Biodegradation of Sulfonated Lignite (SL) by fungi from waste drilling mud

Hanjun Liu1, 2, Qiang Chen2*, Min Huang1, Lirong Chen1, Xuebin Jiang1, Entao Wang1, Yihao Liu2, Xuemei Leng2, Liang Xie2, Ke Zhao2, Yunfu Gu2, Xumei Yu2, Ke Zhang4 and Liangji Deng2*

1 Safety and Environmental Protection Quality Supervision and Testing Research Institute, CNPC Chuanqing Drilling Engineering Co. Ltd., Guanghan 618300, China
2 College of Resource, Sichuan Agricultural University, Wenjiang, Sichuan 611130, China
3 Departamento de Microbiologia, Escuela Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, México D.F. 11340, México
4 College of Civil Engineering, Sichuan Agricultural University, Dujiangyan 611830, China
*Corresponding author’s e-mail: cqiang@sicau.edu.cn (Q. Chen), auh6@sicau.edu.cn (L. Deng)

Abstract. In this paper, three indigenous Sulfonated lignite (SL) degrading fungi were isolated from waste drilling mud (WDM) and were identified, and the mechanism of SL degradation and potential of WDM bioremediation was presented. These SL degrading fungi (WNF15, WNF20, and WNF22) were as Pseudallescheria ellipsoidea, Stachybotrys chartarum, and Scopulariopsis brevicaulis by their physiological characteristics and ITS gene sequencing. SL degradation test results showed that all three species can effectively degrade SL but WNF-20 had the highest SL degradation ratio (47.54%). MnP and Lac were the dominant enzymes used by the fungi for SL degradation. Analysis of SO42- concentration and degradation products suggested that the fungus firstly breaks the C-SO3 bond and release of sulfite assisted. Subsequently, breaks the branched-chain and crosslink bone of the aromatic rings in desulphurisation by-products, and then the broken crosslink bone were transformed into hydrocarbons and aromatic acids. Inoculation of a mixture of the three fungi into a liquid MS medium containing 5% WDM resulted in a chemical oxygen demand removal ratio of 87.65% after 14 d. All rustles suggest that these three fungi are efficient SL degraders and can be used for bioremediation of WDM in gas drilling fields.

1. Introduction
Lignite plays a critical role in the world’s energy supply and its reserves are a third of all coal. But, lignite is regarded as a kind of cheap and less environmentally friendly fuel by reason that low calorific value and high ash content[1]. But lignite has strong thermal stability, thus it was used as filter loss agent and viscosity reducer for drilling fluid in oil and gas drilling[2-3]. Meanwhile, lignite has natural ion exchange characteristic, since it received increasing attention in wastewater treatment and currently offers a very attractive method of heavy metal ions pollution remediation[4-5].
However, the application of lignite in oil and gas drilling is seriously restricted due to its poor water solubility and salt resistance over the last decades. Sulfonated lignite (SL) is mainly obtained by the sulfonated reaction of lignite and vitriol at 120°C to 200°C, which has good water solubility, salt resistance and strong thermal stability due to sulfonate groups was introduced into humic acid molecules, thus it was widely used as filter loss agent and viscosity reducer for drilling fluid in oil and gas drilling[6-7].

In general the dosage of SL is typically 2% - 3% (m:m) in the drilling fluid, and almost of them were remained in wastes drilling mud (WDM) after drilled well. About 2.5 million tons of WDM produced every year in China[8]. The high concentrations of organic pollutants in WDM have detrimental effects on organisms in the soil and water. These pollutants increase the chemical oxygen demand (COD), and are toxic to algae, phytoplankton, and plants[9-10]. The growth of protozoans and plants are affected[11-12] when untreated WDM is deposited onto surrounding soil. In addition, SL is a major contributor to the COD in the WDM. Thus, dispose of SL in WDM is important for oil and gas drilling in China.

Currently, the lignite disposal can be done in either physical method or biological approach[13-14]. Compared with the physical processes, biodegradation of lignite has garnered a great deal of attention due to its mild conditions[15-16]. Despite lignite’s natural recalcitrance, some fungi have been employed to degrade lignite due to their powerful lignin degrading enzymatic systems[1-14,17-18]. However, not any microorganisms were studied for their potential to decompose SL. In addition, the sulfonyl (SO3H) is introduced into the condensing aromatic ring and the fatty side chain of lignite after sulfonated reaction. The presence of sulfonate group in aromatic rings can render such compounds inhibitory towards microbial growth, making their aerobic biodegradation very difficult[19]. For example, the biodegradability of aromatic amines can range from easily degradable to non-biodegradable, depending on the position and number of sulfonate group in the aromatic ring[20]. Therefore, it is necessary to screen high activity of SL-degrading microbial. The purposes of this article were to isolate and screen indigenous fungi for their ability to degrade SL and determined their morphology, physiological characteristics, and the taxonomy, and then studied their capability to degrade SL and WMD.

