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Full Length Research Paper

Identification and biological characteristics of a newly isolated fungus *Hypocrea lixii* and its role in lignite bioconversion

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Fungus, named TZ1, was isolated from coal mine soil at the Fushunxi colliery, Liaoning Province, North of China. The lignite's bioconversion rate was about 23.3% by TZ1. The morphological, biochemical, physiological characterization and analysis based on 18S rDNA gene sequence indicated that the strain TZ1 was most closely related to *Hypocrea lixii* (100% similarity in gene sequence), bioconversion lignite experiments with this new strain showed that it could play an important role in degradation of Chinese lignite.

Key words: Lignite, bio-conversion, *Hypocrea lixii.*

INTRODUCTION

One of the current challenges in the world is how to use the traditional fossil fuels especially low-rank coal (lignite, brown-coal) by clean ways. As we known, coal is a complex combination of organic matter and inorganic ash formed over eons from successive layers of fallen vege-tation (Galetakis et al., 2009). Coals are classified by rank, according to their progressive alteration in the natural metamorphosis from lignite to anthracite. Because of the problems associated with lignite's large water content, porosity, high-volatile, non-bonded, low calorific value and the spontaneous combustion of its by products, it is difficult to utilize it in an effective and clean way by the traditional coal processing methods. It was found that the lignite-fired thermal plants in Turkey exhausted gaseous emissions (e.g. sulfur dioxides, nitrogen oxides, carbon dioxide, etc.) and various organic emissions such as benzene and toluene and some trace metals because of the characteristics and composition of lignite (Vardar and Yumurtaci, 2010). So the clean utilization of lignite become necessarily.

The present utilization of lignite in China is focused in two areas: one in industry, where it's a source of direct energy and the other in agriculture, where it's used as humus (Yuan et al., 2004). Compared with the industrial utilization of lignite: lignite pyrogenation and coking, lignite gasification, liquefaction and extraction of humic acid from lignite (Michael et al., 2009), the microbial degradation of lignite was regarded as a clean and efficient method which provides a good way for lignite's utilization (Elbeyli et al., 2006). Since the phenomenon of bio-solubilization of lignite (brown-coal) had been discovered in 1981, lots of researchers found that certain fungi were able to use coal as sole source of carbon and energy to solubilize solid coal particles (Hofrichter et al.,1 997). However, these fungi could only convert leonardite or oxidative coal into liquid (Willmann and Fakoussa, 1997). Some researchers pointed out that the microorganisms isolated from Spanish coal discard wastes were proved capable of liquefying untreated lignite and the liquefaction would be increased when lignites were pretreated (Monistrol and Laborda, 1994).

Although some fungi could grow specifically on the untreated coals, several researches showed that the brown rot fungi compared with white rot fungi had weakly active or inactive to solubilize lignite under the same test conditions (Catcheside and Mallett, 1991). Other researchers also showed that the fungi and actinomycetes

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were able to solubilize small amounts of 3.2 N nitric acid-oxidized coals in liquid culture (Quigley et al,. 1988). Some researchers point out that the microbial dead cells and their metabolites on the surface of lignite particles will reduce the rate of lignite solubilization; in that case, the microbial cells will be inhibited due to the substrate at higher dense (Tripathi et al,. 2010).

Isolating the microbes of lignite's biotransformation with high efficiency are key point on study of function in fungi bio- transformation. It has been reported that isolated of *situ* microbes was very important to study the bioconversion (Ulrich and Bower, 2008). To understand the TZ1 degra-dation efficiency on lignite, we studied its relationship with oxidized lignite. We also carried out physiological biochemical test of TZ1 and we found out that the strain which was isolated from mining area has a higher activity when it acted on oxidized lignite.

MATERIALS AND METHODS

Sample and strain isolation

The soil samples and lignite were collected from the Fushunxi colliery, Liaoning province, North of China. The PDA (Potato Dextrose Agar) solid medium and liquid medium (without agar) was used for enrichment and isolation of the microbes, and the culture media components were as follows (g/l): potato lixivium 200 g, sucrose 20 g, agar 16 g. The pH of the culture media were adjusted to 8.0 with NaOH (pH = 14.0) and sterilized for 25 min at 121.5°C, then plated when they were cooled to about 60°C. The raw coal preparation referred the method mentioned in our previous report (Yin et al., 2009). After the soil sample solution being deposited for 20 min, 1 ml suspension was inoculated on PDA solid medium plates spread on lignite samples according to the method mentioned in reference (Yin et al., 2009) and the plates were incubated aerobically in constant incubator at 37°C. The bio-solubilization lignite strains were isolated and purified after re-streaking several times.

