Optimization of Weathered Coal Biodegradation by *Penicillium aculeatum* 13-2-1 and UV-visible Spectral Characteristics of Active Degraded Products

Jianguo Ren,[a,b] Chao Zhang,[c] Junli Wang,[b,*] and Jing Cai [b]

Weathered coal is widely distributed in many provinces in China. It has great potential utilization values as soil ameliorant in sustainable agriculture. To find the optimal condition for the biodegradation of weathered coal and obtain the spectral characteristics of active degraded products of coal, the bioactivation of humic acid in weathered coal is essential. In this study, a fungal strain (*Penicillium aculeatum* 13-2-1) capable of degrading weathered coal was isolated from the rhizosphere of *Zelkova serrata*. The experimental results using classical one factor at a time method showed that weathered coal of 1.0 g, inoculum of 150 µL, and sodium nitrate of 0.10 g in 100 mL broth were the optimal conditions for the biodegradation of coal, respectively. The variance analysis of orthogonal tests illustrated that the three factors had little effects on biodegradation of weathered coal, but the weathered coal significantly negatively affected the contents of soluble humic acids in liquid coal products. Different diluted solutions of liquid coal products for all orthogonal combinations promoted the growth of the seeds of *Brassica napus* L., especially on radicles. The UV-visible spectra of the liquid coal products for all combinations presented the differential absorbance closely associated with the quantity of sodium nitrate in medium.

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*Contact information: a: Center of Research and Development of Fine Chemicals, Guizhou University, Huaxi district, Guiyang, Guizhou, China; b: University of Guizhou Medical University, University Town, Guiyang, Guizhou, China; c: Inner Mongolia University of Science and Technology, Baotou, Inner Mongolia, China; *Corresponding author: wjlrjg@126.com*

INTRODUCTION

Coal, as an organic substance of macromolecular structure with high carbon and low hydrogen content, which was formed from ancient plant material by complex geological processes for millions of years, is one of the important energy resources around the world. With the increase of population and energy demand, high rank coal can no longer meet people's needs due to its gradual depletion, which has incurred the mining and utilization of low-value coals such as weathered coal, lignite, sub-bituminous coal, and high ash bituminous coal. Weathered coal, as a low-rank coal, is commonly found in Inner Mongolia Autonomous Region, Shanxi, Guizhou, Hebei provinces in China, and estimated at more than 10 billion tons (Yuan *et al*. 2014). Due to its low calorific value and high ash content, the weathered coal is not suitable for burning which would lead to the pollution of the environment. The development of high value-added bioproducts of weathered coal has been an international focus.

The extraction of humic acid materials, accounting for 30% to 80% of weathered coal weight (Zheng 1991; Qian *et al*. 2015) has become an important area of research. Chemical (Fong *et al*. 2007; Zhumanova *et al*. 2010; Doskočil *et al*. 2014), physical (Gazso 1997; Liang *et al*. 2011), biological (Hofrichter *et al*. 1999; Gao *et al*. 2012), and nanocatalyst (Tang *et al*. 2017, 2020; Song *et al*. 2022) methods have been employed to activate the humic acids in the low-rank coal. Humic acid is beneficial for agricultural production because it improves soil quality (Sun *et al*. 2020) and plant quality (Omer *et al*. 2020; Kishor *et al*. 2021; Çöl Keskin and Akınerdem 2021), increases fertilizer efficacy (Kishor *et al*. 2021), stimulates plant growth (Omer *et al*. 2020; Çöl Keskin and Akınerdem 2021; Kishor *et al*. 2021), and enhances plant resistance to stress (Abdellatif *et al*. 2017; Cha *et al*. 2017; Qin and Leskovar 2020). Compared with chemical and physical methods, biological methods involving microbial, enzymatic, or enzyme-mimetic technology is more suitable at moderate temperatures and pressures (Fakoussa 1992; Faison 1993; Gao *et al*. 2012). Compared with nanocatalysts, biological methods are less complicated and cost saving on the preparation of degraders of weathered coal.

Fungi including *Penicillium waksmanii* (Yang *et al*. 2001), *Penicillium* sp. GRF4, *Fusarium* sp. WF2, *Rhizopus* sp. AHBPF2 (Zhang *et al*. 2002), *etc.*, and bacteria including *Pseudomonas aeruginosa* (Yang *et al*. 2001), *Exiguobacterium* sp., *Serratia* sp., *Proteus* sp., *Citrobacter* sp., *Bacillus* sp., *Escherichia* sp., *Microbacterium* sp. (Olawale *et al*. 2020), *etc.* are responsible for the biodegradation of weathered coal. In-depth analyses of liquid coal products of weathered coal using UV-visible spectra and infra-red spectrum illustrated the releasing of soluble humic acid from weathered coal and its further degradation under the action of fungi, but no definite action mechanisms on coal biodegradation were elucidated in these studies (Yang *et al*. 2001; Zhang *et al*. 2002). However, the bioconversion mechanisms of low-rank coal and lignite indicated solubilization and depolymerization were two main principles for the processing of coal (Fakoussa and Hofrichter 1999). For example, ammonium ions and alkaline substances (Quigley *et al*. 1989; Hofrichter *et al*. 1997; Yuan *et al*. 2006a; Hölker *et al*. 1999), chelators (Cohen *et al*. 1990; Quigley *et al*. 1989; Torzilli and Isbister 1994; Hölker *et al*. 1999), surfactants (Polman *et al*. 1994; Yuan *et al*. 2006a), and hydrolases (Hölker *et al*. 1999; Yang *et al*. 2018) were found to play important roles in the biosolubilization of coal, while ligninolytic enzymes (Hofrichter and Fritsche 1997a), laccase (Pyne Jr *et al*. 1987; Srinivasan *et al*. 1995; Hölker *et al*. 1999; Zavarzina *et al*. 2004; Sekhohola *et al*. 2014; Nsa *et al*. 2022), esterase (Yang *et al*. 2018), lignin peroxidase and manganese peroxidase (Lundell and Hatakka 1994; Steffen *et al*. 2002; Klein *et al*. 2014), laccase and other oxidases (Scott and Lewis 1988; Willmann and Fakoussa 1997; Fakoussa and Frost 1999; Temp *et al*. 1999; Grinhut *et al*. 2007; Dashtban *et al*. 2010; Sekhohola and Cowan 2017) performed the crucial functions in the biodepolymerization of coal. Frequently, multiple solubilising agents are secreted concurrently by microbial organisms for the bioconversion of coal (Quigley *et al*. 1989; Hofrichter and Fritsche 1997b; Hatakka 1994; Ellouze and Sayadi 2016; Ghani *et al*. 2021).

