



Biodegradation of kraft lignin by newly isolated *Klebsiella pneumoniae*, *Pseudomonas putida*, and *Ochrobactrum tritici* strains

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Abstract

Bacterial systems have drawn an increasing amount of attention on lignin valorization due to their rapid growth and powerful environmental adaptability. In this study, *Klebsiella pneumoniae* NX-1, *Pseudomonas putida* NX-1, and *Ochrobactrum tritici* NX-1 with ligninolytic potential were isolated from leaf mold samples. Their ligninolytic capabilities were determined by measuring (1) the cell growth on kraft lignin as the sole carbon source, (2) the decolorization of kraft lignin and lignin-mimicking dyes, (3) the micro-morphology changes and transformations of chemical groups in kraft lignin, and (4) the ligninolytic enzyme activities of these three isolates. To the best of our knowledge, this is the first report that *Ochrobactrum tritici* species can depolymerize and metabolize lignin. Moreover, laccase, lignin peroxidase, and Mn-peroxidase showed high activities in *P. putida* NX-1. Due to their excellent ligninolytic capabilities, these three bacteria are important supplements to ligninolytic bacteria library and could be valuable in lignin valorization.

Keywords Lignin · Biodegradation · *Klebsiella pneumoniae* · *Pseudomonas putida* · *Ochrobactrum tritici* · Ligninolytic enzymes

Introduction

Growing concerns on global climate change and energy security caused by fossil resource consumption increase the demand for renewable fuels and chemicals. Lignocellulose, the largest organic polymer reservoir in ecosystems, is a potential treasury alternative to fossil resources. The main components of lignocellulose are cellulose, hemicellulose, and lignin. Lignin and hemicellulose constitute a complex, three-dimensional structure that wraps cellulose to prevent solvents, catalysts, and organisms from being in contact with the cellulose (Asina et al. 2017; Ragauskas et al. 2014). With the advances of biorefinery technologies via biochemical routes, the cellulose and hemicellulose in lignocellulose can be converted

to biofuels and various chemicals through pretreatment, enzymatic hydrolysis, and microbial fermentation. The remaining lignin is projected to be burned for powering the biorefinery plant (Humbird et al. 2011).

The U.S. Energy Security and Independence Act of 2007 mandated the production of 6×10^{10} gal of lignocellulosic biofuels annually by 2030. In this case, 7.5×10^8 tons of biomass will be consumed, and approximately 2.25×10^8 tons of lignin will be produced (Holladay et al. 2007). Moreover, lignin is also generated in pulp and paper-making processes at approximately 5×10^7 tons per year globally (Bruijninx et al. 2015). Despite this huge production, lignin is still not be efficiently utilized and is mainly burned as a low-quality solid fuel and/or used as adhesive for flakeboard (Chandel et al. 2015). In addition, lignin waste stream is often directly discharged into the environment, thereby causing significant pollution. With further development of biofuels and other chemicals from lignocellulosic biomass, the production of lignin will increase. Thus, efforts should be urgently exerted for its valorization. Lignin valorization is believed to be the key for successful development of lignocellulosic biorefinery (Bruijninx et al. 2015; Ragauskas et al. 2014).

By now, several strategies have emerged for lignin valorization, including thermochemical treatments, homogeneous catalysis, and heterogeneous catalysis (Farag and Chaouki 2015;

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Li et al. 2015). However, the multi-structure of lignin leads to diversiform product streams, which require extensive separation, purification, and recovery processes. Moreover, thermochemical treatments also require large amounts of energy to break the recalcitrant structure of lignin. The conversion of lignin by microorganisms is an alternative option for lignin valorization because this method enables low cost and generates environment friendly products (Hermosilla et al. 2017; Ragauskas et al. 2014). Although lignin is recalcitrant, it still can be decomposed by some microbes. Many studies have focused on lignin biodegradation by wood-rotting basidiomycetes, especially white-rot fungi, which have been investigated for decades with certain research achievements. However, few commercial lignin biodegradation processes by fungi have been developed. New processes are required for lignin valorization.

Recently, bacterial systems have drawn an increasing attention for lignin valorization due to their rapid growth, biochemical versatility, and powerful environmental adaptability. Some researchers even claimed that bacteria may play a leading role in lignin deterioration and degradation in the natural environment (Morii et al. 1995). In fact, bacteria that can depolymerize lignin have already been discovered in compost soil, rainforest, eroded bamboo slips, sludge of pulp paper mill, and intestines of wood-feeding insects (Bandounas et al. 2011; Chai et al. 2014; Mathews et al. 2016; Paliwal et al. 2015; Raj et al. 2007a, b; Suman et al. 2016; Tian et al. 2014). Some of these bacteria have already been used in bio-product synthesis, such as polyhydroxyalkanoate, lipid, vanillin, and other high-value compounds (Cannatelli and Ragauskas 2016; Lin et al. 2016; Shi et al. 2017; Shields-Menard et al. 2017). Lignin-degrading bacteria have also been applied in the treatment of sewages from rayon grade pulp industry (Yadav and Chandra 2015). However, most of these reported bacteria require additional carbon sources for cell growth and ligninolytic enzyme production, and their ligninolytic capability was much lower than that of fungi, which impedes their industrial application. Lignin valorization by bacteria is still in the research stage, and efficient bacteria that can biodegrade and valorize lignin are still needed to enrich the bacterial library (Chen and Wan 2017; Raj et al. 2007b).

Here, we report the isolation and characterization of three potential lignin-degrading bacteria from leaf mold samples. These bacteria exhibited rapid growth in a medium that contains kraft lignin as the sole carbon source. The lignin-degrading capability of these three isolates was determined, and their activities of ligninolytic enzymes were also analyzed. The isolates obtained will be valuable in lignin valorization due to their excellent growth capability and powerful ligninolytic enzymes.

