Bioconversion of lignite humic acid by white-rot fungi and characterization of products

Lei Xiao¹ · Yunyun Li¹ · Yuansong Liao¹ · Huirong Ma¹ · Jianjun Wu¹ · Yixin Zhang¹ · Jinghua Yao¹

Received: 6 January 2018 / Accepted: 7 May 2018 / Published online: 14 May 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Lignite humic acids (LHAs) were sequentially separated from lignite with aqueous NaOH and HCl and biotreated by an isolated fungus WF8. The liquid product (LP), residues (RS) and LHAs were analyzed using a Fourier transform infrared spectrometer (FTIR) and a proton nuclear magnetic resonance (¹H NMR). Three main enzymes in WF8 (i.e., lignin peroxidase, manganese peroxidase and laccase) were also measured and analyzed with and without LHAs. The results show that LHAs can induce the ligninolytic enzymes. The oxidation and hydrogenation reactions proceeded to some extent, aromaticity in LHAs and carboxyl in LP decreased, and LHAs were converted into simpler LP via biochemical reactions by WF8.

Keywords Lignite humic acids · Fungus · Bioconversion · Products analysis

Abbreviations

LHAs	Lignite humic acids
LP	Liquid product
RS	Residues
FTIR	Fourier transform infrared
NMR	Nuclear magnetic resonance
LiP	Lignin peroxidase
MnP	Manganese peroxidase
Lac	Laccase
ABTS	2,2-Azinobis (3-ethylbenzthiazoline-6-sulfonate)
DMSO	Dimethyl sulfoxide-d ₆

Introduction

Lignite reserves in China are about 130 billion tons, accounting for 12.4% of lignite reserves worldwide (Yao et al. 2015). However, lignite hasn't been utilized efficiently and is also a potential threat to the environment (Xiao et al. 2010; Yu et al. 2013; Zhang et al. 2015). Lignite contains up to 30–80% of humic acids. To better utilize lignite, it has been proposed to convert humic acids to chemical products with higher added values (Willmann and Fakoussa 1997).

However, the application of lignite-based humic acids is limited by its high molecular weight, low solubility in water, poor activity and safety concerns. To expand the application range and increase its added value and activity, lignite humic acids (LHAs) can be converted into some simpler products, such as fulvic acids.

Currently, compared to the other conventional conversion procedures, biodegradation/bioconversion is safe, environmentally friendly and energy efficient (Fakoussa and Frost 1999; Gramss et al. 1999; Steffen et al. 2002; Yao et al. 2015; Zavarzina et al. 2004; Ziegenhagen and Hofrichter 1998). Separated from lignite, LHAs are simpler than lignite and have more similarities to lignite than other model compounds (e.g., benzoic acid, phenol, pyrrole, etc.). Thus, bioconversion of LHAs can not only provide useful information for lignite bioconversion, but also have the capability of producing soluble active low-molecular materials.

LHAs are complex mixtures of polydisperse, polyelectrolyte-like molecules with irregular structures (Grinhut et al. 2011). LHAs are hard to be degraded by microorganisms, and the products are difficult to analyze. Currently, systematic studies on LHAs bioconversion mechanism is still lacking, therefore, not fully understood.

In the present investigation, we separated LHAs from lignite, biotreated LHAs using an isolated fungus and examined the bioconversion of LHAs.

✉ Jinghua Yao
yaojhkd@126.com

¹ Key laboratory of Coal Processing and Efficient Utilization, Ministry of Education, China University of Mining and Technology, 1 University Road, Xuzhou 221116, China

Materials and methods

Fungus

The fungus used in this study was isolated from decaying wood and designated as WF8. The isolation and identification of WF8 have been completed and presented in our previous paper (Yao et al. 2015). WF8 was identified as *Ascomycota Hypocrea* on the basis of its 18S rDNA gene sequence (GenBank accession No. JQ806366). In that paper, it was also proved that WF8 can produce polyphenol oxidase, laccase, and peroxidase.