Morphological and physiological characterization

Morphological characteristics of myceliums and spores were observed with optical microscope (COIS XSZ-H and WV-CP240) after staining with loffer's methylene blue. To determine the influence of carbon source and nitrogen source on the ability of utilization lignite of the strain TZ1, the basal salts medium (g/l): carbon source ($NaNO_3$ 3, K_2HPO_4 2, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $CuSO_4{\cdot}5H_2O$ 0.25, agar 16) was supplemented with L-Sodium glutamate (12), glucose (12), L-sodium glutamate(2) + glucose(10) maltose(40) + peptone(10) and saccharose (12); nitrogen source (glucose 12, K_2HPO_4 2, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $CuSO_4{\cdot}5H_2O$ 0.12, agar 16) was supplemented with sodium nitrate (3), ammonium sulfate(3) and carbamide (3) respectively. To analyze the isolated strains TZ1 whether it secrete some extracellular enzymes which can dissolve the lignite, the strain were incubated on the PDA agar plate implemented tanin. The diameter of discoloration circle (Horichter and Fritsch, 1997) and clony of strain were measured as reported.

PCR amplification of 18S rDNA gene sequence

For further identification, the genomic DNA was extracted using genomic DNA purification Kit (Biospin fugus gene extraction kit) according to the manufacturer's instructions and the 18S rDNA gene was amplified by PCR using the universal 18S rDNA primers: reverse primers: (5'-TCCTCCGCTTATTGATATGC-3'),(3'-CCGCAGGTTCACCTACGGA-5') . Amplification reactions were performed in a total volume of 50 l. The reaction mixture contained 5 l of 10 × PCR buffer, 5 l of 25 mmol/l MgCl2, 1 l of 2.5mmol/l dNTPs mixture, 2 l of primers respectively, 0.5 l of Taq polymerase (5 U/ l) and 2 l of template DNA. The PCR reaction conditions were as follows: an initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min 30 s; 72°C for 10 min and 4°C pause. PCR products corresponding to the expected size of amplified 18S rDNA (800 kb) were purified with a Gel Extraction Kit (UNIQ-10) . The product was integrated into pBS-T vector using the pBS- T PCR Products Clone Kit (TAKARA pMD18-T Simple Vector). 3 l of ligation mixture was used to transform high efficiency competent cells of Escherichia coli DH 5 and plated on Luria-Bertani (LB) medium containing ampicillin, X-Gal and IPTG. White colonies including the positive clone were picked out and cultured then sent to Shanghai JieRui Biotechnologies Co. Ltd. for sequencing. The 18S rDNA gene sequence of strain TZ1 was comparatively analyzed with the nucleic acid database of Genbank. The similarity analysis between the strains was conducted using clustal X (1.83) and MEGA 4.0. Phylogenetic trees were constructed based on the similarity analysis (Abdullahi et al., 2005; Wen et al., 2009).

Lignite biosolubilization

Infrared spectroscopy analysis can determine the specific organic chemical groups in the solubilization product (Basaran et al., 2003). For the infrared spectroscopy analysis, the water content of the solubilisation product was removed by using a rotary evaporator, then 0.2 g of the sample was ground well with KBr (1:10) and then pressed to make a slice for analysis. The slices were analyzed using an infrared spectrum analyzer (AVATAR 380, Nicolet, America) with light wave lengths from 4000 to 450 cm^{-1} (resolution 4 cm^{-1}). The infrared spectra of raw and cells processed lignite were analyzed to investigate the transformation of lignite.

RESULTS AND DISCUSSION

Isolation and morphological characterization

A strain named TZ1 was isolated from Fushun colliery, the clonies of TZ1's primary hyphaes on the agar plate are white, translucent and reptant with the growth of the hyphaes, they turned to floss and the colonies produced lots of asexual spores, finally, the surface of the plate turns into bottle green. Single colonies were carefully picked and streak cultures on solid medium were repeated several times (Waite et al., 2003). An optical microscope was used to examine the purity of the culture and the morphology of strains was shown in Figure 1. As shown in Figure 1a, the myceliums of strains were branch, translucence or transparence. The asexual conidiophore were visible, which were branch, upright-ness, thin and bend (Figure 1b) and they also produced lots of spores (Figure 1c), which were rotund, translucent and this did not have septum. From these characteristics, it could be concluded that the strain TZ1 was similar to the species *Trichoderma*, imperfect fungi subphylum