Materials and methods

Isolation and identification of lignin-degrading bacteria

Leaf mold samples of the Purple Mountain (118° 50' E, 32° 04' N) and Laoshan National Forest Park (118° 30' E, 30° 40' N) were collected to screen lignin-degrading bacteria. In brief, 1 g sample was dissolved in 100 mL sterile saline solution (0.9% NaCl) to form a soil solution. Afterward, 1 mL soil solution was added into 50 mL modified M9 medium (MM9 medium), which consisted of Na₂HPO₄ 12.8 g/L, KH₂PO₄ 3.0 g/L, NaCl 0.5 g/L, (NH₄)₂SO₄ 0.5 g/L, and kraft lignin 10 g/L. The culture broth was cultivated aerobically at 30 °C, 200 rpm for 24 h, and then, 1 mL culture broth was trans-inoculated into a fresh MM9 medium. After repeating this process for 7 days, the final culture broth was diluted 10⁻⁹ times for single-colony plotting on a agar plate. The obtained single colonies were re-streaked at least three times to obtain a pure colony for strain identification and ligninolytic activity characterization.

The micro-morphology and 16s rRNA gene of the isolates were determined to verify the identity of the screened isolates. The micro-morphology of the screened isolates was observed via scanning electron microscopy (SEM) (JEOL, JSM-6380, Japan). The target nucleotide fragments for 16s rRNA gene identification were amplified with bacterial universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTACCTTGTTACGACTT). The obtained sequences were aligned with the multiple sequence alignment software CLUSTALW X2 and the BLAST sequence analysis tool (<http://blast.ncbi.nlm.nih.gov/>). The phylogenetic tree was constructed by the neighbor-joining method using Mega 6.0.

Determination of bacterial growth and lignin-degrading capability

Cell growth and lignin degradation were initially determined to test the ligninolytic activity of the screened bacteria. A loop of single colony was inoculated into LB medium and incubated for 12 h at 30 °C, 200 rpm. The culture broth was sequentially centrifuged for 5 min at 4000 rpm to collect the bacterial cells. The cell pellets were washed with sterile saline solution (0.9% NaCl) three times. The washed cells were inoculated into MM9 medium (containing 1 g/L kraft lignin) with the initial OD₆₀₀ = 0.1. The lignin biodegradation experiment was performed at 30 °C, 200 rpm for 7 days. In brief, 1 mL sample was withdrawn every 24 h to measure cell growth and lignin degradation. Cell growth was determined by colony-forming unit (CFU) counts instead of OD₆₀₀ determination because there was still an absorption of the MM9 medium without bacteria at 600 nm (OD₆₀₀ was approximately 0.228

when 1 g/L lignin was applied). Cell growth in MM9 medium that contains glucose or sucrose instead of kraft lignin was used as the control. Lignin degradation was evaluated by monitoring the decrease in A_{280} with a TU-1810 spectrophotometer (Purkinje General Instrument Co., Ltd., Beijing), and an uninoculated medium was used as the control (Chai et al. 2014; Kumar et al. 2015; Raj et al. 2007a). Degradation ratio was calculated by the equation: $\text{Degradation}\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$. All of the experiments were conducted in triplicates.

Characterization of the decolorization capability for aromatic dyes

The capability of the screened isolates to decolorize the aromatic dyes whose structures are close to lignin fragments was determined to further understand their role in lignin degradation. The following dyes were selected: Malachite Green (MG), Methylene Blue (MB), Remazol Brilliant Blue R (RBBR), Toluidene Blue O (TB), and Azure B (AB) (Fig. S1). Dye decolorization by the isolates was conducted following the method of Bandounas et al. with slight modification (Bandounas et al. 2011). Each of the isolates was separately inoculated in LB medium and cultivated at 30 °C, 200 rpm until $\text{OD}_{600} = 1.0$. Different lignin-mimicking dye solutions were added into the culture broth to achieve the final concentration of 50 mg/L. The cultivations were maintained for another 72 h. The LB medium without bacteria was used as the blank control for spontaneous dye decolorization. At the end of the incubation, the samples were centrifuged at 12,000 rpm for 10 min and the supernatants were used to determine aromatic dyes decolorization. The decolorization of a specific dye was measured and calculated at λ_{max} (MG, 470 nm; MB, 665 nm; RBBR, 595 nm; TB 635 nm; AB, 650 nm). The decolorization ratio was calculated with the same method of lignin degradation as follows: Decolorization

$$\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \text{ (Tian et al. 2016).}$$

SEM and Fourier transform infrared spectroscopy analysis

The changes in morphology and chemical bonds of lignin were observed with SEM and Fourier transform infrared spectroscopy (FTIR), respectively, to visually confirm the depolymerization of lignin by the isolates. Samples were withdrawn after 7 days of incubation in MM9 medium. The samples were centrifuged at 12,000 rpm for 10 min to remove bacterial cells. Then, the supernatant was dried to constant weight with a vacuum freeze-drying apparatus (LGJ-10D, Four-Ring Science Instrument Beijing Co., Ltd., China). The dried powder samples were used for SEM and FTIR analysis. The

morphology of related samples was examined via SEM (JEOL, JSM-6380, Japan). FTIR spectra were measured in the 4000–400- cm^{-1} region by a Nicolet iS5 FTIR spectrometer using an iD7 diamond attenuated total reflectance optical base with a resolution of 1.0 cm^{-1} . In the SEM and FTIR analysis, the untreated lignin and the lignin treated by incubation without bacteria were applied as the controls.