Enzymatic assays

Enzymatic assays were performed in the following ways: lignin peroxidases (LiP) by the oxidation of veratryl alcohol to veratraldehyde (Tien and Kirk 1988), manganese peroxidases (MnP) by the oxidation of Mn^{2+} to Mn^{3+} (Peng et al. 2014), and laccases (Lac) by the oxidation of 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Eggert et al. 1996).

Preparation of LHAs

Lignite was freshly collected from Xinjiang Coal Mine, China. It was pulverized to pass through a 90-mesh sieve (<0.16 mm) followed by desiccation in an oven at 80°C for 24 h before use. 50 g lignite was steeped into 300 mL 0.2 mol L^{-1} NaOH solution for 24 h, then centrifuged at 7000g for 15 min. The supernatant was precipitated by adjusting pH to 2.0 with 6 M HCl to obtain LHAs, which were dried in an oven at 80°C , weighed, and stored in a vacuum-dried chamber.

Bioconversion of LHAs

WF8 was inoculated onto the Subaroud plate medium (maltose 40 g, peptone 10 g, agar 20 g, and distilled water 1 L) in Petri dishes of 9 cm diameter and was incubated in the biological incubator for 3 days at 28°C . 1 g sterilized LHAs were manually well distributed onto the dishes. After 7 days, liquid product (LP) and residue (RS) were collected and analyzed, respectively.

Sample analysis

The elemental analyses of selected samples were conducted with a Leco Mac-400 Thermogravimetric Analyzer, a Leco CHN-2000 Elemental Determinator and a Leco SC-132 Sulfur Determinator. A Nicolet Avatar 560 Fourier

transform infrared (FTIR) spectrometer was used to analyze the selected samples using KBr pellets with a resolution of 4 cm^{-1} in the measuring range of 400 cm^{-1} . Nuclear magnetic resonance (NMR) spectra were acquired using a Bruker (Rheinstetten, Germany) AVANCE III HD 600 MHz NMR spectrometer. Samples were dissolved in Dimethyl Sulfoxide- d_6 (DMSO) and 0.6 mL diluted samples were added to 5 mm diameter NMR tubes, and the analysis was performed overnight to enhance the signal-to-noise ratio. Acid groups (total acid groups, carboxyl groups, phenolic hydroxyl groups) were determined by acid–base titration.

E4/E6 analyses

E4 and E6 are the absorption value at 465 and 665 nm, respectively (Zheng 1991). Humic acid solution has no characteristic absorption peaks in the range of 400–700 nm. However, if the humic acid is dissolved in aqueous NaHCO_3 solution, E4/E6 will be constant.

Results and discussion

Enzyme activities in WF8

MnP, LiP and laccase are the three main ligninolytic enzymes in white-rot fungus (Li and Wang 2009). As shown in Fig. 1, MnP activity is the highest and Lac activity is the lowest in WF8. MnP and LiP activities increased rapidly in early culture period. MnP activity reached the maximum on the sixth day, and still remained relatively high. LiP activity peaked around the fifth day, and also remained stable. However, Lac activity hardly changed during the culture period.

WF8 mycelium grew vigorously in the later culture period, which affected the oxygen transfer instead. Oxygen

Fig. 1 Activities of LiP, MnP and laccase in WF8

plays an important role in the bioconversion of LHAs. WF8 is aerobic and need more oxygen during the growth and metabolism. In addition, oxygen participates in the oxidation of glucose to gluconate and H_2O_2 catalyzed by glucose oxidase. H_2O_2 is the activator of ligninolytic enzymes. Therefore, oxygen affects the synthesis of ligninolytic enzymes.

The influence of LHAs to enzymes

Willmann and Fakoussa (1997) indicated that humic acids induce the peroxidase in vivo. However, Zavarzina et al. (2004) drew the conclusion that humic acids were stronger inhibitors of laccase in vitro. In comparison to absence of LHAs, both peroxidase and polyphenol oxidase activities increase significantly with LHAs (shown in Fig. 2). The maximum activities are 211.5 and 43.3 U, respectively. At initial culture, the growth and metabolism of WF8 were on the nutritious medium. After some days, the nutrition was consumed gradually and the fungus began to use other carbon sources (such as LHAs). Therefore, more peroxidase and polyphenol oxidase were produced because of the addition of LHAs, which also are acted as an inducer.