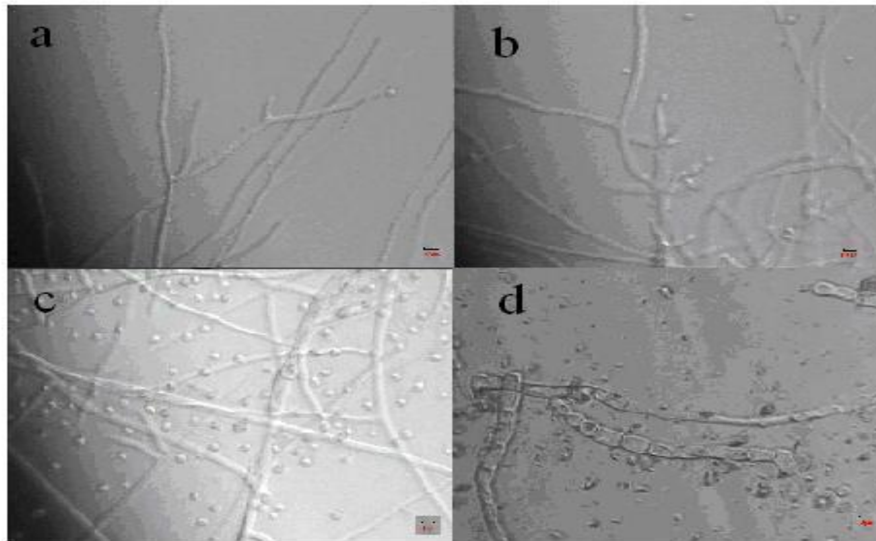

Figure 1. The morphology of strain TZ1 on different growth period (a) mycelium at growth phase, (b) peduncle of special conidiophore, (c) conidium, (d) mycelium at decline phase.

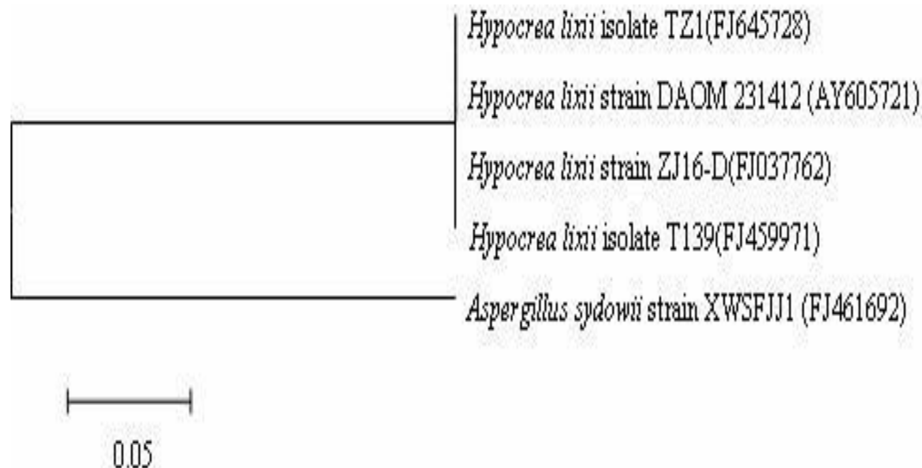

Figure 2. Phylogenetic tree based on the 18S rDNA sequence of strain TZ1.

Phylogenetic analysis of 18S rDNA sequence

The 18S rDNA nucleotide sequence of TZ1 had been submitted to GenBank with the accession number FJ645728. The phylogenetic tree of the strain TZ1 constructed based on the gene sequence is shown in Figure 2. As shown in Figure 2, the phylogenetic tree is mainly divided into 2 branches and the result indicates that the TZ1 has high similariy with *H. lixii* strain DAOM 231412 (AY605721), *H. lixii* strain ZJ16-D(FJ037762) and *H . lixii* isolate T139(FJ459971) and the homology rate is up to 100%. So the strain TZ1 is identified as *H. lixii* TZ1.

Physiological and biochemical characterization

Based on the results in Table 1, the strain TZ1 treated L-sodium glutamate as optimal carbon source to improve lignite bio-solubilization, however, it presented the lowest specific growth rate grown on compared with the other four carbon source. Table 2 shows that the strain TZ1 could only use carbamide among the alternative three nitrogen source to dissolve lignite. From Tables 1 and 2, it shows that different substances have distinct influence on bio-solubilization of lignite. Among these energy sources, the strain TZ1 can dissolve lignite when grows on L-sodium glutamate and carbamide containing media. We

Table 1. The carbon sources ulitization of strain TZ1.