2. Materials and methods

2.1. Sample collection
WDM samples were collected from Deyang (latitude: N 31°12.257’; longitude: E 104°19.545’), Shifang (latitude: N 31°06.398’; longitude: E 104°06.367’), Inner Mongolia (latitude: N 41°03.290’; longitude: E 113°24.205’) and Xinjiang province (latitude: N 45°32.314’; longitude: E 84°58.337’) in China in March 2015. Samples were homogenized, and stored at 4°C until use. A SL sample (technical pure) was get from market.

2.2. Chemicals and medium
Mineral salt (MS) medium was used for enrichment, isolation and screening of the SL-degrading fungi. The MS liquid medium contained 0.6 g NH4Cl, 0.5 g K2HPO4, 0.5 g KH2PO4, 0.003 g CaCl2, 0.003 g FeSO4, 0.003g MnSO4, 0.003 g MgSO4, 0.003 g ZnSO4·7H2O and 1000mL distilled water (pH 7.0–7.2). The MS solid medium was prepared by adding 18.0 g agar into 1000 mL MS liquid medium. Potato dextrose agar (PDA) media was applied to fungal culture. The PDA solid medium contained 200 g potato, 20 g glucose, and 18.0 g agar, and 1000mL distilled water, pH 6.5.

The medium used for SL degradation experiments consisted of 0.2 g Na2HPO4, 1.0 g KH2PO4, 0.3 g ammonium tartrate, 0.5 g MgSO4·7H2O, 0.04 g MnSO4·H2O, CaCl2 0.004 g, Tween 80 0.5 g, and 1000mL distilled water, pH 7.0–7.2.

2.3. Enrichment, Isolation and Screening of the SL-degrading fungi
WDM (5 g) were transferred to 95 mL sterilized MS liquid medium, and cultured shakily for 7 d at
25°C and 120 rpm. Then, 5 mL of the culture media was moved to another flask with 95 mL sterilized enrichment medium (MS liquid medium + 5.0 g L\(^{-1}\) SL), and incubated under the same conditions. After 3 continuous passage culture, 1 mL of culture was taken to isolate fungal strains with modified potato dextrose agar (PDA) plates by the streak plate technique. Based on morphology and microscopic characteristics, the pure culture was picked and inoculated on PDA slants, incubated at 25°C for 72 h, and then stored at 4°C. Colony size was examined after 3 d incubation at 25°C on MS medium plates containing SL. SL free MS medium was used as the control. Strains with large and dense colonies were picked and used for further study.

2.4. Identification of SL-degrading fungi

The morphology of SL degrading fungi was examined by microscopic observation. The biochemical and physiological characteristics, including starch hydrolysis, cellulose hydrolysis, and gelatin hydrolysis, were analyzed using the methods of Simmons[21]. The optimal growth temperature range was tested using the following temperatures: 15°C, 20°C, 25°C, 30°C and 35°C. The optimal pH range for growth was tested at pH 7, 8, 9, 10 and 11. The salt tolerance was examined in liquid potato sucrose medium containing 0%, 2%, 4%, 6%, 8%, and 10% (w/v) sodium chloride. Mycelia of test fungal strains were collected from 5 d incubation liquid potato sucrose medium and ground using liquid nitrogen. Fungi genome DNA was collected by a Fast DNATM Spin kit (MPBIO, USA). ITS fragments were amplified using universal primers ITS4 and ITS5[22]. The reaction mixture was composed of 15 μL mix, 1 μL IT4 primer (10 ppm), 1 μL ITS5 primer (10 ppm), 1 μL template DNA, and ddH₂O adjusted to 30 μL. The PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 60 s, 52°C for 50 s and 72°C for 120 s, and a final 72°C for 10 min. The PCR products were checked in 1.0% agarose gel and purified using a PCR purification kit (Tiangen, China). The ITS sequence analysis was done by the Shanghai Major Bio Technology Co. (Shanghai, China) and compared using BLAST on the NCBI website. A phylogenetic tree was constructed using MEGA 7.0 software using the neighbor-joining method. The ITS sequences of *Pseudallescheria ellipsoidea* WNF-15, *Stachybotrys chartarum* WNF-20, and *Scopulariopsis brevicaulis* WNF-22 were deposited in the GenBank database (No.MG976626 to MG976628).