The production of coal solubilising agents mostly depends on the composition of the culture medium. In terms of coal dissolution by *Phanerochaete chrysosporium*, the oxalate and other chelators (Torzilli and Isbister 1994), rather than ligninolytic enzymes (Odier and Artaud 1992), were found to be responsible for the implementation of low rank coal solubilization under nitrogen sufficient conditions, and results of another trial indicated that the occurrence of low rank coal solubilization was due to the activity of lignin peroxidase induced by veratryl alcohol (VA) added in a nitrogen-limiting liquid

medium. The study on laccase and manganese-dependent peroxidases from the white-rot fungus *Cyathus stercoreus* illustrated the increased activities of all the enzymes, which were both negligible at 1 mM ammonium tartrate and 0.1% glucose, and 4 and 0.9 U/mL at 10 mM ammonium tartrate and 1% glucose in the medium respectively (Sethuraman *et al*. 1999), but no significant amounts of manganese-dependent peroxidases were produced by *Pleurotus eryngii* under the use of ammonium tartrate as the nitrogen source in culture medium (Martínez *et al*. 1996). Ruiz-Dueñas *et al*. (1999) showed that *Pleurotus eryngii*, another white-rot fungal species, produced two peroxidase isozymes in the presence of peptone, both of which showed Mn^{2+} independent activities despite successfully carrying out mono-oxidation of Mn^{2+} to Mn^{3+}. In summary, coal bioconversion by microorganisms is related to the composition of culture medium and the strain used (Ellouze and Sayadi 2016).

This study was designed with weathered coal and sodium nitrate as the carbon and nitrogen sources to optimize the conditions for the degradation of weathered coal by *Penicillium aculeatum* 13-2-1, and meanwhile active degraded products of coal were measured by UV–Visible spectra to probe their active constituents. This data reveals the active substances in the bioconversion products of weathered coal by *Penicillium aculeatum* 13-2-1.

EXPERIMENTAL

Materials

Chemicals and medium

All chemical reagents used were of analytical grade. Potato dextrose agar medium (PDA) was purchased from Shanghai Crown Guide Bioengineering Co., LTD. Czapek Dox broth consisted of 3.0 g/L of $NaNO_3$, 1.0g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.5g/L of KCl, 0.01 g/L of $FeSO_4 \cdot 7H_2O$ and 1.0 g/L of glucose. The broth had a pH of 7.3.

Weathered coal and soil samples

The powder of weathered coal (sieved by 200 mesh sieve) was obtained from Guizhou Haoxiang Humic Acid Factory, Guizhou province, China, and the constituents of elements, determined by a Thermo Scientific Flash 2000 CHNS/O elemental organic analyzer, were nitrogen, oxygen, hydrogen, sulfur, and carbon of 0.803%, 30.086%, 2.646%, 0.216%, and 66.231%, respectively. Five fresh samples of the soils were collected from the rhizosphere of *Cinnamomum bodinieri*, *Zelkova serrata*, *Ginkgo biloba*, *Eucommia ulmoides*, and *Liquidambar formosana*, respectively, in National urban wetland park of Huaxi, Guiyang, China, and transported in ice box to laboratory for the analysis. The physiochemical characteristics of soils, assayed by conventional laboratory methods (Bao 2008), were organic matter of 11.26 to 13.05%, total nitrogen of 0.85 to 1.21%, hydrolysable nitrogen of 211.37 to 241.06 mg/kg, available phosphorus of 18.82 to 19.32 mg/kg, available potassium of 86.21 to 87.98 mg/kg, and pH of 5.93 to 6.02.

Enrichment Culture and Isolation of Fungus Degrading Weathered Coal

The fresh soil of 10 g was transferred into 100 mL sterile water in conical flask with six glass beads of diameter 3 mm, and shaken (200 rpm) for 12 h at room temperature and precipitated for 30 min. The soil suspension of 100 µL was spiked into 150 mL sterile water in conical flask having 15 g weathered coal. After a transient shaking by hands, the

enrichment culture for weathered coal-degrading microorganisms was carried out at 28 °C for incubation of 14 days under static condition using soil suspension as the inoculum. None of the inoculum specimens was looked upon as the control. The prominently coralloid mycelium appearing on the deposition of weathered coal was regarded as the target fungus of enrichment, and the sterile inoculation loop was used to pick it up for purification.

The mycelia obtained from the enrichment were transferred into sterile water in a petri dish, and they were vigorously agitated using a sterile glass rod. The mycelial suspensions of 50 µL were spread evenly on the surface of PDA medium in petri dish (ϕ9 cm) using a triangular glass rod. A total of 30 replicates were prepared for isolation of the enrichment. After four days of incubation at 28 °C, a uniform colony in morphology was selected for further purification on PDA medium. The purified strains were stored at -20 °C.