Carbon source	Bio-solubilization	Specific growth rate/cm·d [1]*
Maltose +peptone	+	2.2
Glucose+L-sodium glutamate	++	2.0
L-Sodium glutamate	+++	1.6
Glucose	-	2.4
Saccharose	-	2.2

Note " "represents bio-solubilization " "represents not bio-solubilization * The specific growth rate is the rate of colonies' diameter at the exponential period.

Table 2. The nitrogen sources ulitization of strain TZ1.

Nitrogen source	Bio-solubilization	Specific growth rate/cm·d [1]*
Sodium nitrate	-	2.4
Ammonium sulfate	-	2.2
Carbamide	++	1.8

Note " "represents bio-solubilization " "represents not bio-solubilization * The specific growth rate is the rate of colonies' diameter at the exponential period.

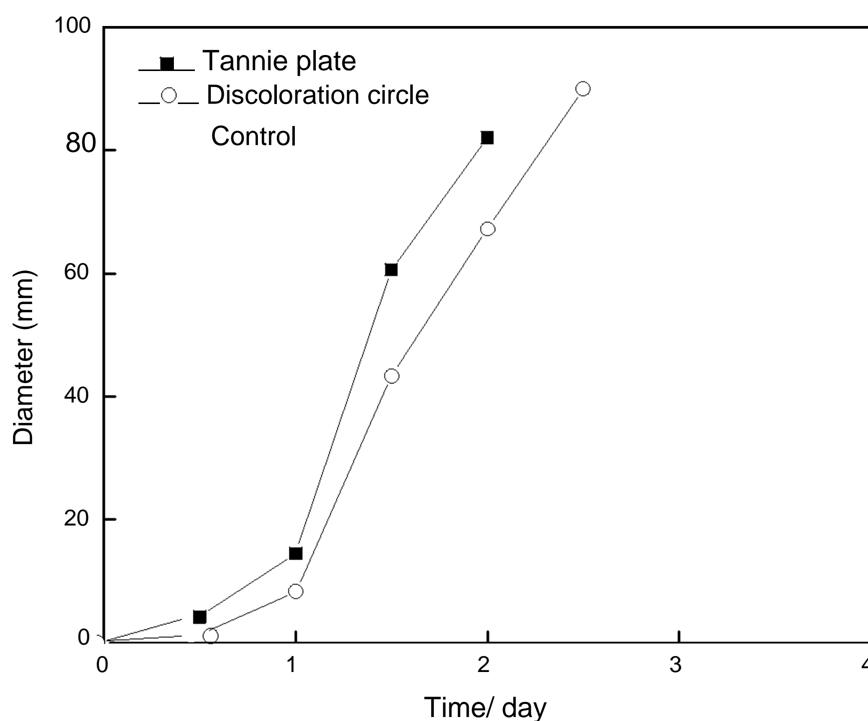

Figure 3. Growth characteristics of strain TZ1 on Tannin plate media.

speculated that the strain TZ1 could produce extracellular enzymes such as polyphenol oxidase as reported (Hölker et al., 2002), which could utilize these active groups, such as $-NH_2$, $-COO^-$ and -COOH. It has been reported that the extracellular enzyme of cells such as: laccase, lignin peroxidase and manganese peroxidase play an important role in bio-solubilization of lignite (Deepak et al., 2007). After 1 day incubation of the strain, the tanin plate changed its color (Data not shown here) and the diameter of discoloration circle and clony of strain gradually increased (Figure 3). The phenomenon indicated that the strain TZ1 could react with lignite and form discoloration