2.5. SL degradation experiments

2.5.1. SL degradation ratio. To evaluate the SL degradation capacity of the fungi strain, 100 mL mineral salt basal medium in a 250-mL Erlenmeyer flask containing 1 g SL[23] was prepared, the tested strain was inoculated, and the un-inoculated medium was the control. All strains were cultured shaking for 14 d at 25°C and 160 rpm, and then the fungi mycelia in liquid culture was separated by nylon net, dried at 80°C and weighed. The SL residue in liquid culture was separated using filter paper, and weighed after drying at 80°C for 8 h. The degradation ratio was determined by the net weight loss method[14].

2.5.2. GC-MS analysis of SL degradation products. For analysis of degradation products, the culture medium was first separated by centrifuging at 9000 rpm for 10 mins and the supernatant was extracted by hexane. The hexane extract was analyzed by GC-MS method[18]. Compounds were separated using a capillary column (RTX-1, 30 m × 0.25 mm × 0.25 μm). The temperatures of the injector and detector were 280°C. N₂ was used as the carrier gas at a flow rate of 1.2 mL min\(^{-1}\). The total injection volume of each sample was 1 μL. The following temperature program was used: hold at 60°C for 2 min, incremental temperature increases at 5°C per min, finally hold at 300°C for 10 min. A quadrupole analyzer (Agilent 7890A/5795C, USA) with an m/z range from 15 to 500 was used and runed in electron impact (70 eV) mode. Data were terated using Chemstation software. Compounds were identified by comparing their mass spectra to those in the NIST14 library.
2.5.3. **Enzymatic activity analysis.** Manganese peroxidase (MnP), Laccase (Lac), and lignin peroxidase (LiP) activity of the tested fungal strains was performed at different culture times. A 20 mL sample of liquid culture medium (described in 2.5.1) was spun at 10,000 rpm for 10 min at 4 °C, collected the supernatant and used as crude enzyme extract. Manganese peroxidase (MnP) activity was determined by monitoring the formation of the Mn$^{3+}$-malonate complexes in 50 mM sodium malonate buffer (pH 4.5) with 0.5 mM MnSO$_4$ ($\varepsilon$ 270 = 11,590 M$^{-1}$ cm$^{-1}$)[24]. Laccase (Lac) was assayed by following the oxidation of 2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) ($\varepsilon$ 420 = 9,300 M$^{-1}$ cm$^{-1}$) in 0.1 M sodium acetate buffer at pH 4.5[25]. Lignin peroxidase (LiP) activity was measured by determining the oxidation rate of veratryl alcohol to veratraldehyde ($\varepsilon$ 310 = 9,300 M$^{-1}$ cm$^{-1}$) in 250 mM sodium tartrate buffer (pH 3.0) at 30°C[26]. All reactions were started by the addition of 1 mM H$_2$O$_2$. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per minute. Each experiment was conducted in triplicate.

2.5.4. **Sulfate radical concentration analysis.** A 10 mL sample of liquid culture medium (described in 2.5.1) was spun at 10,000 rpm for 10 min at 4 °C, collected the supernatant and used as test sample. Sulfate radical concentrations of supernatant were detected using the ion chromatography[27].

2.6. **Degradation of WDM**
Tested fungi strains were inoculated into sterilized 100 mL of MS liquid medium containing 5.0 g WDM, and shaken at 160 rpm for 14 d at 25°C. Meanwhile co-culture experiment using the three strains was performed. The culture was spun at 10000 rpm for 10 min at 4°C and collected the supernatant. The chemical oxygen demand (COD) of the supernatant was determined at different culture times and used to evaluate the degradation ability of WDM by the fungal strain. Un-inoculated medium was used as the control. The COD was tested by using the silver nitrate precipitation method[28].

2.7. **Statistical analysis**
All data are presented as the mean ± standard deviation (SD). Statistical analysis was completed using SPSS for Windows (Ver. 18.0). Statistical significance of treatments was tested at P<0.05 using the Tukey multiple comparisons test. Graphics were plotted using Excel 2010.

3. **Results**

3.1. **Isolation, screening and identification of SL-degrading fungi**
A total of 24 fungi strains (designated WNF-1 to WNF-24) were isolated. The colonies of strain WNF-15, WNF-20 and WNF-22 were larger and denser than the other strains. They were regarded as the best SL-degrading candidates and selected for further study. Morphological and biochemical characteristics of three fungal strains are shown in Table 1 and Figure 1. The colonies of strains WNF-15 and WNF-20 were white, flocy, and loose. WNF-22 colonies were dense and loose. WNF-15 formed orbicular-ovate arthrosporic spores; WNF-20 and WNF-22 formed rod conidiophores. WNF-15 and WNF-20 tested positive for starch and cellulose hydrolysis, but negative for gelatin hydrolysis. WNF-22 was positive for starch, gelatin, and cellulose hydrolysis (Table 1). WNF-15 and WNF-22 grew well in medium containing 2% sodium chloride. The optimum growth temperature was 25°C and optimum growth pH was pH 10, respectively (Figure 1). WNF-20 had a wide growth pH range (pH 7.0 to 11), and the optimum growth temperature was 25°C.