Confirmation and Classification of Fungus Degrading Weathered Coal

All the purified fungi were inoculated on PDA medium in petri dishes for incubation of four days at 28 °C. The weathered coal sterilized by dry heat at 160 °C lasting for 2 h was scattered on the mycelial mats on PDA medium in petri dish, and the phenomena of biosolubilization of weathered coal were observed after incubation of seven days at 28 °C. The darkening of agar medium and brown droplets appearing on the mycelia mats (Cohen and Gabriele 1982) were regarded as the evaluation criteria of weathered coal biodegradation in the present study. The taxonomic status of fungus was identified according to the morphological characteristics (Kong 2007) and molecular typing of internal ITS region of 18S rRNA gene (Silva-Stenico *et al*. 2007). A phylogenetic tree was constructed by neighbor joining method using MEGA5.0 software. The partial ITS/18S rRNA gene sequences were submitted to GenBank to get accession number (OQ048276).

Optimization of Cultural Conditions for Fungus Degrading Weathered Coal

To eliminate the possibility of fungus utilization of other organic matters besides weathered coal, a modified Czapek Dox broth (Moolick *et al*. 1990) was used by substituting weathered coal for glucose in the present study. In view of the need for carbon and nitrogen sources for microbial growth, weathered coal and the constituent sodium nitrate in Czapek Dox medium were used as two of the growth factors; the fungal inoculum was another factor. The classical one factor at a time method was employed to investigate the effects of the amount of weathered coal (0.5, 1.0, 1.5, 2.0, 2.5 g/100 mL), the quantity of sodium nitrate (0.05, 0.10, 0.15, 0.20, 0.25 g/100 mL), and the dose of inoculum (3×10^9 spores/mL) (100, 150, 200, 250, 300 µL/100 mL) on the degradation of weathered coal. The incubation conditions of broth culture were as follows: constant temperature of 28 °C, incubation period of 7 days, and shaking velocity of 120 rpm. The biodegradation extent of weathered coal was determined by the gravimetric method as the weight loss. After the incubation, the culture broth was centrifuged (10600 × g, 10 min) to separate the residues of weathered coal from the supernatant. The precipitating residues were thoroughly washed to remove fungal material and liquid products until pH 5.0 of the washings, and they were dried to constant weight at 60 °C in a thermostatic drier and weighed. The weight loss of weathered coal was calculated as the following formula,

$$\text{Weight loss (\%)} = \frac{W_0 - W_t}{W_0} \times 100 \quad (1)$$

where W_0 is the dry weight before degradation and W_t is the dry weight after biodegradation. The experiments were done in triplicate.

Orthogonal Experiment for Biodegradation of Weathered Coal

On the basis of the experimental results of the one factor at a time method, a test of orthogonal design was used to optimize the biodegradation of weathered coal (Table 1). The incubation conditions of broth culture for all the biodegradation trials were those described in the above section. After the shaking culture, the culture broth was centrifuged (10600 × g, 10 min) to separate the residues of weathered coal from the supernatant. The precipitating residues were thoroughly washed to remove fungal material and liquid products until pH 5.0 of the washings, and dried to constant weight at 60 °C in a thermostatic drier and weighed, and the contents of soluble humic acids in the liquid coal products were determined by the volumetric method (Agricultural Chemistry Committee of Soil Society of China 1983). All the experiments were done in triplicate.

Growth-promoting Effect of Liquid Coal Products on Rapeseed (*Brassica napus* L.)

The seeds of *Brassica napus* L. were sterilized with 5% sodium hypochlorite for 10 min and washed with distilled water. The liquid coal products, *viz.* the supernatant samples of the orthogonal design experiments diluted by 1, 2, 4 and 10 times, were used for the growth-promoting trials. At least 10 seeds (100% of germination rate) were laid on the filter paper (7 cm in diameter) saturated with 300 μL of different concentrations of liquid coal products in petri dish (9 cm in diameter), and the same volume of different concentrations of liquid coal products was added for the corresponding treatment each day. The culture broth of modified Czapek Dox medium (substituting 15 g weathered coal for glucose) autoclaved at 121 °C for 15 min was regarded as the control (HWP). The experiment was replicated three times. After 2 days incubation at day/night temperatures of 28/15 °C under a 12 h photoperiod, the lengths of radicle and plantule were determined.

Analysis of UV Spectrum of Liquid Coal Products

The UV-visible absorbance spectra of liquid coal products of all the samples of nine different combinations and HWP of were measured by using a UV–Vis spectrometer (Shimadzu UV-2600) from 200 to 700 nm at an interval of 1 nm in a 1-cm quartz cell at 28 °C with milli-Q water as the blank. All sample spectra were first smoothed and then corrected by baseline correction with OriginLab software. Specific peaks were picked to quality the structural category of the components in liquid coal products.

Statistical Analyses

The difference on biodegradation ratio of weathered coal (%) and contents of soluble humic acid (%) of different combinations were compared by one-way analysis of variance (ANOVA) followed by Tukey test (SPSS 13.0, SPSS Inc. Illinois, 1989-2004, USA).

RESULTS AND DISCUSSION

Isolation and Confirmation of Fungus Degrading Weathered Coal

As being the higher oxygen content in forms of aromatic cluster linkages, weathered coal was used to screen the fungal biodegraders from soil. After the enrichment culture of 14 days with the soil inocula from five different locations, only on the weathered coal in the conical flask spiked with the soil suspension from the rhizospheric soil of *Zelkova serrata* were there conspicuous coralloid-like mycelia present, while others did not share the same phenomenon.

