from soil, straw, and silage. The humus-rich soil samples were collected from the campus of Shanxi Agricultural University. The straw compost (no additive was added) was collected from a harvested cornfield in Jinzhong, Shanxi Province, and the silage samples were taken from a husbandry cooperative, which made whole-plant corn silage (fermented without additive) in Taigu, Shanxi Province. The samples (500 g) were taken and placed in a sample box (ESKY, 12 l) containing an icepack and brought back to the laboratory.

10 g of each sample was placed in a sterile Erlenmeyer flask, and 50 ml of 0.9% physiological saline was added, then stirred violently by a vortex mixer. The mixed liquids were diluted by five levels (1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}), and 100 µl was inoculated into the ligninolytic selection medium. The medium contained 2.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 1.0 g K_2HPO_4, 0.5 g NaCl, 5.0 g alkaline lignin, 20.0 g agar powder, 1.0 l H_2O. All the chemicals were purchased from Solarbio Biotechnology Co., Ltd. In the ultra-clean bench, it was coated with a triangular glass rod, and finally, the plate was inverted and cultured at 30°C for 48 h. Finally, single colonies were inoculated into LB tube medium.

The decolorization of aniline blue proved that bacteria could produce ligninolytic enzymes. Also, the decolorization of brilliant blue was considered as the laccase production in the plate. Therefore, 1% aniline blue or brilliant blue was added into the alkaline lignin medium by a sterile filter (0.45 µm), respectively. Afterward, the bacteria, for which had the hydrolysis circle was observed, were selected and purified. Finally, they were stored at –20°C with liquid paraffin.

The total DNA of the strains was extracted using the Omega Bacterial Genomic DNA Extraction Kit, and then the 16S rDNA sequence of the bacterial PCR was amplified by the 27F and 1492R primers as follows: 27F: (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R: (5'-TACGGCTACCTTGTTACGACTT-3'). The 16S rRNA amplicons were sequenced by the Nanchang Kechang Biotechnology Company.

The 16S rDNA sequences of the identified strains were imported into MEGA 7.0 for phylogenetic analysis, and the phylogenetic tree was constructed by the Neighbor-joining method.

Characterization of ligninolytic bacteria. The colony size and OD_{600} value of the strain were determined separately. The colony diameter was measured by the cross method, while OD_{600} value in alkaline lignin was measured using an ultraviolet spectrophotometer.

The shape of bacteria was observed via scanning electron microscopy. 2 ml of the fermentation broth was added to the centrifuge tube, centrifuged at 8,000 g for 3 min at 4°C, the supernatant liquid was discarded, the precipitate was washed by adding a phosphate buffer solution (pH 7.2), and then centrifuged again. After repeating three times, the supernatant was discarded. 2.5% glutaraldehyde was added and fixed in a refrigerator at 4°C for 24 h. The ethanol was used for the gradient (30%, 50%, 70%, 80%, 90%) dehydration treatment. After gradient dehydration centrifugation, it was eluted twice with absolute ethanol, and the supernatant was discarded by centrifugation, and the bacteria were resuspended in absolute ethanol. The coverslips were immersed in 1 M HCl solution for 12 h, and the coverslips were washed with absolute ethanol, sonicated for 30 min and dried. 5–10 µl of the resuspended bacterial liquid was pipetted and added to cover glass. After drying, the sample was observed by scanning electron microscopy.

The Lip (lignin peroxidase) and Mnp (manganese peroxidase) of the bacteria were determined at 24 h, 48 h, and 72 h, respectively.

Lip activity was detected with the lignin peroxidase kit (Beijing Solabao Technology Co., Ltd.). Lip oxidized resveratrol to produce veratraldehyde with a specific absorption peak at 310 nm (Yang et al. 2017; Zhou et al. 2017). The bacterial suspension was centrifuged at 10,000 g for 10 min, and the supernatant was placed in a 2 ml centrifuge tube on ice for testing. The reaction system contained 1 mM resveratrol, 50 mM phosphate buffer (PBS), pH 7.2, and 0.1 mM hydrogen peroxide, and the supernatant was added in a volume of 100 µl. Ultrapure water was used as a control to measure the 10 S and 310 S absorbance at 310 nm which was recorded as A_1 and A_2, and $\Delta A = A_2 - A_1$. The one enzyme activity unit was defined as the amount of enzyme required to oxidize 1 nmol of resveratrol per liter of culture suspension, and the molar extinction coefficient of veratraldehyde, ε_1 was equal to $9{,}300\ l \cdot mol^{-1} \cdot cm^{-1}$.