ITS sequence analysis showed that WNF-15, WNF-20, and WNF-22 were 99% similar to *Pseudallescheria ellipsoidea* 161-CC (KJ607262), *Stachybotrys chartarum* ATCC 9182 (AF081468), and *Scopulariopsis brevicaulis* FMR 12216 (KP132731), respectively (Figure 2). The phylogenetic tree (Figure 2) showed that WNF-15, WNF-20, and WNF-22 were located in three different phylogenic branches.
Table 1. Physiological and biochemical characterization of three candidate fungi strains.

| Characteristics       | WNF-15 | WNF-20 | WNF-22 |
|-----------------------|--------|--------|--------|
| Physiological         |        |        |        |
| Colonies              | Flocky | Flocky | Blanket|
| Shape                 |        |        |        |
| Texture               | Loose  | Loose  | Dense  |
| Color                 | White  | White  | Incanus|
| Spore                 |        |        |        |
| Asexual spore         |        |        |        |
| Spore morphology      | Orbicular-ovate | Conidiospore | Rod |
| Biochemical           |        |        |        |
| Starch hydrolysis     | +      | +      | +      |
| Gelatin hydrolysis    | -      | -      | +      |
| Cellulose hydrolysis  | +      | +      | +      |

Figure 1. Mycelium growth rate of candidate fungi strains at different pH levels (A), salt concentrations (B), and temperatures (C).

Figure 2. Phylogenetic tree created using the ITS gene sequences of three candidate fungi species.

Scopulariopsis brevicaulis FMR 12216 (KP132731)
WN-22 (MG976628)
Scopulariopsis stercoraria MUCL 14213(LM652479)
Scopulariopsis insectivore MUCL 9035(LM652477)
Scopulariopsis flava CBS 334.35(LN850790)

Pseudallescheria angusta CBS 254.72(AY228114)
Pseudallescheria boydii RKI 2956 (AY228123)
Pseudallescheria ellipsoidea 161-CC(KJ607262)
WN-15F (MG976626)
WN-20 (MG976627)

Stachybotrys chartarum ATCC 9182(AF081468)
Stachybotrys chlorohalonata CBS 136194(KU846733)
Stachybotrys xanthohalonata CBS 136160(KU846748)

Penicillium sumatrense DI16-76(LT558898)
3.2. SL degradation and enzymatic activity of the three fungi
The mycelial dry weights of WNF-15, WNF-20, and WNF-22 were 0.083 g, 0.713 g and 0.250 g. The SL degradation ratios of WNF-20, WNF-22 and WNF-15 were 47.54%, 29.29% and 5.31%, respectively.
These strains had different enzymatic activity (Figure 3). They all lacked LiP activity. The MnP activity of WNF-20 and WNF-22 increased from 1 to 5 d, reached a maximum of 19.12 U/ml and 17.14 U/ml at 5 d, and then declined. For WNF-15, the highest MnP activity of 38.71 U/mL appeared at 9 d (Figure 3A). Strain WNF-20 had the highest Lac activity (38.27 U/mL), followed by WNF-22 (8.89 U/mL) and WNF-15 (< 2.22 U/mL) (Figure 3B).

Figure 3. MnP (A) and Lac (B) activities of three fungi strains under in vivo culture conditions at different times after SL addition.

Figure 4. GC-MS analysis of SL biodegradation after a 14 day incubation period.
Table 2. Produced from three strains degrading of SL. ‘+’ indicates that a characteristic peak was observed. ‘-’ indicates no characteristic peak was observed.

| No | Parent product                                         | Treatments |
|----|--------------------------------------------------------|-------------|
| 5  | Propanedioic acid                                      | CK WNF-15 WNF-20 WNF-22 |
| 10 | 2-hydroxymethyl-6-methoxymethylpyran                   | - + + +     |
| 11 | 9-Octadecene, 1,1-dimethoxylpyran                      | + + + +     |
| 12 | Sulfurous acid, hexadecyl penty ester                  | + + + +     |
| 13 | Docosane                                               | + + + +     |
| 14 | Tricosane                                              | + + + +     |
| 15 | Sulfurous acid, butyl heptadecyl ester                | + + - -   |
| 16 | Erucylamide                                            | + + + +     |
| 17 | Tetracosane                                            | - + + +     |
| 18 | Pentacosane                                            | + + + +     |
| 19 | Hexacosane                                             | + + + +     |
| 20 | Hentriacontane                                         | - + + +     |
| 21 | Beta-Sitosterol acetate                                | + + + +     |