Another thing worth mentioning was that sporadic mycelial masses occurred on the surface of the enrichment broth for all the spiking of soil suspensions, which indicated the utilization of fungi in soils towards thermally stable polycondensed aromatics of the mobile phase in weathered coal (Grint *et al*. 1985). The purified strain named 13-2-1, growing on the deposition of weathered coal, was further confirmed as the biodegrader of weathered coal on PDA plates, with brown droplets forming in the middle of mycelial mats and black diffusion areas around coal particles (Fig. 1a to 1d) demonstrating its ability of coal degradation. Gupta *et al*. (1988), Quigley *et al*. (1989), Cohen *et al*. (1990), Willmann and Fakoussa (1997), Hofrichter and Fritsche (1997a,b), Laborda *et al*. (1999), Fakoussa (1988), Tao *et al*. (2009), Kwiatos *et al*. (2018) and Ghani *et al*. (2021) attributed this phenomenon to the metabolic production of extracellular enzymes, alkaline substances and/or chelating agents and surfactants for the coal-solubilizing microbes.

Finally, strain 13-2-2 was identified as *Penicillium aculeatum* (homotypic synonym-*Talaromyces aculeatus*) according to the cultural characteristics on PDA medium (Fig. 1a and 1b), microscopic examination under a microscope (Fig. 2) and molecular identification based on the phylogenetic tree (Fig. 3). As for genus *Penicillium*, it has been reported to degrade coals through the action of extracellular enzymes, alkaline compounds or biosurfactants, *etc*. (Stewart *et al*. 1990; Yuan *et al*. 2006a; Li *et al*. 2023).

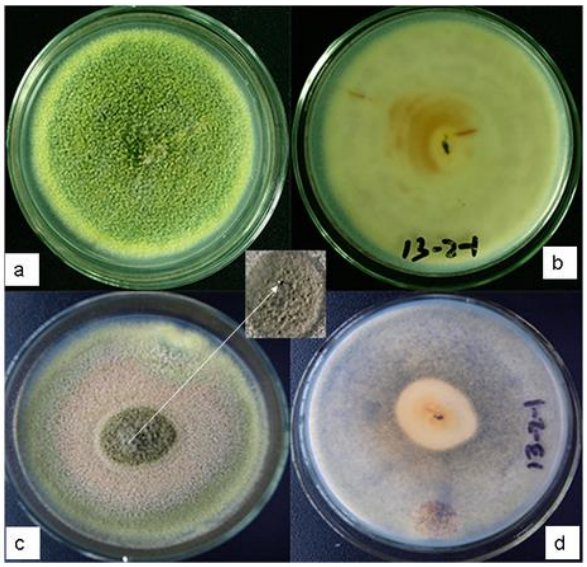

Fig. 1. Bio-solubilization experiment on PDA plate. a and b represent the frontal and converse observation of strain 13-2-1 growth after 4 days incubation at 28 °C, respectively (Control); c and d represent the biosolubilization of weathered coal by strain 13-2-2 after 4 days incubation at 28 °C (viewed from the frontal and converse faces, respectively)

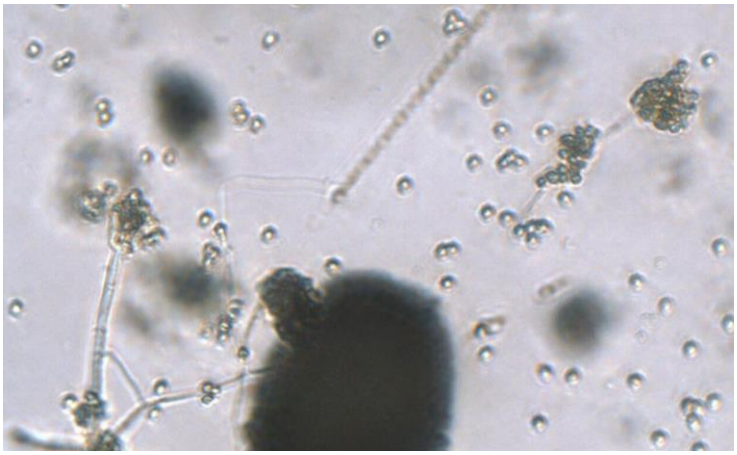

Fig. 2. Microscopic observation of strain 13-2-1 (10×40)

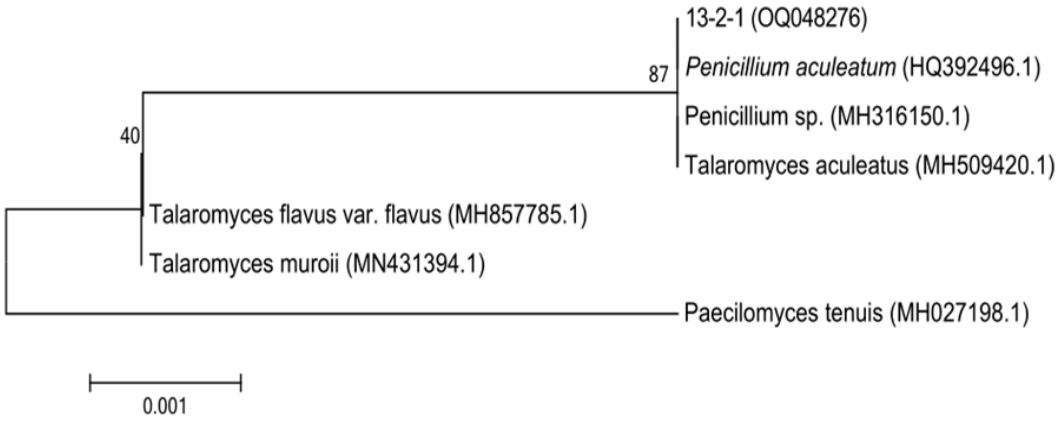

Fig. 3. The phylogenetic tree based on 18S rRNA gene sequence with neighbour-joining method. Bar, 0.001 substitution per nucleotide position

Optimization of Biodegradation Efficacy of Weathered Coal Using Classical One Factor at a Time Method

In terms of the main factors priming the biodegradation of weathered coal, three factors, *i.e.*, inoculum, weathered coal, and sodium nitrate were selected for the degradation experiments during the incubation of seven days in the present study.