Enzyme assays

As the isolates were all cellular bacterium, the kraft lignin cannot be taken up into the cell directly. Therefore, these bacteria had to secrete extracellular ligninolytic enzymes to degrade lignin. Thus, the extracellular enzyme activities of the isolates were assayed. Laccase (Lac) activity was determined by monitoring the oxidation of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS radical at 420 nm ($\epsilon_{420} = 36,000 \text{ mol}^{-1} \text{ cm}^{-1}$). The reaction mixture (1 mL) contained 50 mM HAc-NaAc (pH 4.5), 1 mM ABTS, and 100 μL cell-free supernatant (Sakamoto et al. 2008). Lignin peroxidase (LiP) activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde at 310 nm ($\epsilon_{310} = 9300 \text{ mol}^{-1} \text{ cm}^{-1}$). The reaction mixture (1 mL) contained 50 mM HAc-NaAc (pH 4.5), 1 mM veratryl alcohol, and 100 μL cell-free supernatant (Kapich et al. 2004). Mn-peroxidase (MnP) activity was determined by monitoring the oxidation of 2,6-dimethyl phenol (2,6-DMP) to coerulignone at 469 nm ($\epsilon_{469} = 49,600 \text{ mol}^{-1} \text{ cm}^{-1}$). The reaction mixture (1 mL) contained 50 mM HAc-NaAc (pH 4.5), 1 mM MnSO_4 , 0.5 mM 2,6-DMP, and 100 μL cell-free supernatant (Kapich et al. 2004). The reactions of LiP and MnP were started with 0.1 mM H_2O_2 . One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol product per min. All assays were performed in triplicate.

Results and discussion

Isolation and identification of lignin-degrading bacteria

The microorganisms living in leaf molds have been identified as fast decomposers of plant materials. Rapid nutrient cycling of dead wood and leaf litter by microorganisms is thought to support the bio-diversity of forests (Carles et al. 2017). Thus, we attempted to screen for bacteria with high ligninolytic capacity from the leaf mold samples. To obtain lignin-degrading bacteria, 10 g/L kraft lignin was added in MM9 medium. The microorganisms that cannot depolymerize kraft lignin and utilize the degradation compounds as the carbon and energy sources would be weeded out because no other carbon source exists in the medium. Furthermore, the high concentration of

kraft lignin can also eliminate most microorganisms that cannot tolerate aromatic compounds derived from lignin decomposition because these aromatic compounds can induce membrane disruption, enzyme inhibition, and DNA damage (Zeng et al. 2014). After 7-day repeated cultivation, culture broth was diluted for 10^{-9} times and plated on a MM9 agar plate and the three most abundant bacteria were obtained based on colonial morphology difference. To identify the species of the screened isolates, the 16S rRNA gene sequences were determined and submitted to NCBI with accession numbers of MF093730, KY593170, and MF093751. On the basis of 16S rRNA gene sequence homology, these three isolates were closely matched to *Klebsiella pneumoniae* strain CAV1042 (99%), *Pseudomonas putida* strain PC2 (99%), and *Ochrobactrum tritici* TA93 (99%) (Table 1). To further confirm the species of the screened bacteria, the micro-morphologies of the isolates were observed by SEM (Fig. S2), and the result was consistent with that of 16S rRNA gene blast. Thus, these three isolates were identified and named as *Klebsiella pneumoniae* NX-1, *Pseudomonas putida* NX-1, and *Ochrobactrum tritici* NX-1 (Fig. 1).

K. pneumoniae, a gram-negative bacterium that belongs to γ -Proteobacteria, is widely distributed in the environment. In industrial biotechnology, *K. pneumoniae* is an important strain for many bio-products, such as 2,3-butanediol, muconic acid, and some exopolysaccharides. To the best of our knowledge, only one study was conducted on lignin degradation by *K. pneumoniae*, which was co-cultivated with a *Bacillus subtilis* (Yadav and Chandra 2015). However, several studies reported its degradation capability of aromatic substances, such as N,N'-dimethyl-p-phenylenediamine, herbicide bromoxynil, and methyl red (Wong and Yuen 1996, 1998). The degradation capability of *K. pneumoniae* for aromatic substances indicated that this species contains abundant oxidoreductases, which are also important components for lignin degradation. *P. putida* is commonly considered as an innocuous environmental microorganism with an enormous potential for biotechnological applications due to its well-developed genetic system and metabolic versatility. *P. putida* has been applied to produce various bio-products, such as arginine deiminase, biosurfactant, and indigoids (Patil et al. 2017). Except for synthesizing many valuable bio-products, *P. putida* has also been applied to degrade multitudinous

hazardous compounds, especially heterocyclic compounds and aromatic compounds, for example, nicotine, lignin, phenol, polycyclic pesticide, methyl parathion, and γ -hexachlorocyclohexane (Lin et al. 2016; Ravi et al. 2017). Despite limited available reports, some researchers have found the powerful capability of *O. tritici* to biodegrade pyrethroids and nicotine (Wang et al. 2011; Yu et al. 2015). As an opportunistic pathogen of wheat, the pathogenicity of *O. tritici* may be greatly correlated with its lignin-degrading capability because lignin is a critical protective layer for protecting plants from being damaged by microorganisms and insects. To the best of our knowledge, this is the first report with *O. tritici* species capable of degrading lignin.