3.3. GC-MS analysis of SL degradation products
SL degradation products analysis showed that the three fungi strains can effectively degrade SL. More than 88 components were detected, and 21 of them were highly concentrated (Figure 4). The abundance of 21 components were compared between tested strains and control and revealed that the abundance of 19 components (1, 2, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 21) increased while 2 components (6 and 7) remained the same in the WNF-15 group. Meanwhile the abundance of 11 components in the WNF-20 group increased (1, 2, 5, 10, 13, 14, 17, 18, 19, 20 and 21), and the rest of 10 components decreased. The abundance of 10 components (2, 10, 11, 13, 14, 16, 17, 18, 19 and 20) increased, while 4 components (5, 7, 9, and 21) remained the same, and 7 components (1, 3, 4, 6, 8, 12 and 15) decreased in the WNF-22 group. Thirteen of the 21 components were identified. Seven components (11, 13, 14, 17, 18, 19 and 20) were hydrocarbons, 3 components (12, 15, and 21) were esters, and component 5, 10, and 16 was propanedioic acid, pyran, and erucylamide, respectively (Table 2).

3.4. Sulfate radical concentration of medium
The concentration of SO$_4^{2-}$ ions of three fungi strains under in vivo culture conditions at different times showed that the concentration obviously increased (Fig 5). The concentrations of SO$_4^{2-}$ ion of WNF-20 and WNF-22 obviously increased from 1 to 5 d, and then increased slowly. For WNF-15, the concentrations of SO$_4^{2-}$ ion obviously increased from 1 to 9 d, and then increased slowly. Strain WNF-20 had the highest of SO$_4^{2-}$ concentration (1307.69 mg/L), followed by WNF-22 (1287.45 mg/L) and WNF-15 (1271.56 mg/L), all of them significantly higher than initial SO$_4^{2-}$ concentration (1216.35 mg/L) in medium (P<0.05).
Figure 5. Sulfate radical Concentration of three fungi strains under in vivo culture conditions at different times after SL addition.

3.5. COD removal of WDM

WDM degradation capacity analysis showed the COD removal ratio among the fungi strains differed and ranged from 24.32% to 70.71% after 14 d incubation. The highest ratio was strain WNF-20 (70.71%), followed by WNF-15 (36.67%) and WNF-22 (24.32%). The COD removal ratio in a co-culture of the three strains was 87.65%.

4. Discussion

Many degrading microorganisms have been isolated from WDM[29-31], and shown to decontaminate petroleum hydrocarbons in WDM but few SL degrading microorganisms have been studied. To enhance the efficiency of SL removal from WDM, the degraders should be able to (i) utilize SL, and (ii) adapt to WDM conditions.

Organic pollutant contamination play selective pressure on microorganisms, bring about a development of the microbial community towards those species with enhanced pollutant degradation ability[32]. In this study, WDM collected from an old storage pool (> 3 years) was used for the enrichment and isolation of the SL degradative fungi. In total, 24 fungi strains were separated and three strains with good SL degradation ability were identified. This showed that indigenous fungi capable of degrading SL existed in the WDM.

Several studies have reported that strains of S. brevicaulis were PAHs and cellulose degraders[33-34], and some strains of Stachybotrys and Pseudallescheria can digest crude oil and PAHs[35-37]. In this study, the SL degrading fungal strains were identified as Pseudallescheria ellipsoidea, Stachybotrys chartarum and Scopulariopsis brevicaulis on the basis of their phylogenetic position of ITS sequences (Figure 3). This was the first report that strains of these species can degrade SL. In addition, WNF-15, WNF-20 and WNF-22 grew well in medium containing 2% sodium chloride and had acceptable growth under a wide range of pH values (pH 7.0 to 11), suggesting that these degraders were capable of adapting to WDM conditions (Figure 2 and Table 1).

In previous studies, a lot of fungi have been identified for being capable of lignite degradation/solubilization[13-14,16,18,38], and fungi strains solubilized lignite with weight loss of 5.8% and 31.83%. SL is a lignite humic acid derivative and its chemical structure is similar to lignite. However, the sulfonyl (SO$_3$H) is introduced into it. The presence of sulfonate group in aromatic rings can render such compounds inhibitory towards microbial growth, making their aerobic biodegradation very difficult[19]. Therefore, it is necessary to screen high activity of SL-degrading microbial. In this investigation, strains WNF-15, WNF-20 and WNF-22 showed different degradation ability to SL. WNF-20 had the highest SL degradation ratio (47.54%), and was more effective than previously reported strains [13-14,18,38].