Effect of Inoculum

The results of the effects of the different dose of fungus inoculum of *Penicillium aculeatum* 13-2-1 on biodegradation of weathered coal are shown in Fig. 4a. The broth inoculated with 150 μL/100 mL exhibited the highest degradation of 22.6%, and a higher dose of inoculum did not significantly express enhanced efficacy of coal biodegradation for *Penicillium aculeatum* 13-2-1. This might imply that the biodegradation extent of weathered coal was dependent on the accumulative biomass of mycelia feeding on defined amount of weathered coal (2.0 g/100 mL).

Effect of Weathered Coal

The effects of the amount of weathered coal on coal biosolubilization by *Penicillium aculeatum* 13-2-1 were indicated in Fig. 4b. The results showed that the highest ratio of biodegradation on weathered coal by *Penicillium aculeatum* 13-2-1 was 54.1% for

the loading dose of 1.0 g of weathered coal in 100 mL broth. The experimental results of the loading dose of 0.5 g weathered coal in 100 mL did not exhibit the highest ratio of coal biodegradation as the report of Sabar *et al*. (2019) for the degradation of low rank coal with the dose of 0.5% by *Rhizopus oryzae*. While those of other researchers (Yuan *et al*. 2006b; Haider *et al*. 2015) illustrated the optimal coal loading concentration of 1.0% for low rank coal degradation, which coincided with the present results on biodegradation of weathered coal by *Penicillium aculeatum* 13-2-1. Additionally, the results of Li *et al*. (2023) showed that a coal loading dose of 1.5% was optimal for biodegradation of lignite using *Penicillium ortum* MJ51. In the present study, coal loading dose of over 1% could not enhance the biodegradation of weathered coal by *Penicillium aculeatum* 13-2-1, which might be ascribed to the detrimental effects of coal on the growth of fungus (Nemati and Harrison 2000), or the non-availability of the surface area on coal particles owing to clogging/blockage of spores by the debris of dead cells and their metabolites (Tripathi *et al*. 2010).

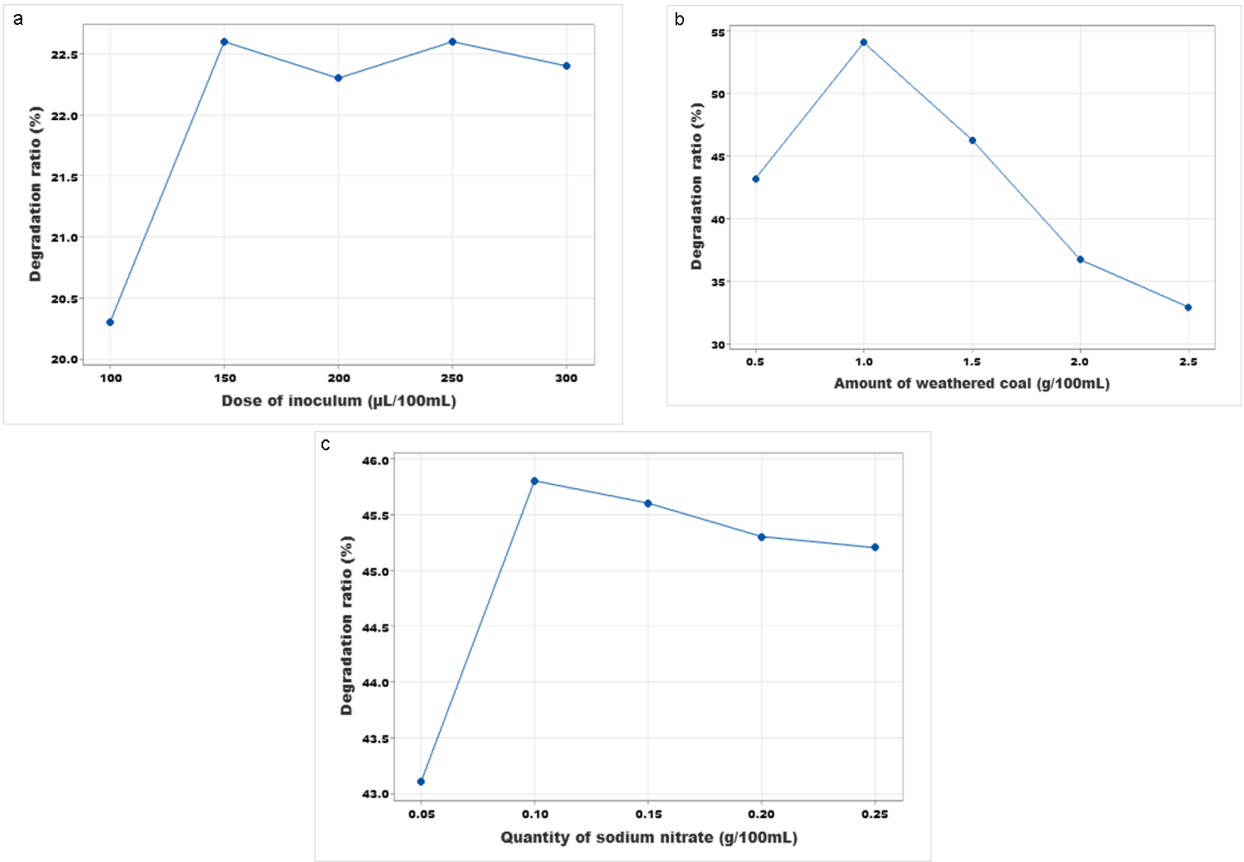

Fig. 4. Effects of dose of inoculum (a), amount of weathered coal (b), and quantity of sodium nitrate on coal biodegradation by strain 13-2-1