Mnp is also an oxidase that contains heme, which is oxidized with guaiacol to tetra-o-methoxyphenol in the presence of Mn^{2+} and has a characteristic absorption peak at 465 nm (Hwang et al. 2008). Mnp activity was detected by the manganese peroxidase kit (Beijing Suo Laibao Technology Co., Ltd.). The culture was centrifuged at 10,000 g for 10 min. The supernatant was placed on ice to be tested as a crude enzyme solution. A 100 µl sample and 900 µl of the substrate were thoroughly mixed in a 1 ml glass cuvette as a reaction system. After incubation at 37°C for 10 min, the absorbance at 465 nm was measured to calculate the difference ΔA, and ultrapure water was used as a control. An enzyme activity unit was defined as the amount of enzyme required to oxidize 1 nmol of guaiacol per minute per liter of the culture medium. The guaiacol extinction coefficient ε_2 was equal to 12,100 $l \cdot mol^{-1} \cdot cm^{-1}$, and the calculation formula was as follows:

$$\text{Lip}\ (nmol \cdot min^{-1} \cdot l^{-1}) = \Delta A \div (\varepsilon_1 \times d) \times V_A \div V_S \div T$$
$$\text{Mnp}\ (nmol \cdot min^{-1} \cdot l^{-1}) = \Delta A \div (\varepsilon_2 \times d) \times V_A \div V_S \div T$$

d – cuvette light path, VA – total reaction volume,
VS – sample volume in the reaction, T – reaction time.

Analysis of degradation product. The strains were inoculated in a liquid medium, placed on a constant temperature shaker, and cultured for 72 h at the optimum temperature. The fermentation broth of different strains was centrifuged at 10,000 g for 5 min at a temperature of 4°C. After centrifugation, the supernatant was placed in a new centrifuge tube for measurement.

The pH meter was calibrated, and the pH of the supernatant was determined and recorded. The supernatant's pH was adjusted to about 2.0 with a concentrated hydrochloric acid (38%). After adjusting the pH, each broth was extracted using accelerated solvent extraction (ASE300). Using diatomaceous earth solidified one ml of the liquid, and solids were added. In the extraction vessel, ethyl acetate was used as an extractant, and after extraction, ethyl acetate was blown off using a nitrogen purifier to obtain an extracted product. After nitrogen drying, the product was dissolved in 100 µl of cyclohexane, dioxane, and ethyl acetate. At the same time, 50 µl of a trimethylsilyl trifluoroacetamide (BSTFA (N,O-bis(trimethylsilyl)) was added to facilitate measurement.

The dissolved sample was analyzed by GC-MS. The liquid was injected using Thermo Trace 1300 ISQ, the injection volume was 1 µl on the OM-5MS capillary column, the carrier gas was He gas, and the flow rate was controlled at 1 $ml \cdot min^{-1}$. The inlet temperature was set to 200°C, the column temperature was kept at 50°C for 4 min, then raised to 220°C for 25 min, the solvent delay time was 3 min, the transmission line and ion source temperature were set to 230°C, and 250°C, respectively (Barros et al. 2013). Electron ionization mass spectra were recorded in a Full Scan mode. The degradation product's chemical structure was presumed based on the material retention time, electron mass spectrometry, and the NIST database.

Statistical analysis. The experimental data were statistically sorted via Office 2016 (data presented as mean ± SD), and curves were plotted by Origin 2018. Enzyme activity was analyzed by one-way ANOVA, and multiple comparison analysis was performed by Duncan method ($p < 0.05$).