Previously, enzymology of the lignite degradative pathway in basidiomycetes has been studied. And the pathway is that fungus firstly breaks branched-chain and crosslink bone of the aromatic rings in lignite, and then the broken cross bone were transformed to hydrocarbons and aromatic acids [18].
However, there contain sulfonyl (SO$_3$H) in SL, and desulfonation was the essential step before sulfonated compounds were degraded by microbial[39]. Therefore, the concentration of SO$_4^{2-}$ ion of three fungi strains under in vivo culture conditions were tested, and the results showed that the concentration obviously increased in treatment process (Fig 6). In addition, GC-MS analysis of SL degradation products produced by WNF-15, WNF-20 and WNF-22 identified 13 degradation components (Table 1); 9 components were propanedioic acid (component 5), 2-hydroxymethyl-6-methoxytetrahydropyran (component 10), hydrocarbons (components 13, 14, 17, 18, 19, 20), and erucylamide (component 16), which have been previously reported to be the biosolubilization matter of lignite[14,40], suggesting that three test strains degradation SL by biosolubilization and had same degradation pathway with reported strains. Based on the result of SO$_4^{2-}$ concentration and degradation products, we proposed a possible pathway of SL degradation. The fungus firstly breaks the C-SO$_3$ bond and release of sulfite assisted. Subsequently, test strain degradation the desulphurisation by-products by biosolubilization with the same degradation pathway reported by Yin[18]. The abundance of most components increased in WNF-15. But the abundance of nearly 50% of the components decreased and the others increased in the WNF-20 and WNF-22 group (Figure 5), suggesting their ability to degrade SL was different.

MnP, LiP and Lac are major lignite degrading enzyme systems[41], and several fungi strains can solubilize lignite coals using MnP, LiP and Lac[17,42-43]. Simultaneously, their enzymatic activity was different. For example, *Phanerochaete chrysosporium* had LiP and MnP[17] only, and *Ganoderma applanatum*, *Perenniporia tephropora* and *Merulius tremellosus* could secrete MnP and/or Lac, but not secrete LiP[15-16]. In this study, all the strains had MnP and Lac activity but LiP activity was not detected. Therefore, MnP and Lac appear to be the major enzymes involved in SL degradation. In addition, WNF-20 and WNF-22 have relatively high MnP and Lac activities. WNF-15 had the highest MnP activity but low Lac activity (Fig 4).Mnp is a key enzyme for initial degradation of lignin, which can break the cross link of aromatic rings in the lignin, and breaks up big molecules to little molecules[44]. The degradation substrates of Lac are polyphenols and aromatic amines[44]. And there have a synergy between MnP and Lac in degradation of lignin. All of these may help explain why these strains had different SL degradation ratios. The COD removal rates of WDM by WNF-15, WNF-20 and WNF-22 were 36.67%, 24.02%, and 70.71%, respectively. COD removal was 87.65% in a mixture of the three strains after 14 d incubation. This suggests that these three SL degrader fungi strains could be effective in WDM bioremediation.

5. Conclusions

Three different indigenous fungi species capable of degrading SL were isolated from WDM and identified as *P. ellipsoidea* WNF-15, *S. chartarum* WNF-20, and *S. brevicaulis* WNF-22. All three strains effectively degraded SL in MS medium. After a 14 d treatment, WNF-20 had the highest SL degrading efficiency (47.54%), followed by WNF-22 (29.29%). MnP and Lac are the dominant SL degrading enzymes, and Lac enzymatic activity is correlated with the SL degradation ratio. All three strains showed effective degradation of WDM, and the COD removal ratio reached 87.65% in a mixture of the three strains. These three fungi strains have the potential for efficient SL pollutants environments and WDM bioremediation.

Acknowledgments

This work was financed by the project of waste disposal and utilization technology (No. 2016ZX05040003), the Sichuan Agricultural University (No. 03571873), and the Safety Environmental Protection Quality Supervision and Testing Research Institute, CNPC Chuanqing Drilling Engineering Co. Ltd (No. CQ2019B-42-1-7).

References

[1] Hüttiger, K.J., Michenfelder, A.W. (1987) Molecular structure of brown coal. Fuel, 66: 1164-1165.
[2] Zhang, G.B., Liu, D.H., Zhao, Q.M., et al. (1999a) Development and evaluation of lignite drilling fluid additive with cation. Drilling & Production Technology, 22: 60-68.