Effect of Sodium Nitrate

Sodium nitrate was reported to be the most suitable nitrogen source for the biosolubilization of low rank coal (Selvi *et al*. 2009). In this study, the effect of different quantities of sodium nitrate (g) on the extent of coal dissolution is presented in Fig. 4c. The maximal ratio of biodegradation by *Penicillium aculeatum* 13-2-1 was 45.8% for the adding of 0.1 g sodium nitrate in 100 mL of broth. With the increasing quantity of sodium

nitrate in the broth, the extent of coal biosolubilization presented a gradual decreasing trend, which might be associated with the suppression of the activities of oxidative enzymes decomposing the coal lignin due to the increase of nitrogen concentrations in cultural medium (Ürek and Pazarlioğlu 2005; Rivera-Hoyos *et al*. 2013).

Optimization of Biodegradation Efficacy of Weathered Coal Using Orthogonal Test ($L_9 3^4$)

To improve the biodegradation of weathered coal and enhance the release of humic acid components from coal, the biodegradation of weathered coal by fungus *Penicillium aculeatum* 13-2-1 was optimized. Based on the experimental results of classical one factor at a time method, three levels (*i.e*. 1, 2, and 3) for inoculum (A), weathered coal (B), and sodium nitrate (C) were designed as shown in Table 1.

Table 1. Experimental Conditions Based on Orthogonal Design Form $L_9(3^4)$ and Contents of Soluble Humic Acid in Biodegradation Liquids

Experiment Number	Dose of Inoculum (μL)(A)	Amount of Weathered Coal (g)(B)	Quantity of Sodium Nitrate (g)(C)	Biodegradation Ratio of Weathered Coal (%)	Contents of Soluble Humic Acid(%)
1	100(A1)	0.5(B1)	0.05(C1)	19.58	3.50
2	100(A1)	1.0(B2)	0.10(C2)	32.44	1.47
3	100(A1)	1.5(B3)	0.15(C3)	41.45	0.52
4	150(A2)	0.5(B1)	0.10(C2)	55.76	3.32
5	150(A2)	1.0(B2)	0.15(C3)	17.20	1.93
6	150(A2)	1.5(B3)	0.05(C1)	39.74	0.58
7	200(A3)	0.5(B1)	0.15(C3)	38.03	3.69
8	200(A3)	1.0(B2)	0.05(C1)	81.98	2.40
9	200(A3)	1.5(B3)	0.10(C2)	42.97	0.58

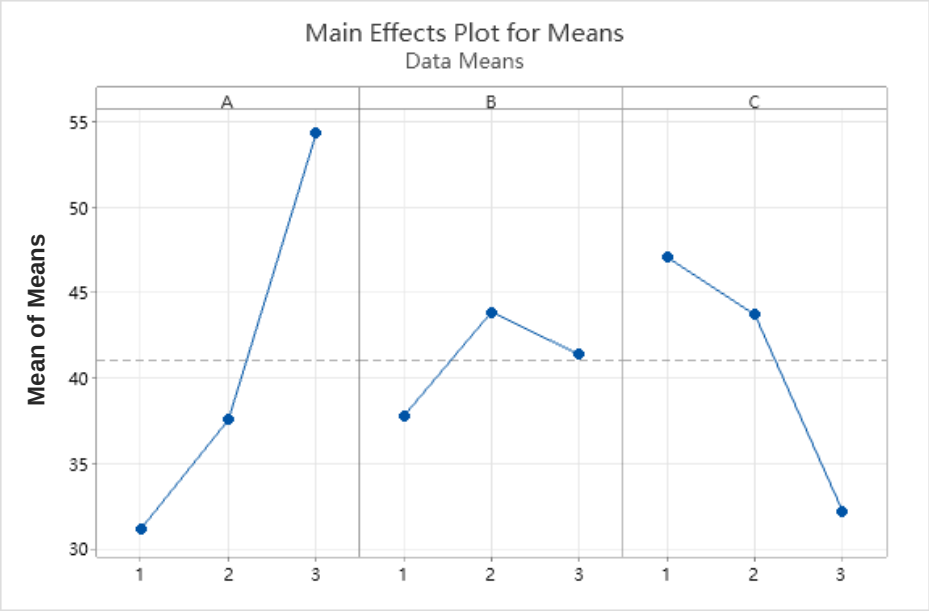

Fig. 5. Level effects of inoculum (A), weathered coal (B), and sodium nitrate (C) on coal biodegradation

The results of the orthogonal test of three factors three levels illustrated the highest degradation efficacy (81.98%) of weathered coal and was presented for the combination of A3B2C1, which was followed by those of A2B1C2 (55.76%), A3B3C3 (42.97%), A1B3C3 (41.45%), A2B3C1 (39.74%), A3B1C3 (38.03%), A1B2C2 (32.44%), A1B1C1 (19.58%), and A2B2C3 (17.20%) (Table 1). In terms of biodegradation of weathered coal, a high dose of inoculum (200 µL/100 mL), median dose of weathered coal (1.0 g/100 mL), and low dose of sodium nitrate (0.05 g/100 mL) were found to be beneficial for coal biodegradation by *Penicillium aculeatum* 13-2-1 (Fig. 5).