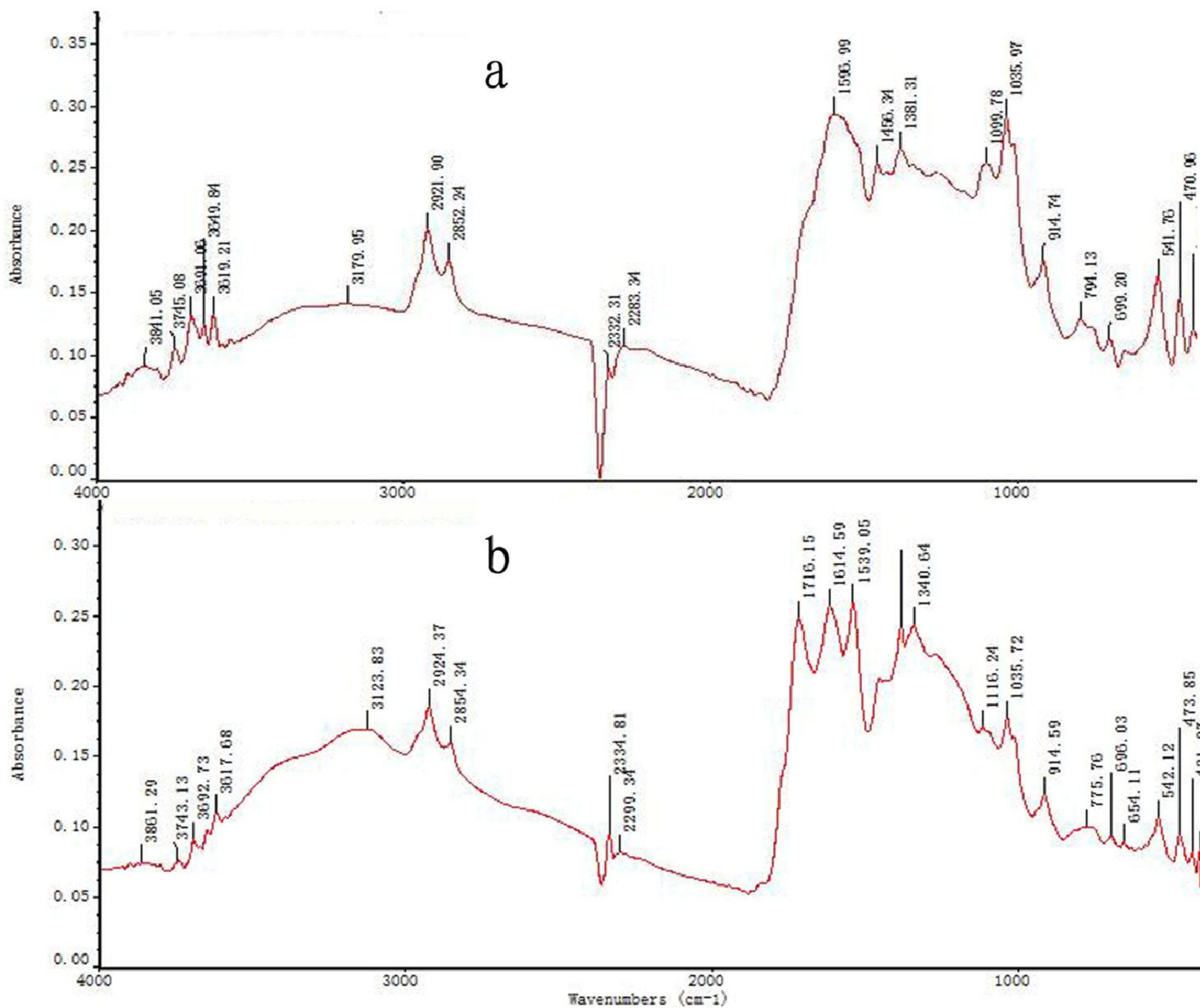

Figure 4. FT-IR Spectra of (a) oxidized lignite (b) bio-conversed lignite.

circle on the surface of solid agar.

Lignite bio-solubilization analyse

The infrared spectra of the raw and bio-converted lignite are shown in Figure 4. Compared with the spectra of raw lignite, the latter has a narrow absorption bands at 3400 cm^{-1} which are due to the decrease of hydroxyl group. The strong absorption bands at 2921 and 2852 cm^{-1} are assigned to the aliphatic hydrocarbon. However, the intense absorption band at 1716 and 1539 cm^{-1} which are assigned to carboxyl group distinctly disappeared, in contrast, the absorption band of alkyl ether at 1035 cm^{-1} increased. It indicated that the chemical composition of lignite varied significantly when treated by strain TZ1 (Shi et al., 2007). Thus we speculated that the complicated chemical bonds such as carboxyl and hydroxyl groups of lignite were broken by metabolites secreted by the strain TZ1.On the other hand, the degradation of lignite by the strain might form new other bonds (Maboeta et al., 2006).

Conclusion

In this study, a fungus was isolated from mining area. The fungus culture condition, the utilization characteristics of different carbon source and nitrogen source were investigated. And we obtained the fungus's 18S rDNA sequence, which were analyzed through GenBank. Based on the information, we found the evolutionary relationship of this strain, which was finally identified as *H. lixii* TZ1. The bio-conversion results showed that the fungus could solubilize the oxidized lignite.

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