[3] Kelessidis, V., Marinakis, D., Tsamantaki, C. (2007) Laboratory Assessment Of Drilling Fluid Formation Damage In Sandstone Cores And Mitigation With Lignite Additives for High Temperature Fields. Society of Petroleum Engineers, pp. 1-12.

[4] Zhang, H. (1999b) Studies on the adsorption characteristics of lignite activated by sulphonation of alkalization for heavy-metal ions. Environmental Chemistry, 18: 482-487.

[5] Uçurum, M. (2009) A study of removal of Pb heavy metal ions from aqueous solution using lignite and a new cheap adsorbent (lignite washing plant tailings). Fuel, 88: 1460-1465.

[6] Wang, Z. (2009) Ultra-High Temperature Drilling Fluid System Study(2): Synthesis and Evaluation of a Polymer Fluid Loss Additives. Petroleum Drilling Techniques, 37: 1-6.

[7] Niu, S.C., Wang, J.C., Yuan, L.H. (2016) Optimization of High Temperature-resisting Drilling Fluid in Dingbei Block. Exploration Engineering, 43: 23-27.

[8] Sun, L., Yao, C.S., Zhang, T., et al. (2016) High efficient and harmless drilling waste mud treatment process. Petrochemical Industry Technology, 2: 93. (In Chinese)

[9] Zhang, H.Y., Lu, R.H., Guo, S. (2007) Treatment of drilling wastewater correlated with three-sulfonated mud system by coagulation-ozone oxidation process. Chinese Journal of Process Engineering, 7: 718-722.

[10] Zhang, A., Wu, S., Liu, D. (2011) Characteristics and control of drilling waste in gas field. Industrial Safety and Environmental Protection, 37: 29-31.

[11] Dalmazzone, C., Blanchet, D., Lamoureux, S., et al. (2004) Impact of drilling activities in warm sea: recolonization capacities of seabed. Oil and Gas Science and Technology, 59: 625-647.

[12] Wang, L., & Shi, X. (2011) Investigation of sulige natural gas reserve and analysis of the composition and harmfulness of its drilling fluid. Northern Environment, 23: 62-64.

[13] Machnikowska, H., Pawelec, K., Podgórska, A. (2002) Microbial degradation of low rank coals. Fuel Processing Technology, 77: 17-23.

[14] Yao, J., Xiao, L., Wang, L. (2012) Separation and analysis of lignite bioconversion products. International Journal of Mining Science and Technology, 22: 529-532.

[15] Hofrichter, M., Bublitz, F., Fritsche, W. (1997) Fungal attack on coal: II. Solubilisation of low-rank coal by filamentous fungi. Fuel Process Technol, 152: 55-64.

[16] Yuan, H.L., Yang, J.S., Wang, F.Q., et al. (2006). Degradation/solubilization of Chinese lignite by Penicillium sp. P6[J]. Applied Biochemistry & Microbiology, 42: 52-55.

[17] Yuan, H.L., Yang, J.S., Chen, W.X. (2006). Production of alkaline materials, surfactants and enzymes by Penicillium decumbens, strain P6 in association with lignite degradation/solubilization. Fuel, 85: 1378-1382.

[18] Yin, S., Tao, X., Shi, K.Y., et al. (2009) Biosolubilisation of Chinese lignite. Energy, 34: 775-781.

[19] Tan, N.C.G. & Field, J.A. (2000) Biodegradation of sulfonated aromatic compounds. In: Environmental technologies to treat sulfur pollution. Principles and engineering. P. Lens and L.W. Hulshoff-Pol (eds.), IWA Publishing, London, pp. 377-392.

[20] Okey, R.W., Stensel, H.D. (1996) A QSAR-based biodegradability model—A QSBR. Water Research, 30: 2206-2214.

[21] Simmons, E.G. (2008) Alternaria: An Identification Manual. Utrecht: CBS Fungal Biodiversity Centre. ASM Press p. 38.

[22] White, T.J., Bruns, T.D., Lee, S.B., et al. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications. Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (eds). San Diego (CA): Academic Press pp. 315-322.

[23] Saparrat, M.C.N., Cabello, M.N., Arambbari, A.M. (2002) Extracellular laccase activity in Tetraploa aristata. Biotechnology Letters, 24: 1375-1377.
[24] Wariishi, H., Valli, K., Gold, M.H. (1992) Manganese(ii) oxidation by manganese peroxidase from the basidiomycete phanerochaete chrysosporium. kinetic mechanism and role of chelators. Journal of Biological Chemistry, 267: 23688-23695.