The lignin-degrading bacteria reported in the literature fall into several classes: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Archaea*, and some *Bacteroidetes*. These bacteria were isolated from compost soil, sediments, sludge of pulp paper mill, eroded bamboo slips, and insect guts. Among these strains, *Bacillus subtilis*, *Paenibacillus* sp., *K. pneumoniae*, and *Aneurinibacillus aneurinilyticus* have been applied for the treatment of pulp mill effluent (Mathews et al. 2014; Raj et al. 2007a; Yadav and Chandra 2015). Some strains have already been applied in pretreatment to accelerate cellulase performance (Bugg et al. 2011; Chang et al. 2014), and some bacteria can even produce valuable compounds from lignin (Johnson and Beckham 2015; Vardon et al. 2015; Zhao et al. 2016). These studies illustrated the abilities of bacteria in lignin treatment. We believe that the screened isolates in this study are excellent additions to the knowledge of lignin valorization by bacteria.

Cell growth of the isolates using lignin as the sole carbon source

As reported, the lignin-degrading bacteria could be classified into two categories based on their carbon source utilization: those that could utilize lignin as the sole carbon source for cell growth and other physiological metabolic activities, and those that require additional carbon sources for cell growth and lignin decomposition (Bandounas et al. 2011). In this study, no additional carbon source in MM9 medium is available, and thus, all isolates could utilize lignin as the sole carbon source. To determine the cell growth of the isolates on lignin as the

Table 1 Identification of isolates by 16S rRNA gene sequencing

Bacterial isolates	Closest phylogenetic relative	Bacteria subdivision	Sequence identity	GenBank accession number	Sequence length (nt)
Strain A	<i>K. pneumoniae</i> ATCC 13883	γ -Proteobacteria	99%	MF093730	1441
Strain B	<i>P. putida</i> IAM 1236	γ -Proteobacteria	99%	KY593170	1427
Strain C	<i>O. tritici</i> TA 93	α -Proteobacteria	99%	MF093751	1382

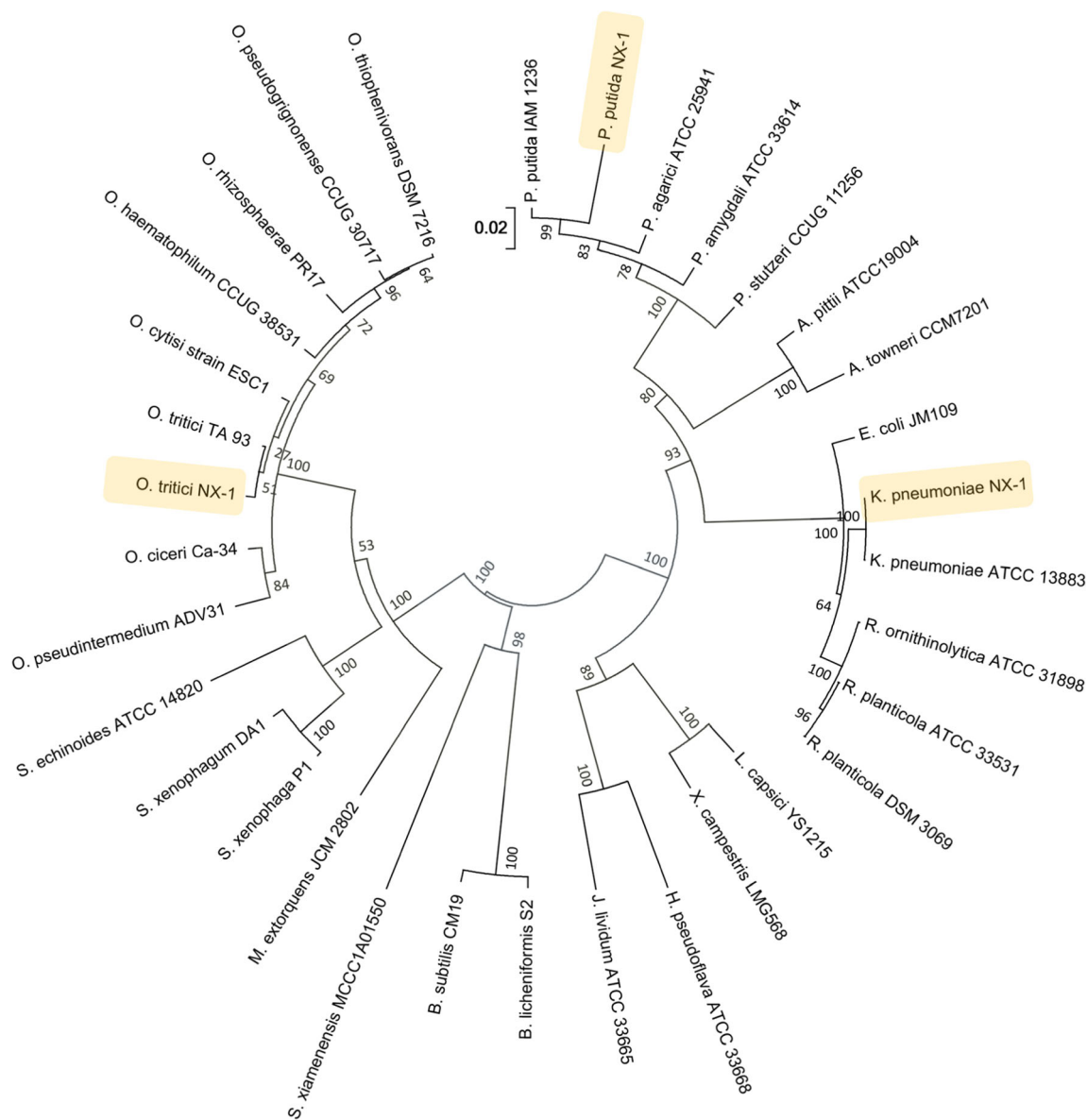


Fig. 1 Phylogenetic relationships of the isolated bacteria and other strains based on the neighbor-joining tree analysis of the 16S rRNA gene

sole carbon source, the bacterial counts were determined in the 3rd and 7th days. As shown in Table 2, all of the three isolates reached 10^{10} – 10^{11} CFUs/mL in the MM9 medium. In previous study, lignin-degrading bacteria achieved only 10^5 – 10^9 CFUs/mL in the lignin media. For example, *Rhodococcus*

opacus DSM 1069R achieved approximately 10^7 CFUs/mL in a medium that contains ethanol organosolv lignin (Kosa and Ragauskas 2013). *Rhizobium* sp. strain YS-1r achieved approximately 10^7 CFUs/mL in alkali lignin medium (Jackson et al. 2017). *Pseudomonas* sp. LD002 and *Bacillus*