Fig. 2 Activities of peroxidase and polyphenol oxidase in WF8 with and without LHAs

Table 1 Elemental analysis of LHAs, LP and RS

Sample	Ash	N	C	H	S	O	O/C	H/C	C/N
LHAs	8.89 (0.1) ^b	0.83 (0.1)	57.13 (0.2)	3.50 (0.1)	0.08 (0.4)	38.46 (0.7)	0.50	0.74	80.30
LP	2.29 (0.2)	3.16 (0.1)	48.88 (0.2)	4.76 (0.2)	0.03 (0.3)	43.17 (0.8)	0.66	1.17	18.05
RS	10.43 (0.1)	2.90 (0.1)	55.80 (0.3)	4.32 (0.1)	0.02 (0.4)	36.95 (0.7)	0.49	0.93	22.45

^aAsh is the percentage (w/w) of inorganic residue in a dry sample. C, H, O, N and S are the elemental composition in percentage (w/w) of a dry, ash-free sample

^bThe figure in parentheses refers to the standard deviation

Table 2 Acidic groups in LHAs, LP and RS

Sample	Total acid groups (mmol g ⁻¹)	Carboxyl (mmol g ⁻¹)	Phenolic hydroxyl (mmol g ⁻¹)
LHAs	11.83 (0.1) ^a	1.35 (0.3)	10.48 (0.2)
LP	11.63 (0.1)	0.38 (0.4)	11.25 (0.1)
RS	4.78 (0.2)	2.50 (0.2)	2.28 (0.2)

^aThe figure in parentheses refers to the standard deviation

Elemental analysis

As listed in Table 1, LP has lower carbon but higher hydrogen, oxygen and nitrogen content than LHAs and RS. The possible reason for the decrease in carbon content could be that WF8 break C–C, C–O–C and C–O bonds during the bioconversion of LHAs so that some carbon atoms were left in the RS and the others were kept in LP. Hydrogen and oxygen content in LP increased as a result of the hydrogenation of the unsaturated groups (e.g., double bond of carbon, benzene, etc.) and the oxidation of some functional groups (Yuan et al. 2009). The increases of nitrogen in LP and RS come from nitrogen sources in the medium (Amir et al. 2010). Therefore, C/N value in LP and RS was much lower than that in LHAs.

H/C is an important parameter on aromatization. The smaller H/C is, the higher aromatization and unsaturation is and the more stable chemical bonds are (Zavarzina et al. 2004). As exhibit, H/C in LHAs is the smallest. It is clear that LHAs have the highest aromatization and the most stable structures before bioconversion. With the help of the enzymes in WF8, the unsaturated structures were converted into saturated ones. O/C shows the content of carboxyl and carbohydrates (Song et al. 2009). O/C in LP is the highest in Table 1, which indicates that LP contains more carbohydrates and oxygen-containing functional groups.

Acidic groups in LHAs

As exhibited in Table 2, total acidic groups in RS (4.78 mmol g⁻¹) are less than that in LHAs (11.83 mmol g⁻¹) and LP (11.63 mmol g⁻¹). In particular, phenolic hydroxyl

groups in RS are much less. It is possible that WF8 could attack carboxyl and aromatic ring in macromolecules, resulting in less carboxyl and more phenolic hydroxyl in LP.

E4/E6

E4/E6 reflects degree of aromatic condensation with negative correlation between them (Kononova 1966). Furthermore, the higher E4/E6 is, the smaller molecular weight (Chen and Senesi 1977; MacCarthy and Rice 1985; Moreda-Piñero et al. 2006). Therefore, Zheng (1991) indicated that there was a positive correlation between degree of aromatic condensation and molecular weight. In addition, the ratio also included the information of conjugated carbonyl groups. Thus, during the bioconversion, the molecular weight reduces with the decrease of aromaticity (shown in Fig. 3).