Result

Screening and identification of ligninolytic bacteria. Four strains, which could grow in selective medium and use lignin, were isolated from humus-rich soil samples (Table I). Both YB5 and CL32 strains presented a decolorizing circle in the aniline blue medium. Besides, not only could LN2 and LN4 decolorized aniline blue in the selective medium, but also the hydrolyzed circle was the largest. None of those strains could decolorize brilliant blue in the alkaline lignin selective medium and it indicated that they might not be able to produce laccase.

The phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 1A. As it is visible

Table I
Screening and identification of bacterial isolates based on different media.

Name	Species	Accession number	Alkaline lignin	Aniline blue	Brilliant blue
YB5	*Bacillus aryabhattai*	MT745877	o	+	–
CL32	*Micrococcus yunnanensis*	MT745880	o	+	–
LN2	*Acinetobacter johnsonii*	MT745878	o	++	–
LN4	*Acinetobacter lwoffii*	MT745879	o	++	–

"o" – diameter is over 0.2 cm, "+" – indicates that a hydrolyzed circle appears,
"++" – indicates that the hydrolyzed ring diameter is over than 1 cm, and
"–" – indicates that the hydrolyzed ring diameter is less than 0.5 cm

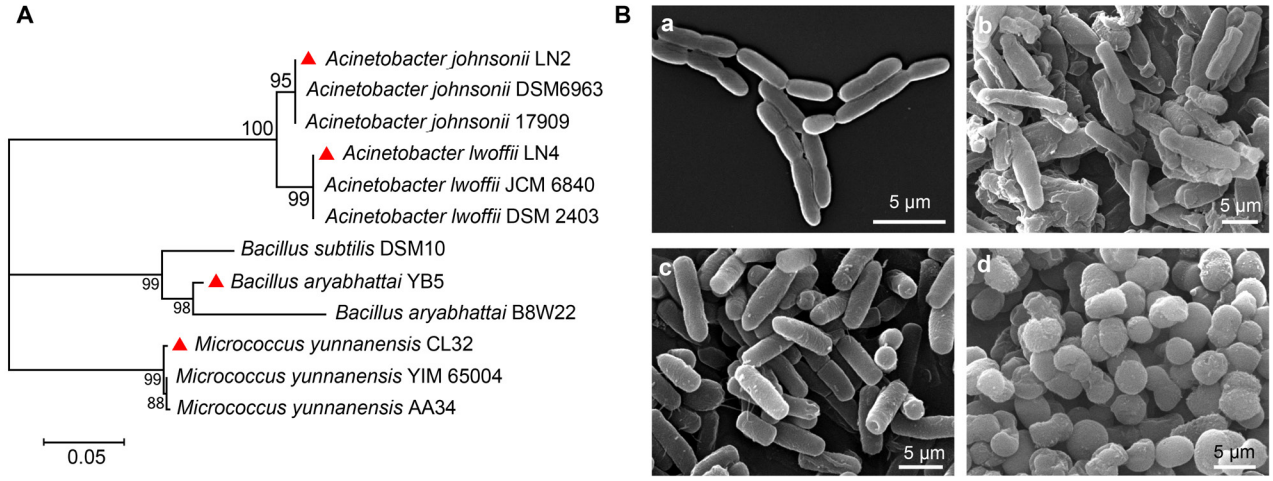

Fig. 1. (A) Phylogenetic tree of four strains. Strains in this study were marked with a red triangle (▲). (B) Scanning electron micrograph of lignin-degrading bacteria; "a" – *B. aryabhattai* YB5, "b" – *A. johnsonii* LN2, "c" – *A. lwoffii* LN4, "d" – *M. yunnanensis* CL32.