Table 2 Growth of bacterial isolates on different carbon sources

Carbon sources	Day 3 (CFUs/mL)			Day 7 (CFUs/mL)		
	<i>K. pneumoniae</i> NX-1	<i>P. putida</i> NX-1	<i>O. tritici</i> NX-1	<i>K. pneumoniae</i> NX-1	<i>P. putida</i> NX-1	<i>O. tritici</i> NX-1
Glucose	$(3.0 \pm 0.7) \times 10^{17}$	$(7.8 \pm 3.4) \times 10^{15}$	$(3.1 \pm 1.2) \times 10^{15}$	$(3.0 \pm 1.2) \times 10^{15}$	$(1.3 \pm 0.4) \times 10^{15}$	$(4.6 \pm 2.8) \times 10^{16}$
Sucrose	$(4.2 \pm 1.5) \times 10^{13}$	$(8.3 \pm 2.1) \times 10^8$	$(1.8 \pm 0.7) \times 10^{11}$	$(1.6 \pm 0.5) \times 10^{13}$	$(1.0 \pm 0.7) \times 10^8$	$(2.7 \pm 1.3) \times 10^{12}$
Lignin	$(1.4 \pm 0.8) \times 10^{11}$	$(4.5 \pm 1.8) \times 10^{10}$	$(2.0 \pm 0.4) \times 10^{10}$	$(6.0 \pm 3.7) \times 10^9$	$(3.2 \pm 1.7) \times 10^{11}$	$(3.7 \pm 1.8) \times 10^{10}$

sp. LD003 achieved 10^9 – 10^{10} CFUs/mL in the lignin medium (Bandounas et al. 2011). Compared with those in previous studies, the three isolates in the present study exhibited advantage in cell growth when using lignin as the carbon source. The growth superiority of these three isolates indicated their potential industrial application. However, there is still a great room for improvement because these isolates' cell density could reach as high as 10^{15} – 10^{16} CFUs/mL when glucose was used as the carbon source (Table 2).

Lignin and aromatic dye degradation by the screened bacteria

The decrease in A_{280} could be used to characterize the degradation of aromatic compounds due to the intense absorption of benzene ring at 280 nm. Thus, the A_{280} of incubation was detected daily to determine the rate of lignin degradation (Chai et al. 2014; Kumar et al. 2015; Raj et al. 2007a) (the standard curve of “Abs₂₈₀ and lignin concentration” was presented in the supplementary materials (Fig. S3)). The lignin degradation results, which are illustrated as the reduction of A_{280} , are shown in Fig. 2. A_{280} was reduced by 23.8, 28.5, and 19.4% during the 7-day incubation with *K. pneumoniae* NX-1, *P. putida* NX-1, and *O. tritici* NX-1, respectively. All of these three isolates rapidly degrade kraft lignin in the initial 4 days. This finding indicated that the isolates could break down lignin in the primary metabolic stage. The same result is also mentioned in the study of lignin degradation by *Comamonas* sp. (Chai et al. 2014). The loss of lignin content can be directly correlated with the cell growth of the isolates because no additional carbon source exists in the medium. Although the growth rates of the isolates slowed down in the 5th–7th days, considerable amount of lignin was degraded. This phenomenon occurred because these bacteria utilized lignin not only for their growth but also for other metabolic activities. Moreover, the slow degradation rates in the 5th–7th

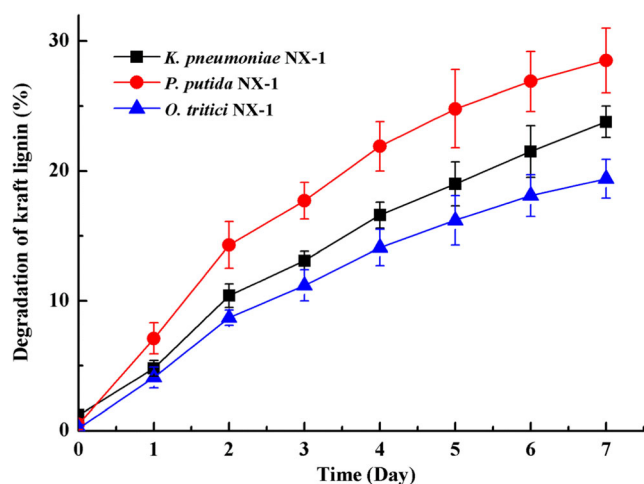


Fig. 2 Kraft lignin degradation with the treatment of the three isolates

days are probably caused by the special obstinate components which cannot be degraded directly by the screened isolates.