As shown in Fig. 3, E4/E6 increases with culture time and remains constant after 7 days. E4/E6 of LHAs, LP and

RS were also analyzed. The ratios are 2.40, 2.93 and 2.27, respectively. E4/E6 of LP is the biggest. However, E4/E6 of RS is a little smaller than LHAs. It is possible that WF8 destroyed the aromatic rings of LHAs into smaller substances in LP. However, some substances that are hard to be utilized by the fungus in RS repolymerized into some more complex structures. Therefore, RS has a higher degree of aromatic condensation and larger molecular weight (Grinhut et al. 2007; Zavarzina et al. 2004).

FTIR analysis

FTIR spectra of LP and LHAs are shown in Fig. 4. The absorbance at 3416 cm^{-1} attributed to alcohol or phenolic hydroxyl group stretching is stronger in LP than in LHAs, implying that ether bonds in LHAs may be hydrolyzed by WF8. Relatively stronger absorbance around 1700 cm^{-1} (carbonyl and carboxyl C=O stretching vibrations) in LHAs than in LP showed the formation of hydroxyl or CO_2 via hydrogenation or decarboxylation by the action of microorganism on LHAs. This conclusion is in line with analysis of acidic groups in Table 2. The intensity of aromatic structures at 1600 cm^{-1} in LHAs is almost the same as that in LP. The absorbance at $1300\text{--}1000\text{ cm}^{-1}$ is assigned to symmetric and asymmetric stretching of ester, phenol, ether and acetoxyl groups. In this range, there are a strong at 1230 cm^{-1} and a weak peak at 1036 cm^{-1} (C–O–C stretching vibrations) in LHAs. However, the absorbances at 1262 cm^{-1} (carboxyl C–O and O–H stretching vibrations) and at 1036 cm^{-1} (alcohol C–O groups) (Yang et al. 2013) are present in LP. It was deduced that the microorganism destroyed the ether bonds into hydroxyl or further oxidized into traces of carboxyl.

FTIR spectra of RS and LHAs are shown in Fig. 5. Some of hydroxyl groups are interacted with each other by hydrogen bond, which makes peak shift. Therefore, a new shoulder appears at 3543 cm^{-1} in RS. The absorbance of aliphatic

Fig. 3 E4/E6 of LP during the bioconversion

Fig. 4 FTIR spectra of LHAs and LP

Fig. 5 FTIR spectra of LHAs and RS

C–H stretching at 2926 cm^{-1} is weak in LHAs and almost absent in RS, indicating that the amount of aliphatic structures reduce and even some cyclic structures are formed. The absorbance at 1700 cm^{-1} attributed to carboxyl/carbonyl C=O stretching is present in LHAs and not in RS. However, the intensity of the aromatic structure (1600 cm^{-1} , C=C) is weak in RS. It was deduced that aromatic structures are destroyed by the microorganism. Relatively stronger absorbance at 1139 cm^{-1} (aliphatic ether C–O–C) in RS showed the polymerization of the fragments by the action of microorganism on LHAs. The absorbance at $1000\text{--}400\text{ cm}^{-1}$ (aromatic C–H out-of-plane stretching vibration) is not present in LP. However, a strong absorbance at 614 cm^{-1} is present in RS. It is deduced that some structures are hard to be degraded by microorganism and re-attach to the aromatic side chains, resulting in the new peak.