The contents of the soluble humic acids in liquid coal products displayed changing tendencies collated with the biodegradation ratio of weathered coal. The highest contents of soluble humic acids were 3.69% of the combination A3B1C3, followed by 3.50% of A1B1C1, 3.32% of A2B1C2, 2.40% of A3B2C1, 1.93% of A2B2C3, 1.47% of A1B2C2, 0.58% of A2B3C1, 0.58% of A3B3C2, and 0.52% of A1B3C3. Regarding the production of soluble humic acid, high dose of inoculum (200 µL/100 mL), low dose of weathered coal (0.5 g/100 mL), and low dose of sodium nitrate (0.05 g/100 mL) were conductive to the production of soluble humic acid during coal biodegradation by *Penicillium aculeatum* 13-2-1 (Fig. 6).

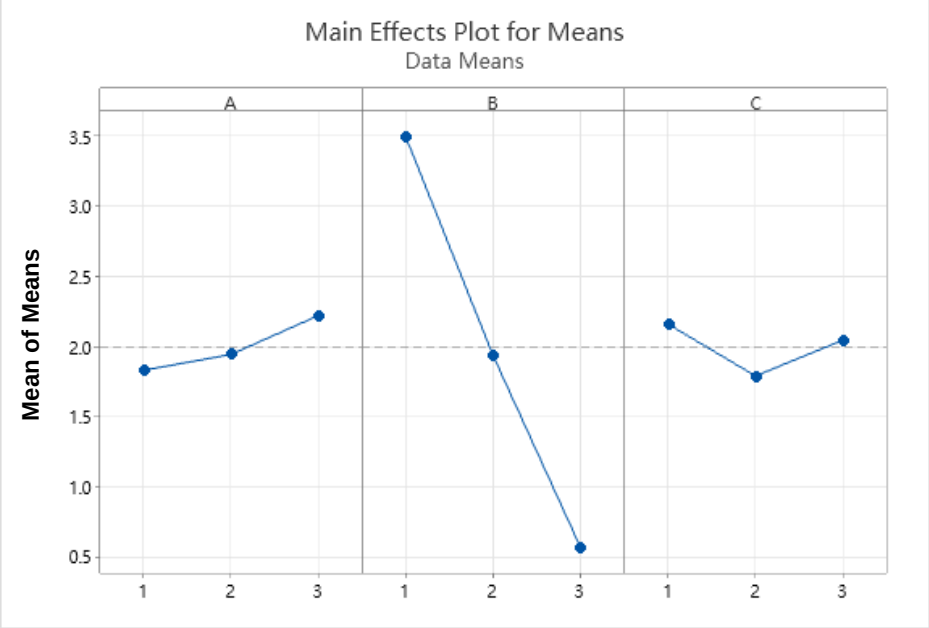

Fig. 6. Level effects of inoculum (A), weathered coal (B) and sodium nitrate (C) on production of soluble humic acids

The variance analysis of single factor showed that only the amount of weathered coal negatively affected the contents of soluble humic acids in liquid coal products, which suggested the inhibition of coal towards extracellular enzymes of fungi functioning in the depolymerization of macromolecular humic substances. None of the factors significantly affected the biosolubilization of weathered coal (Table 2), but the dosage effects of sodium nitrate on coal biosolubilization were contradictory to the findings of Hofrichter *et al*. (1997) for the enhancing coal biosolubilization under nitrogen-rich medium. This might imply the low molecular weight lignin fragments could be split off enzymatically from solid coal at the low nitrogen concentrations, incurring the acidic environments in the present study.

Table 2. ANOVA Results Showing Effects of Three Factors on Weathered Coal Biodegradation and Contents of Soluble Humic Acid in Biodegradation Liquids Based on $L_9(3^4)$ Orthogonal Design

Factor	Biodegradation Ratio of Weathered Coal (%)		Contents of Soluble Humic Acid (%)	
	F	p-value	F	p-value
Inoculum (A)	0.50	0.668	5.89	0.145
Weathered Coal (B)	0.03	0.969	311.67	0.003
Sodium Nitrate (C)	0.21	0.826	5.16	0.162

Effects of Liquid Coal Products on Rapeseed (*Brassica napus* L.) Growth

The effects of different combinations on growth of rapeseed (*Brassica napus* L.) seeds are illustrated in Fig. 7. Compared with the efficacy of the pyrolysis products of weathered coal (HWP) on rapeseed growth, all of the combinations could promote the growth of rapeseed (*Brassica napus* L.) seedlings, especially on the radicles, which suggested the activation of humic acids from weathered coal by *Penicillium aculeatum* 13-2-1. A striking promotion of growth on the radicles of rapeseed indicated the auxin-like activities of soluble humic acids in liquid coal products existed for all the treatments, which corresponded to the reports of Trevisan *et al*. (2010) and Dobbss *et al*. (2007) about the auxinic activity of humic substances on root development. The combinations of A2B1C2 and A1B1C1 significantly improved the growth of radicle and plantule of rapeseed (*Brassica napus* L.), which conformed to the higher contents of soluble humic acids in liquid coal products. In contrast, A3B1C3 of the highest contents of soluble humic acids did not exhibit the strongest promotion on the growth of rapeseed (*Brassica napus* L.) collated with other combinations (A2B1C2 and A1B1C1), which meant that there were differential functions of active components of soluble humic acids in liquid coal products on plants for different combinations. Nardi *et al*. (2007) ascribed this phenomenon to the particular cause that different humic fractions affected the enzyme activities related to glycolysis and tricarboxylic acid cycle (TCA) in different ways, depending on their molecular sizes, molecular characteristics, and concentrations. Additionally of note is that not all the liquid coal products of the combinations, besides HWP, showed a dose-dependent promotion effects on the growth of rapeseed seedlings, which resembled the findings of Hou *et al*. (2022) on growth-promoting effects of humic acids from weathered coal on mung bean.