[25] Wolfenden, B.S., & Wilson, R.L. (1982) Radical cations as reference chromogens in studies of one-electron transfer reactions: pulse radiolysis studies of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate). J. Chem. Soc. Perkin Trans. II: 805-812.

[26] Tanaka, H., Koike, K., Itakura, S. (2009) Degradation of wood and enzyme production by ceriporiopsis subvermispora. Enzyme & Microbial Technology, 45: 384-390.

[27] Živojinović, D.Z. & Rajaković, L.V. (2011) Application and validation of ion chromatography for the analysis of power plants water: Analysis of corrosive anions in conditioned water-steam cycles. Desalination, 275: 17-25.

[28] Mamais, D., Jenkins, D., Prrr, P. (1993) A rapid physical-chemical method for the determination of readily biodegradable soluble COD in municipal wastewater. Water Research, 27: 195-197.

[29] Okparanma, R.N., Ayotamuno, J.M., Davis, D.D., et al. (2011) Mycoremediation of polycyclic aromatic hydrocarbons (PAH)-contaminated oil-drill-cuttings. African Journal of Biotechnology, 10: 5149-5156.

[30] Rusjanto, J., Asmaradewi, G., Safitri, D., et al. (2011) Enhancing bioremediation of oily waste by bioaugmentation method. Abstract, International Petroleum Technology Conference, pp. 125-137. doi:10.2523/IPTC-15316-MS.

[31] Ma, B., Pu, X.L., Zhang, S. (2017) Research progress and development trend of drilling waste treatment technology. Xiandai Huagong/Modern Chemical Industry, 37: 42-45, 47.

[32] Patel, V., Cheturvedula, S., Madamwar, D. (2012) Phenanthrene degradation by pseudoxanthomonas sp. dmvp2 isolated from hydrocarbon contaminated sediment of amlakhadi canal, gujarat, india. Journal of Hazardous Materials, 201: 43-51.

[33] Alba, J., Conde, E., Pérez-Guevara, F. (2003) Degradation of the main components of cellulose-paint thinner by the mould Scopulariopsis brevicaulis cultured on rice hulls. Letters in Applied Microbiology, 37: 7-11.

[34] Mao, J., & Guan, W. (2016) Fungal degradation of polycyclic aromatic hydrocarbons (PAHs) by Scopulariopsis brevicatiulis and its application in bioremediation of PAH-contaminated soil. Acta Agriculturae Scandinavica, 66: 1-7.

[35] April, T.M., Abbott, S.P., Foght, J.M., et al. (1998) Degradation of hydrocarbons in crude oil by the ascomycete Pseudallescheria boydii (Microascales). Canadian Journal of Microbiology, 44: 270-278.

[36] Ishii, K., Furuichi, T., Tanikawa, N., et al (2009) Estimation of the biodegradation rate of 2,3,7,8-tetrachlorodibenzo-p-dioxin by using dioxin-degrading fungus, Pseudallescheria boydii. Journal of Hazardous Materials, 162: 328-332.

[37] Jia, H.B., Qu, L.N., Wang, Q.Y. (2013) Selection of crude oil-degrading filamentous fungi and their degradation properties. Research of Environmental Sciences, 26: 678-683.

[38] Reiss, J. (1992) Studies on the solubilization of German coal by fungi. Applied Microbiology and Biotechnology, 37: 830-832.

[39] Cook, A.M., Laue, H., Junker, F. (1999) Review: Microbial Desulfonation[J]. Fems Microbiology Reviews, 22: 399-419.

[40] Elbeyli, İ.Y., Palantöken, A., Piskin, S., et al. (2006) Liquefaction/Solubilization of low-rank Turkish coals by white-rot fungus (Phanerochaete chrysosporium). Energy Sources, 28: 1063-1073.

[41] Martinez, A.T., Speranza, M., Ruiz-Dueñas, F.J., et al. (2005) Biodegradation of lignocelluloses: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. International Microbiology, 8: 195-204.

[42] Ralph, J.P., Graham, L.A., Catcheside, D.E.A. (1996) Extracellular oxidases and the transformation of solubilised low-rank coal by wood-rot fungi. Applied Microbiology & Biotechnology, 46: 226-232.
[43] Chen, H., Tao, X.X., Shi, K.Y., et al. (2008) Lignite bioconversion and research prospect. Clean Coal Technology, 3914: 4239-4242.

[44] Tang, J. (2011) Characteristics of Ligninolytic Enzymes of White-rot Fungus and Their Interactions in the Process of Lignin Degradation. Biotechnology Bulletin, 21: 32-36.