¹H-NMR analysis

To further study the bioconversion of LHAs, ¹H-NMR analysis was performed on LHAs, LP and RS. LHAs are complex mixtures. Therefore, most of the signal peaks can not form single peak, but are linked to each other, as shown in Fig. 6. This result is supported by Fan et al. (2013). Peaks in the range of 0.8–1.0 and 1.0–1.4 ppm represent methyl in the terminal and in side chain (Sh et al. 2012), respectively. The peak at around 1.2 ppm in LHAs is obvious, but shifts to 0.98 ppm in LP. The possible reason is that microorganism broke the side chains to form more terminal methyl groups. The peaks between 1.8 and 3.0 ppm are attributed to hydrogen bonded to highly electronegative groups (such as carbonyl, hydroxyl, aromatic or double bonds) (Malcolm 1990; Peuravuori et al. 2007). The peaks at 3.0–4.4 ppm are attributed to α -C hydrogen such as hydrocarbons, polyethers or methoxy group (Fan et al. 2013). The two strong peaks at 3.41 and 4.31 ppm in LP indicate more oxygen-containing

Fig. 6 ¹H-NMR spectra of LHAs, LP and RS

functional groups. The peaks in the region of 6.5–10.0 ppm are thought to be the hydrogen on complex functional groups, such as aromatic rings, substituted aromatic rings, phenol or amino groups (Francioso et al. 2001, Sh et al. 2012). The peak around 8.13 ppm attributed to highly substituted aromatic rings is present in both LHAs and RS, which is broad and shades other characteristic peaks of phenol and alkyl benzene.

In comparison to LHAs and RS, peaks in LP are hardly present at 6.5–10.0 ppm, which demonstrates that WF8

could destroy the complex aromatic network structure so that some local structures are exposed. Peaks in LHAs and RS are almost similar, which indicates that complex structures remain in RS.

Conclusions

The fungus WF8 can produce ligninolytic enzymes during the incubation, and LHAs can induce the peroxidase and polyphenol oxidase. During the bioconversion of LHAs by WF8, the oxidation and hydrogenation reactions proceeded to some extent, aromaticity in LHAs and carboxyl in LP decreased, and the molecular weight of LP is smaller than that of LHAs. The fact further indicates that the bioconversion of LHAs proceeded via biochemical reactions by WF8.

Further studies will analyze the structure of LHAs before and after bioconversion in detail, clarify the role of ligninolytic enzymes (laccase, peroxidase), and gain a better understanding of the bioconversion mechanisms for LHAs.

Acknowledgements This work was supported by the Fundamental Research Funds for the Central Universities (2017CXNL04) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