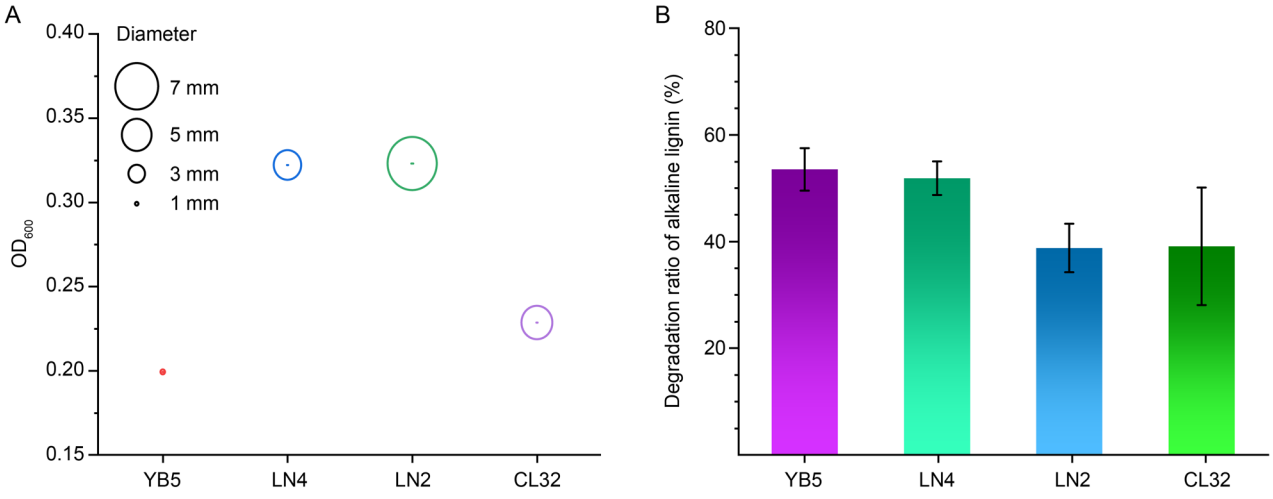

Fig. 2. (A) Bubble chart about colony size on alkaline lignin medium and OD_{600} in the alkaline lignin liquid medium of four strains. The bubble center points indicate the OD_{600} values, and the bubble size indicates the colony size, but it is not an isometric diagram. (B) The degradation rate of alkaline lignin. After the bacteria were cultured in an alkaline lignin medium for three days, the alkaline lignin degradation rates of strains were calculated (the OD value determined the degradation rate).

in Fig. 1B, the bacteria of YB5 strains were long rod-shaped under SEM and with a length of 2.5 μm, and seemed to be splitting. The cells of both LN2 and LN4 strains were long rod-shaped, with a length of 1–1.5 μm. However, CL32 was a globular bacterium with a sphere diameter of fewer than 1 μm.

Characterization of ligninolytic bacteria. The carbon source would affect the metabolism and growth of the strains in the medium. The colony size and OD_{600} were determined for the evaluation of the growth of bacteria on alkaline lignin as the only carbon source in a solid or liquid medium. The utilization of alkaline lignin was shown in Fig. 2A. LN2 strain has the largest colony diameter and highest OD_{600} value, and the colony size of CL32 strain was the second only to LN4 strain, but its OD_{600} value was lower. OD_{600} values of LN2 and LN4 strains were almost the same, although the colony diameter of LN2 was only 4.5 mm. The colony size of the YB5 strain was only 0.75 mm, and the OD_{600} value was 0.209.

Degradation of alkaline lignin is a biological process involving a reduction in carbon atoms and a decrease in molecular weight. It includes both incomplete degradation of macromolecules into small molecules, and the complete degradation of macromolecules into carbon dioxide and water. In this study, alkaline lignin was decomposed into small molecules or fully utilized to reduce the content of alkaline lignin in the environment. The histogram of the degradation rate of alkaline lignin was shown in Fig. 2B. The degradation rate of strains was determined after 72 h incubation in the alkaline lignin liquid medium. The results showed that the degradation rate of the YB5 strain reached 53.5%, it was slightly lower for LN4 than YB5 strain, and the degradation rate