The decolorization of dyes, whose structure is close to that of lignin fragments, was used as indicator of lignin-degrading activity because dye decolorization is direct and easy to visualize. Among the five types of the aromatic dyes used in the experiments, MG showed a great extent of spontaneous dye decolorization (54.58%) in 72 h even without any bacteria. Spontaneous dye degradation was also observed by Bandounas (Bandounas et al. 2011; dos Santos et al. 2014), and the spontaneous dye degradation may be caused by pH and light fluctuation. The decolorization rates of the screened bacteria are shown in Fig. 3. In general, each isolate exhibited a certain degradation capability for MB, MG, TB, and AB. *K. pneumoniae* NX-1, *P. putida* NX-1, and *O. tritici* NX-1 degraded MG with maximum decolorization rate of 93.9, 87.7, and 93.1%, respectively. In particular, only *K. pneumoniae* NX-1 significantly decolorized RBBR (17.3%), indicating that some special enzymes exist in *K. pneumoniae* NX-1 as compared with *P. putida* NX-1 and *O. tritici* NX-1. The undecolorization for RBBR of *P. putida* NX-1 indicated that it lacked related enzymes for RBBR degradation. However, *P. putida* NX-1 must have other powerful enzymes for lignin degradation because it exhibited the most powerful degradation capability for lignin as compared with *K. pneumoniae* NX-1, and *O. tritici* NX-1. As mentioned in the introduction part, some bacteria display versatile metabolic pathways and various enzymes to degrade and metabolize heterocyclic compounds and aromatic compounds, from simple phenols to xenobiotic substances and complex lignins, and these bacteria are great treasures for target enzyme mining. Therefore, further research may focus on investigating the related genes and enzymes involved in the decolorization of lignin and related dyes in these three isolates. Moreover, these

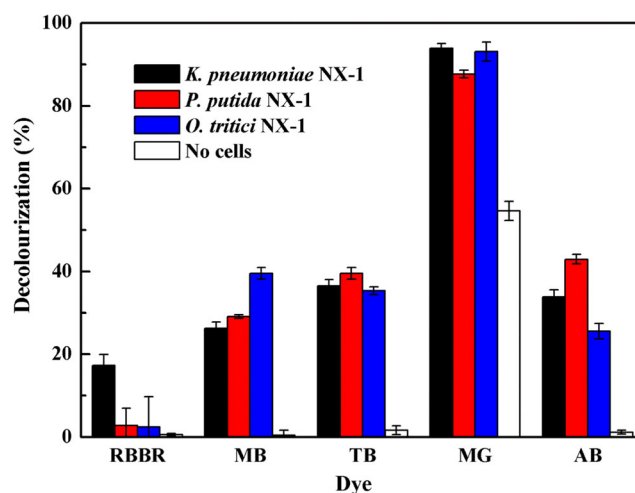


Fig. 3 Decolorization of ligninolytic indicator dyes. Dyes: Remazol Brilliant Blue R (RBBR), Toluidene Blue O (TB), Methylene Blue (MB), Malachite Green (MG), and Azure B (AB)

three isolates could also be applied in the decolorization and degradation of reactive textile dyes, similar to *Phanerochaete chrysosporium* (Koyani et al. 2013).

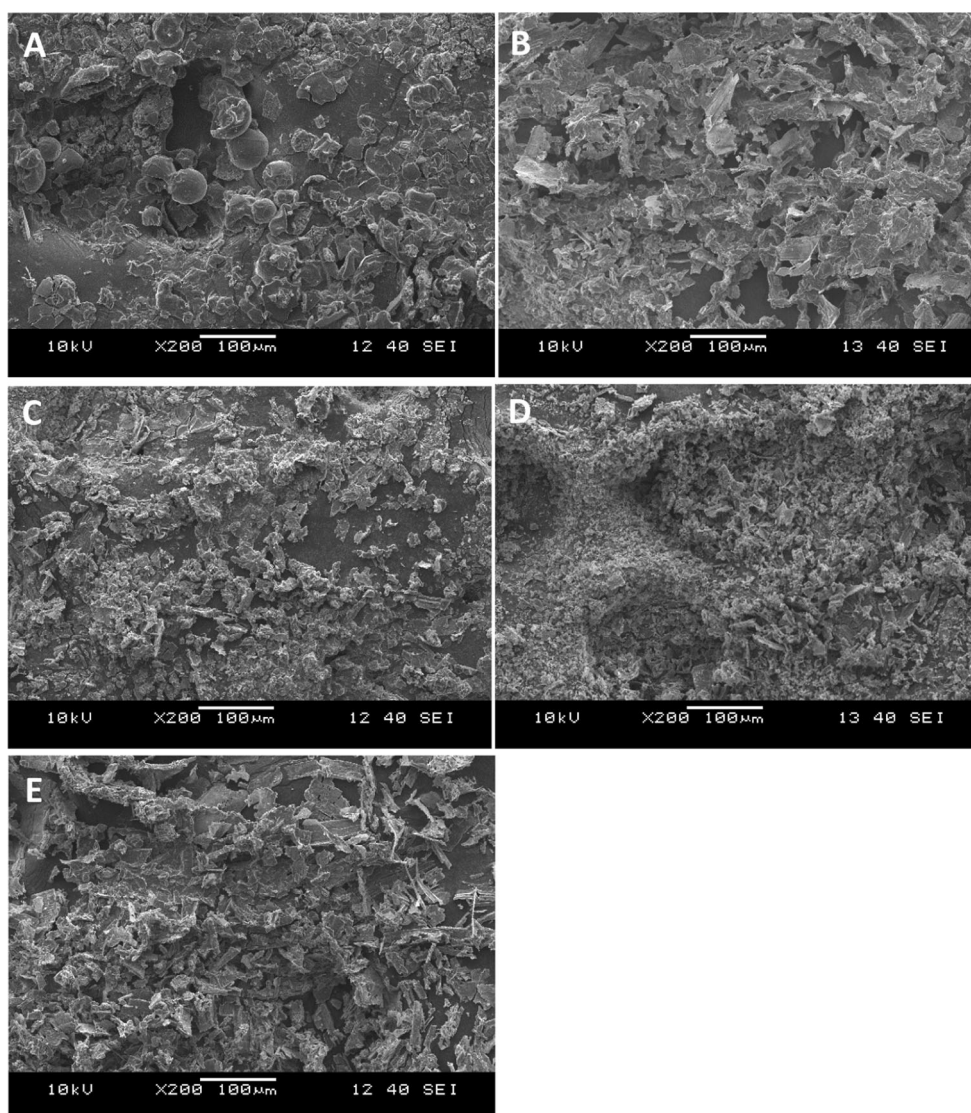
Micro-morphology and chemical group analysis for lignin degradation