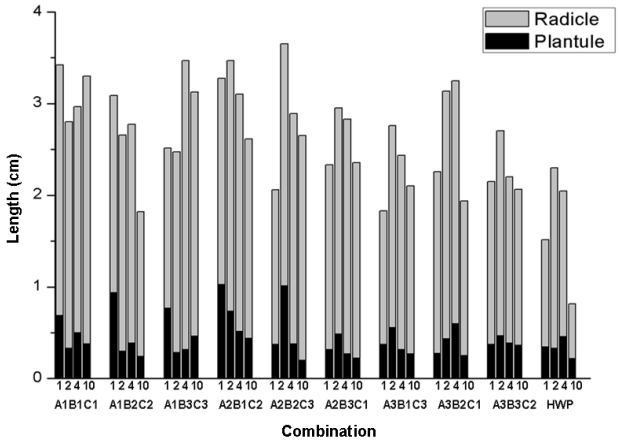

Fig. 7. Effects of liquid coal products diluted by different times (1×, 2×, 4× and 10×) on the growth of rapeseed

Spectral Characteristics of Liquid Coal Products

The UV spectra of the liquid coal products from different combinations are shown in Fig. 8. First-order derivative spectra were used to analyze the spectral bands. The major absorbance in the range of 200 to 300 nm indicated that unsaturated chemical bonds were present in the coal solubilisation products (Shevla 1976; Yin *et al*. 2009). In particular, the appearance of chief peaks in the range 220 to 330 nm indicated the presence of aromatic and humic materials released from coals under the fungal attack (Sabar *et al*. 2019), while the absorbance strength of the released organics in the coal-containing control (HWP) was only detectable at about 240 nm. For the combination of A1B3C3, A2B2C3, and A3B1C3, absorbance at about 240 and 300 nm indicated that alkyl substituted unsaturated aldehyde and ketone compounds and probable hydrophilic humic contents were present in the biosolubilisation products of weathered coal, which might be attributed to the coal bioconversion by phenol-oxidases of fungus (Tao *et al*. 2009) and the solubility of water-based media (Haider *et al*. 2015) or alkaline compounds produced by fungus (Miszkiewicz *et al*. 2016), respectively. For those of A1B1C1, A2B3C1, and A3B2C1, absorbance at about 230 and 250 nm implied that most probably low-molecular-weight phenolic compounds (Yakimenko *et al*. 2018) and unconjugated phenolic lignin (Gärtner and Gellerstedt 1999) appeared in the biosolubilisation products, which might be ascribed to the coal lignin decomposition through microbial extracellular enzymes-lignin peroxidase, manganese peroxidase, laccase, or others (Reid 1995; Kamimura *et al*. 2019). For other combinations, absorbance at about 240 nm for A1B2C2 and A3B3C2, and 250 nm for A2B1C2 suggested that not exactly similar biosolubilisation products were present in liquid coal products.

Sodium nitrate appeared to be the crucial factor determining the dissolved organic components in liquid coal products, which indirectly reflected the differential metabolic modes of *Penicillium aculeatum* 13-2-1 towards weathered coal at different cultural conditions.

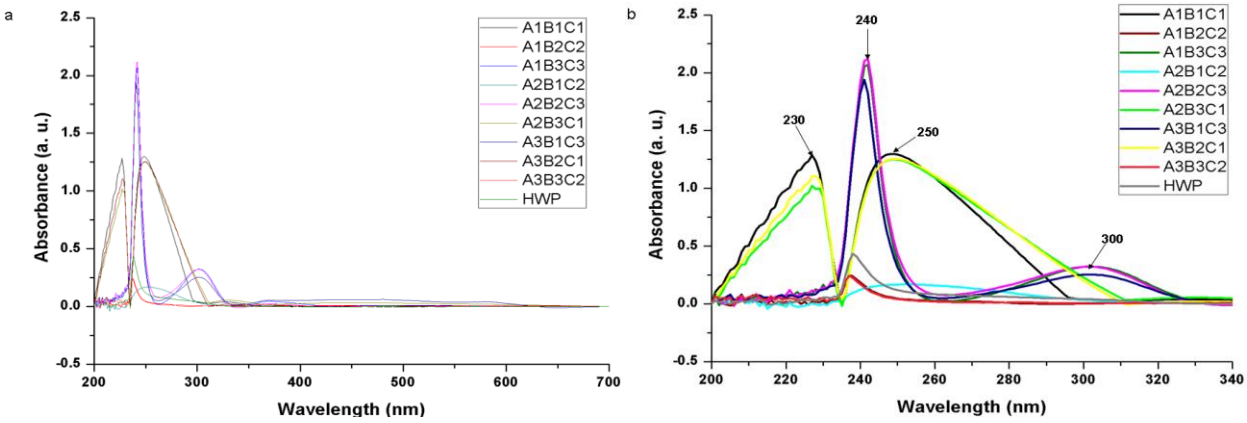

Fig. 8. The spectra of liquid coal products of different combinations and HWP in the range 200 to 700 nm (a) and 200 to 340 nm (b)

CONCLUSIONS

1. *Penicillium aculeatum* 13-2-1 isolated from the rhizospheric soil of *Zelkova serrata* showed the ability to degrade the weathered coal.
2. The extent of coal biodegradation and soluble humic acid production was affected by the dose of the inoculum, the amount of weathered coal, and the quantity of sodium nitrate. The results of orthogonal experiment indicated that 200 ìL of inoculum, 1.0 g of weathered coal, and 0.05 g of sodium nitrate (A3B2C1) was most optimal for coal degradation, and 200 ìL of inoculum, 0.5 g of weathered coal, and 0.15 g of sodium nitrate (A3B1C3) was most suitable for the production of soluble humic acids.
3. The liquid coal products derived from coal bioconversion by *Penicillium aculeatum* 13-2-1 could promote the growth of *Brassica napus* L. seedlings.
4. The components of liquid coal products associated with the soluble humic acids were determined by the quantity of sodium nitrate in broth culture.

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