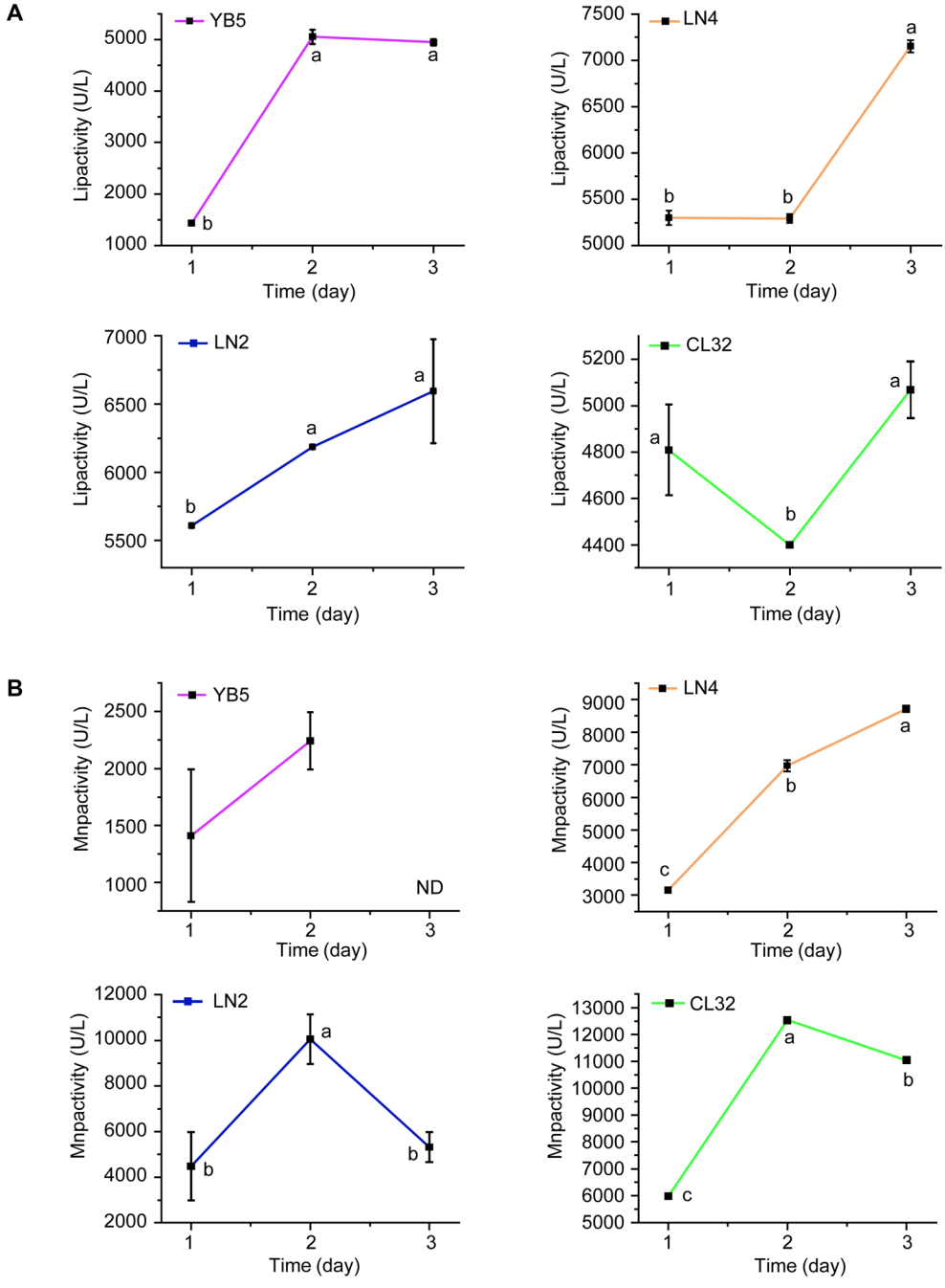

Fig. 3. Lip (A) and Mnp (B) activity dynamic of four strains. The color band around the line indicates the standard deviation. The width indicates the level of the standard deviation value. "a, b, c" indicates that the enzyme activity in the different periods was significantly different, and the same letter indicates any significant difference. "ND" – not detected. All activity assays were obtained from triplicate experiments.

of CL32 was less than 40%, but the alkaline lignin degradation of LN2 strains was as low as 38.8%.

Both Lip and Mnp were essential oxidases in the lignin degradation process. The strains in this study failed to decolorize brilliant blue in the alkaline lignin selective medium. So, the enzyme activity of Lac was not determined. The dynamics of Lip and Mnp enzyme activity during three days were shown in Fig. 3.