To confirm the degradation of lignin visually, the change in its morphology was observed by SEM. The SEM images of the related kraft lignin are presented in Fig. 4. The figure exhibited that the untreated kraft lignin consisted of small balls, ovals, and spherical fragments (Fig. 4(a)), which have become irregular fragments after 7 days of incubation even without any bacterium (Fig. 4(b)). The particle sizes of lignin treated with the isolates were smaller compared with that of lignin without microbial treatment. The lignin incubated without isolates has large bigger particle sizes (within the range of 100–200 μm). When incubated with the isolates, the fragment size ranges of

lignin were reduced evidently by different degrees. In particular, the fragment size of the lignin treated with *K. pneumoniae* NX-1 was reduced to 10–30 μm (Fig. 4(c)). The change in lignin fragment size indicated that lignin was physically and/or chemically decomposed into small parts. Thus, these three isolates were visually confirmed as capable of lignin degradation.

FTIR could exhibit a visualized and rapid indication of the changes in chemical bonds, and many researchers have investigated the structural changes of lignin given its infrared absorption characteristics (Chen et al. 2015; Liu et al. 2014). The FTIR spectra of related kraft lignin samples are presented in Fig. 5. The figure shows indicated that the appearance of lignin was changed from round to flakes when incubated in the liquid medium for 7 days. However, the change in its chemical bond was slight. Moreover, the FTIR spectra of kraft lignin changed evidently under the action of the isolates and the main change ranged from 1600 to 500 cm^{-1} , especially at

Fig. 4 SEM images on lignin treated with the three isolates. a Lignin incubation without strains; b lignin was not treated by incubation; c–e lignin treated by 7 days with *K. pneumoniae* NX-1, *P. putida* NX-1, and *O. tritici* NX-1, respectively



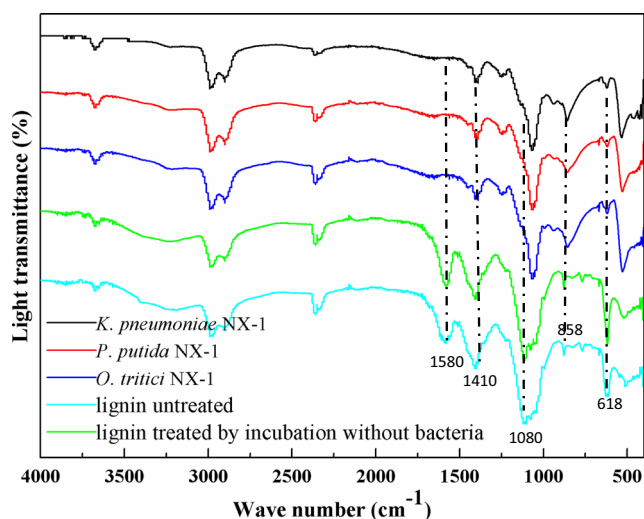


Fig. 5 The FTIR spectrum of treated and untreated kraft lignins

1580, 1410, 1080, 858, and 618 cm^{-1} . The band at 1580 cm^{-1} was attributed to the stretching vibration of aromatic rings (Chai et al. 2014); the band at 1410 cm^{-1} was attributed to carboxylic anionic groups, carbonate, and CH_2 (Cohen and Gabriele 1982); the band at 1080 cm^{-1} may be attributed to C–O vibrations in aliphatic ethers and/or in secondary alcohols (Carvalho et al. 2008); the band at 858 cm^{-1} was assigned to the C–H out-of-plane vibrations of guaiacyl units (Wang et al. 2015); the band at 618 cm^{-1} was assigned to the stretching vibrations of the C–S bond linked to the aromatic ring (Rumyantseva et al. 1994). The variation in sizes and chemical groups is consistent with the decrease in A_{280} in the above experiment, further illustrating that lignin was chemically broken by the screened bacterial strains.

Analyses of enzyme activities related to lignin biodegradation

With the decrease in A_{280} of culture broth, the decolorization of aromatic dyes, and the variation in lignin sizes and chemical groups, there must be related enzymes secreted by the isolates. It is commonly accepted that Lac, LiP, and MnP are the three major enzymes involved in lignin-degrading processes. Lac is a class of multicopper oxidases that can catalyze the oxidation of a variety of organic compounds, including oxidative coupling of phenolic compounds, aryl-ring cleavage, and degradation of polymers (Huang et al. 2013). LiP is a hemeprotein which catalyzes the H_2O_2 -dependent oxidation of lignin derivative-based polymers. Compared with the common LiP, MnP is a specific heme enzyme, whose catalysis depended on the oxidation of Mn^{2+} to Mn^{3+} (Xu et al. 2017). In this research, the cell-free supernatant was used to measure the ligninolytic enzyme activity. The activity of related ligninolytic enzymes from these three isolates is presented in Fig. 6. The results show that all three isolates secreted