References

- Amir S, Jouraiphy A, Meddich A, Gharous ME, Winterton P, Hafidi M (2010) Structural study of humic acids during composting of activated sludge-green waste: Elemental analysis, FTIR and ^{13}C NMR. *J Hazard Mater* 177:524–529
- Chen Y, Senesi N (1977) Information provided on humic substances by E4/E6 ratios. *Soil Sci Soc Am J* 41:352–358
- Eggert C, Temp U, Eriksson KEL (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl Environ Microbiol* 61:1151–1158
- Fakoussa RM, Frost PJ (1999) In vivo-decolorization of coal-derived humic acids by laccase-excreting fungus *Trametes versicolor*. *Appl Microbiol Biotechnol* 52:60–65
- Fan XJ, Song JZ, Peng PA (2013) Comparative study for separation of atmospheric humic-like substance (HULIS) by ENVI-18, HLB, XAD-8 and DEAE sorbents: elemental composition, FT-IR, ^1H NMR and off-line thermochemolysis with tetramethylammonium hydroxide (TMAH). *Chemosphere* 93:1710–1719
- Francioso O, Sánchez-Cortés S, Tugnoli V, Marzadori C, Ciavatta C (2001) Spectroscopic study (DRIFT, SERS and ^1H NMR) of peat, leonardite and lignite humic substances. *J Mol Struct* 565–566:481–485
- Gramss G, Ziegenhagen D, Sorge S (1999) Degradation of soil humic extract by wood- and soil-associated fungi, bacteria, and commercial enzymes. *Microb Ecol* 37:140–151
- Grinhut T, Hadar Y, Chen Y (2007) Degradation and transformation of humic substances by saprotrophic fungi: processes and mechanisms. *Fungal Biol Rev* 21:179–189
- Grinhut T, Hertkorn N, Schmitt-Kopplin P, Hadar Y, Chen Y (2011) Mechanisms of humic acids degradation by white rot fungi explored using ^1H NMR spectroscopy and FTICR mass spectrometry. *Environ Sci Technol* 45:2748–2754
- Kononova MM (1966) Soil organic matter, 2nd edn. Pergamon Press, Oxford, pp 400–404
- Li CF, Wang LS (2009) Application research in biodegradation of lignin by white-rot fungi. *Mod Agric Sci Technol* 1:274–275
- MacCarthy P, Rice JA (1985) Spectroscopic methods (other than NMR) for determining functionality in humic substances. In: Aiken GR, McKnight DM, Wershaw RL, MacCarthy P (eds) Humic substances in soil sediment and water. Wiley, New York, pp 527–559
- Malcolm LR (1990) The uniqueness of humic substances in each of soil, stream and marine environments. *Anal Chim Acta* 232:19–30
- Moreda-Piñero A, Seco-Gesto EM, Bermejo-Barrera A, Bermejo-Barrera P (2006) Characterization of surface marine sediments from Ría de Arousa estuary according to extractable humic matter content. *Chemosphere* 64:866–873
- Peng X, Yuan XZ, Zeng GM, Huang HJ, Wang H, Liu H, Bao S, Ma YJ, Cui KL, Leng LJ, Xiao ZH (2014) Synchronous extraction of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* fermentation broth. *Sep Purif Technol* 123:164–170
- Peuravuori J, Simpson AJ, Lam B, Žbáňková P, Pihlaja K (2007) Structural features of lignite humic acid in light of NMR and thermal degradation experiments. *J Mol Struct* 826:131–142
- Sh T, Liu CQ, Wang L (2012) Antimony coordination to humic acid: Nuclear magnetic resonance and X-ray absorption fine structure spectroscopy study. *Microchem J* 103:68–73
- Song HY, Yin YY, Song JZ (2009) The chemical composition and structure of humic acids from different environments. *J S China Normal Univ* 1:61–66
- Steffen KT, Hatakka A, Hofrichter M (2002) Degradation of humic acids by the litter-decomposing basidiomycete *Collybia dryophila*. *Appl Environ Microbiol* 7:3442–3448
- Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods Enzymol* 161:38–249
- Willmann G, Fakoussa RM (1997) Biological bleaching of water-soluble coal macromolecules by a basidiomycete strain. *Appl Microbiol Biotechnol* 47:95–101
- Xiao L, Yao JH, Wan YZ, Tao XX, Liu JT (2010) Briquetting mechanism and parameters optimization of the lignite/biomass. *J China Univ Min Technol* 39:352–356
- Yang H, Li YS, Zhang YG (2013) Characterization of humic acid molecular structure by method of infrared spectrum and nuclear magnetic resonance. *Coal Convers* 36:72–76
- Yao JH, Wei XY, Xiao L, Ji HM, Zong ZM, Liu FJ (2015) Fractional extraction and biodepolymerization of Shengli lignite. *Energy Fuel* 29:2014–2021
- Yu JL, Tahmasebi A, Han YN, Yin FK, Li HC (2013) A review on water in low rank coals: the existence, interaction with coal structure and effects on coal utilization. *Fuel Process Technol* 106:9–20
- Yuan HL, Jiang F, Gao GT (2009) Study on biotechnology application in producing fulvic acid by degraded lignite. *Humic Acid* 5:1–5
- Zavarzina AG, Leontievsky AA, Golovleva LA, Trofimov SY (2004) Bio-transformation of soil humic acids by blue laccase of *Panus tigrinus* 8/18: an in vitro study. *Soil Biol Biochem* 36:359–369
- Zhang YX, Wu JJ, Ma J, Wang BB, Shao XL, Si CD (2015) Study on lignite dewatering by vibration mechanical thermal expression process. *Fuel* 130:101–106
- Zheng P (1991) Production and application of coal humic acid. Chemical Industry Press, Beijing, pp 72–112
- Ziegenhagen D, Hofrichter M (1998) Degradation of humic acids by manganese peroxidase from the white-rot fungus *Clitocybula dusenii*. *J Basic Microbiol* 4:289–299