The Lip activity of four strains was the highest on the third day (Fig. 3A). The YB5 strains enzyme activity continued to rise, and the difference between the third day and the second day was not significant ($p > 0.05$), and was close to 5000 $U \cdot l^{-1}$. The enzyme activity of the LN4 strain increased slowly at the beginning, and it was close to 6500 $U \cdot l^{-1}$ on the third day that was significantly different from the previous two days ($p > 0.05$). The activity of the LN2 strain enzyme increased linearly, and the difference of enzyme activity between the second and third days was not significant ($p > 0.05$). The enzyme activity of the CL32 strain showed a downward

Table II
The compounds identified in three solvents extracts from the alkali lignin degraded by the bacterial strains and the control sample.

RT (min)	Compounds	CK	YB5	LN4	LN2	CL32
Hexane as solvent						
19.24	Pentonic acid lactone*	–	+	+	+	–
22.65	Hexadecanoate*	–	+	+	+	+
24.46	Octadecanoate*	–	+	+	+	+
28.20	Propyl hexadecanoate*	–	+	+	+	+
Dioxane as solvent						
8.45	2-Ethoxyethanol	+	+	+	+	+
11.67	2,4-Hexadienal	+	+	+	+	+
12.83	Di (ethylene glycol) vinyl ether	+	+	+	+	+
21.92	Dimethylbiphenyl	+	–	–	–	–
24.07	1,2,3-trimethyl-4-prop-1-enylnaphthalene	+	–	–	–	–
26.87	Diisobutyl phthalate	+	–	–	–	–
29.32	Dibutyl phthalate	+	–	–	–	–
Ethyl acetate as solvent						
21.54	Acetosyringone	+	+	+	+	+
22.93	Diisooctyl phthalate	+	–	–	–	–
23.6	Palmitoleic acid	+	–	–	–	–
23.79	Dibutyl phthalate	+	–	–	–	–
25.51	Cis-Vaccenic acid	+	–	–	–	–

"RT" – retention time, "+" – present, "–" – absent,
"*" – indicates that the derivatization reagent a silane group was determined,
"YB5" – *B. aryabhattai* YB5, "LN4" – *A. lwoffii* LN4, "LN2" – *A. johnsonii* LN2, "CL32" – *M. yunnanensis* CL32

trend on the second day and was significantly lower than on the first and third days ($p > 0.05$). The difference between day one and day three was not significant ($p > 0.05$).

The Mnp activity dynamic trend of YB5 and LN4 strains continued to increase and were the highest on the second and third day, respectively. This activity was significantly higher than on the first day ($p > 0.05$). However, the changing trends of enzyme activities in LN2 and CL32 strain were similar, and both increased first and then decreased and were significantly higher on the second day than on the first and third day ($p > 0.05$).

Ethyl acetate, hexane and 1,4-dioxane are common chromatographic solvents. Hexane is a non-polar solvent, but dioxane is a polar solvent. Finally, the ethyl acetate polarity is normal. Due to different chemical polarity, these solvents are often selected for the detection of products of different-polarity. Characteristics of the products measured in different polar solvents are shown in Table II. Four strains were cultured for 72 h in the alkaline lignin medium and then treated to determine degradation products.

Clearly, 2-ethoxyethanol, 2,4-hexadienal, di(ethylene glycol) vinyl ether, and acetosyringone were not degraded by the strains and were detected in both the CK group (only medium, no strain, as a control group), and in the treatment group. Hexadecanoate, octadecanoate, and propyl hexadecanoate were not detected in the CK group but were detected in the YB5, LN4, LN2 and CL32 strains. Pentonic acid lactone was detected in the YB5, LN4 and LN2 strains, but not in the control group and in CL32 strain.

Dimethylbiphenyl, 1,2,3-trimethyl-4-prop-1-enylnaphthalene, diisobutyl phthalate, dibutyl phthalate, palmitoleic acid, cis-vaccenic acid was detected in the CK group. It is worth noting that in YB5, LN4, LN2 and CL32 strains the compounds that appeared in CK group were not detected. The chromatographs of compounds extracted with ethyl acetate, hexane, and 1,4-dioxane are showed in Supplementary Fig. 1–3.