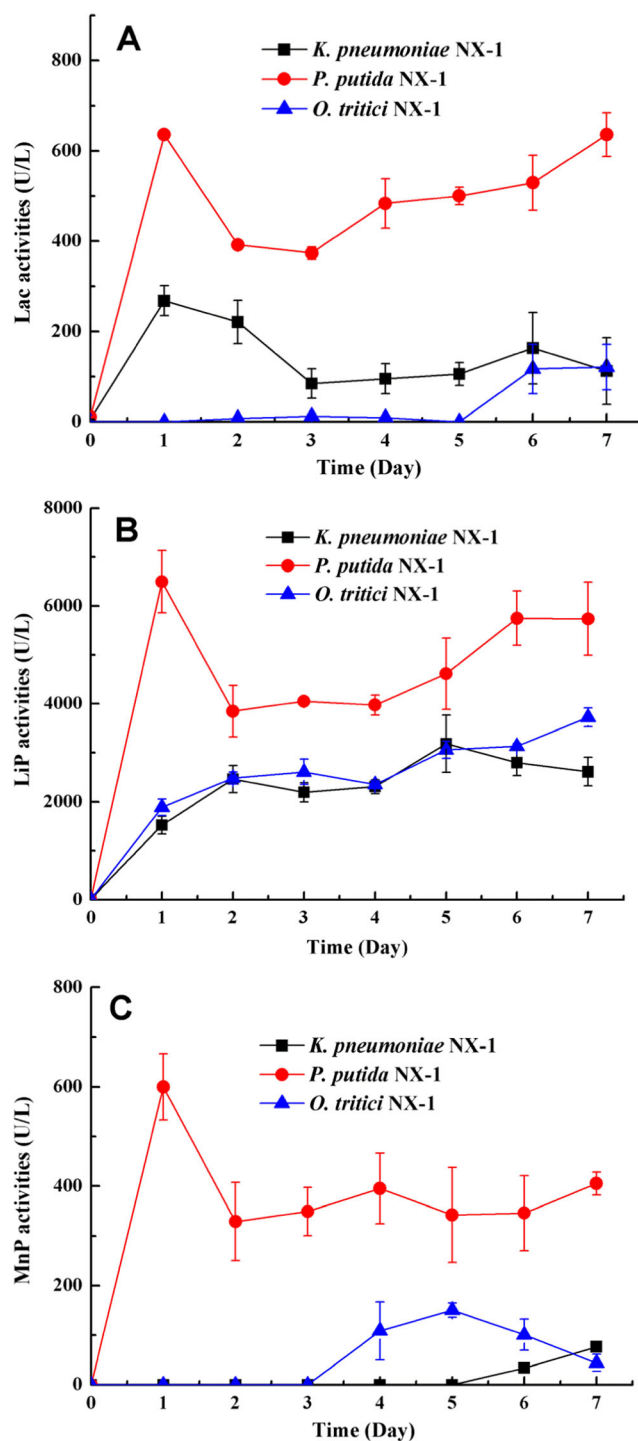


Fig. 6 Activities of the Lac, LiP, and MnP during the incubation

considerable reactive LiP under tested conditions. However, only *P. putida* NX-1 secreted Lac and MnP efficiently. For *P. putida* NX-1, the activities of Lac, LiP, and MnP reached the maximum values of 635.9, 6497.2, and 599.7 U/L, respectively, during the first day followed by evident decrease in the second day. Then, the related activity fluctuated in a definitive range in the 3rd–7th days, indicating that ligninolytic enzymes were secreted during the entire life cycle of *P. putida* NX-1,

including the primary and secondary metabolism stages. However, *P. putida* NX-1 did not exhibit any capability to decolorize RBBR (Fig. 3), which could be degraded by Lac secreted by cyanobacteria and white-rot fungi. For *K. pneumoniae* NX-1, the activities of Lac and LiP were detected during the first 5 days, whereas low MnP activity was only detected in the 6th and 7th days. In contrast to that in *P. putida* NX-1 and *K. pneumoniae* NX-1, only high LiP activity was detected in *O. tritici* NX-1, and the Lac and MnP activities were extremely low during incubation. The enzyme activity assays revealed that *P. putida* NX-1 exhibited the best ligninolytic capability among the three isolates. The result of lignin morphology also yielded the same conclusion, because the lignin treated with *P. putida* NX-1 had the smallest particle size (Fig. 4(d)).

In previous studies, some research results indicated that bacteria cannot secrete extracellular ligninolytic enzymes and thus could only use the low molecular weight portion of lignin instead of depolymerizing lignin. However, in 1988, Ramachandra et al. reported that *Streptomyces viridosporus* T7A could secrete extracellular peroxidases for lignin degradation (Ramachandra et al. 1988). Since then, other ligninolytic enzymes, such as oxygen-utilizing Lac and MnP from *Rhodococcus jostii* RHA1 and *Comamonas* sp. B-9, were found successively (Chai et al. 2014; Chen et al. 2012). In some cases, the characteristics, such as temperature-, pH-, and salt-tolerance, of bacterial-origin ligninolytic enzymes are superior to their fungal analogs and confirm that their potential industrial applications are intriguing. For example, some Lac found in *Bacillus halodurans* exhibited excellent characteristics in alkaline- and chloride-contaminated environment (Ruijssenaars and Hartmans 2004). Thus, it is interesting to characterize the ligninolytic enzymes of these three isolates and evaluate their applications in bio-bleaching, bio-pulping, bio-remediation, and other industrial processes in the future studies.

Conclusion

In this study, three lignin-degrading bacteria were isolated from leaf molds. All three isolates exhibited substantial growth on kraft lignin. The ligninolytic capabilities of the three isolates were evaluated, and their related ligninolytic enzyme activities were detected. The results showed that *P. putida* NX-1 exhibited the best lignin-degrading capability and could efficiently secrete all of Lac, LiP, and MnP. We believe that these three bacteria are important supplements to ligninolytic bacteria library due to their excellent ligninolytic capabilities, and these isolates may also be valuable in lignin valorization.

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