Discussion

The objective of this study was to screen the functional bacteria, which could degrade lignin in soil. There are many plant residues on the black soil in the forest, rich in humus and they potentially contain many lignin-degrading microorganisms. The screened bacteria were Bacillus, Acinetobacter, and Actinomycetes, whose ability to decolorize aniline blue and degrade lignin was reported. So, they could synthesize lignin

peroxidase and manganese peroxidase, although none of them can synthesize laccase. So, they could synthesize lignin peroxidase and manganese peroxidase, although none of them can synthesize laccase. *B. aryabhattai* is a Gram-positive bacterium found early in the 21st century (Shivaji et al. 2009). In recent years, many studies paid attention to the degradation of biomass macromolecules, including the degradation and decolorization of lignin and its by-products (Min et al. 2015). Researchers have performed degradation tests on various materials, including the treatment of residual dyes from the textile-processing industry (Paz et al. 2016b). Therefore, *B. aryabhattai* DC100 can be used as a bio-decontaminant in the textile industry wastewater treatment and bio-decontamination and also can inevitably degrade lignin from plant tissue. *B. aryabhattai* BA03 has the ability to convert ferulic acid to vanillin and 4-vinylguaiacol, and can partially or fully degrade lignin (Paz et al. 2016a; Paz et al. 2017). *B. aryabhattai* BA03 can transform lignocellulosic waste or an industrial aromatic compound waste of agriculture into a new product. Also, it can be useful in reducing pollution from pulp and paper wastewater. *B. aryabhattai* MG966493 reduced 67% and 54% color and lignin, respectively, from the pulp and paper mill wastewater (Zainith et al. 2019). At present, many studies have been carried out on the degradation ability of lignocellulose, but most of the research objects are alkaline lignin, sodium lignosulfonate, and other lignin model compounds (Zhu et al. 2017). *Bacillus* species such as *B. licheniformis*, *B. subtilis*, *B. thuringiensis*, *B. megaterium,* and *B. aryabhattai* have been found to degrade lignin or participate in the degradation of lignin (Iyer and Mahadevan 2002).

It had been reported that many *Acinetobacter* spp. could degrade aromatic hydrocarbons or phenolic substances (Yu et al. 2004; Chen et al. 2008). Polycyclic aromatic hydrocarbons (PAHs), naphthalene, and crude oil could be degraded by *Acinetobacter* spp. *A. lwofii* NCIB 10553 was one of the species which could use aromatic and aliphatic carboxylic esters as a sole carbon source (Grant 1973). A long time ago, *A. lwoffii* K24 was reported to be capable to degrade aniline and monocyclic aromatic hydrocarbon, although under the name of *Burkholderia* sp. K24 (Kim et al. 1999; Lee et al. 2014; Lee et al. 2016). Similarly, in this study, *A. lwoffii* LN4 decolorized aniline-blue and degraded dibutyl phthalate, which was also a monocyclic aromatic compound. Jiang isolated *A. johnsonii* strain from waste oil. It can decompose naphthalene and phenanthrene while purifying sewage via sedimentation (Jiang et al. 2012; Jiang et al. 2020). Crude oil can also be co-degraded by *A. johnsonii* XM-02 and *Pseudomonas* sp. XM-01, which was isolated from the soil polluted by crude oil at China's Bohai Bay (Chen et al. 2014). Lignin was composed of a wide variety of aromatic compounds; the basic unit was a phenylpropanoid group (Cragg et al. 2015). *Acinetobacter* spp. could also degrade lignin, because of their substrate has a similar monocyclic aromatic structure.

Lignin peroxidase is an important lignin-degrading enzyme. Interestingly, although the enzyme from the YB5 strain has a higher degradation rate, the Lip enzyme activity was lowest on the first day. Bharagava and coworkers studied *Aeromonas hydrophila* and measured lignin peroxidase activity when studying its ability to degrade fuel. The maximum enzyme activity did not exceed 2,000 $U \cdot l^{-1}$ (Bharagava et al. 2018). It is similar to YB5 strain, but as time goes by, the enzyme activity approached 5,000 $U \cdot l^{-1}$ on the third day. Vandana optimized the culture conditions and increased lignin peroxidase activity by adjusting the carbon source of white-rot fungus medium, nitrogen source, culture temperature, and pH (Vandana 2009). Lip activity of white-rot fungi is much higher than the bacteria screened in this study. White-rot fungi have been extensively studied for lignin degradation, and the low rate of bacterial degradation did not mean that bacteria did not play a role in lignin degradation. They all showed higher degradation rates on the third day. Although alkaline lignin was used as a substrate in a culture medium, they have great potential for future application in agricultural straw, paper mill black liquor, and industrial lignin waste (Yi et al. 2018).

In contrast, lignin-degrading bacteria could also be found in black liquor. *B. aryabhattai* MG966493 was isolated from pulp and paper mill wastewater, and its Mnp activity peak at 24 h showed 6.1 $U \cdot l^{-1} \cdot ml^{-1}$ (Zainith et al. 2019). In addition, Chen and coworkers isolated *Comamonas* sp. B-9 from Soochow bamboo slips and studied its lignin manganese oxide enzyme. It was found that the enzyme activity was highest after 96 h, which was 2,903.2 $U \cdot l^{-1}$ (Chen et al. 2012). In this study, the Mnp activities of different species were diverse, and their values in LN4, LN2, and CL32 strains were higher than in YB5. Compared with *Comamonas* sp. B-9, there was no significant correlation between the Mnp activity and degradation rate of bacteria in this study (Fig. 3). Despite this, it almost had the same rate (45%) of degradation of Kraft lignin, which compared to alkaline lignin degradation rate (38.8–53.5%) in this test (Chai et al. 2014).

Sonoki and coworkers discovered that *Sphingobium* sp. SYK-6 can degrade biphenyls (Masai et al. 2007; Sonoki et al. 2009). The biphenyl metabolic pathway is a metabolic pathway that degrades biphenyls by a series of enzymes (Xu et al. 2008). In this study, biphenyl was a class of organic compounds with a benzene ring contained in the CK group. However, after three days of inoculation, biphenyl was not detected in the

YB5, LN4, LN2, and CL32 strains. Four strains might degrade lignin by such a metabolic pathway, which was replaced by the demethylase LigX to remove the biphenyl group methyl group that was replaced by the phenolic hydroxyl group, the monocyclic benzene was broken by dioxygenase LigZ. LigY enzyme cleaves the C-C bond to form a carboxylic acid and an aromatic acid for further metabolism (Sonoki et al. 2002). Dibutyl phthalate (DBP) and its similar organic matter were degraded in this study, possibly through the DBP aerobic metabolic pathway. First, a phenolic compound such as syringyl or protocatechuic acid is formed, and then converted into pyruvic acid or the like into the tricarboxylic acid cycle, and finally wholly degraded into carbon dioxide and water.

Why are the degradation characteristics of these four strains so similar? The degradation ability of microbial communities after incubation in alkali lignin, which tends to be consistent, is also called a functional convergence. Carlos and coworkers mentioned it in their research, due to an enrichment of genes involved in benzoate degradation and catechol ortho cleavage pathways (Carlos et al. 2018). Go and coworkers isolated *Acinetobacter* spp. from hydrospheres in Tokyo, that could metabolize dibutyl phthalate. Interestingly, commercially available strains of *Acinetobacter* were also found to degrade DBP (Ogawa et al. 2009). Richard (1982) reported a similar metabolism pathway a long time ago. *Micrococcus* sp. 12B leads dibutyl phthalate to monobutyl phthalate, finally transforms it to protocatechuate (Eaton and Ribbons 1982). It also confirmed that strains screened in the present study most likely possess these metabolic pathways; moreover, they also have great potential for future applications in the lignin industry and agriculture recycling.

Conclusions

In this study, four strains of lignin-degrading bacteria from soil, straw compost, and silage were screened. *B. aryabhattai* BY5, *A. johnsonii* LN2, *A. lwoffii* LN4, and *M. yunnanensis* CL32 could produce Lip and Mnp enzymes to degrade lignin in an aerobic environment. The degradation products of alkaline lignin were determined via GC-MS, and speculative pathways were analyzed. Therefore, based on the bacteria degradation capabilities and degradation pathways, it can be concluded that they could be used in industrial lignin treatment and agricultural recycling processes.

Acknowledgments

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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