Development of a saprophytic fungal inoculum for the biodegradation of sub-bituminous coal

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Received: 15 May 2021 / Accepted: 13 December 2021

Published online: 12 January 2022

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Abstract

The rhizospheres of the weeds *Ageratum conyzoides*, *Axonopus compressus*, *Emilia coccinea*, *Synedrella nodiflora*, *Urena lobata* and *Sida acuta* from a sub-bituminous coal mining site and a control site, without coal discards, were screened for new fungi with ability to degrade sub-bituminous coal in the laboratory. The isolates were identified by cultural and molecular methods. Seventeen out of the sixty-one fungal isolates tested could utilize sub-bituminous coal as an energy source. Upon further evaluation, only seven of these were promising candidates for coal biodegradation, and they were assayed for their biosolubilization and depolymerization activities to determine their mechanisms of coal biodegradation. Based on the accumulation of humic acid (HA), which is the marker for biosolubilization, *Mucor circinelloides* and *Aspergillus tubingensis* were the most active. On the other hand, *Cunninghamella bertholletiae*, *Simplicillium subtropicum*, *Penicillium daleae* and *Trichoderma koningiopsis* were the highest producers of fulvic acid (FA), the indicator of depolymerization. *Purpureocillium lilacinum* produced the lowest yields of both HA and FA compared to the other six coal-degrading candidates. The presence of laccase in *Trichoderma koningiopsis*, *Penicillium daleae* and *Simplicillium subtropicum* suggests a role for this enzyme in the enhancement of the coal biodegradation process. However, the inability to amplify the laccase gene in *Cunninghamella bertholletiae* indicates that another enzyme probably aids its coal bioconversion. The current investigation highlights the potentials of these strains in harnessing biotechnological processes of sub-bituminous coal conversion into value-added products, which could be extended to the bioremediation of coal-polluted soils. The fungi with the highest coal bioconversion capabilities belonged to Ascomycota and Zygomycota and were found in the rhizospheres of the weeds *Emilia coccinea*, *Ageratum conyzoides* and *Axonopus compressus*.

Keywords Sub-bituminous coal · Fungi · Humic acid · Fulvic acid · Biosolubilization depolymerization

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42452-021-04925-6>.

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1 Introduction

Coal mining has considerable impact on the environment since coal production, its utility and disposal cause significant agricultural and environmental concerns [1–4]. The environmental impact includes loss of vegetation as well as the replacement of valuable indigenous agricultural plant species by grasses and other weeds [5–8]. Sub-bituminous coal is a lower-ranked coal in calorific terms (8500–13,000 Btu/lb) in comparison with anthracite (13,000–15,000 Btu/lb) and bituminous coal (11,000–15,000 Btu/lb) but higher than lignite (4000–8,300,000 Btu/lb) [9–11]. Sub-bituminous coal constitutes a significant percentage of Nigeria's coal reserves, and it is located in both the Lower and Upper Benue Trough as well as in many parts of Enugu [12–14]. The subsequent abandonment of the Enugu mines and the indiscriminate disposal of coal discards led to environmental degradation in the area as evidenced by the loss of agricultural species [15].

Microbial coal conversion can be executed through any of these bioprocesses: depolymerization, decolorization, liquefaction and solubilization. Depolymerization is defined as the catabolic reduction in higher-molecular-mass coal to smaller fractions which could be accompanied by a loss of chromophores, whereas decolorization (bleaching) is the loss of chromophores without any change in the molecular size [16, 17]. Liquefaction is defined as a change of physical state (solid to liquid) and should not be confused with solubilization, which is the dissolution of all or part of the coal molecule [18]. Coal-degrading microbes naturally occur in coal dumpsites where coal bioconversion has been shown to occur in situ. A deuteromycete, *Neosartorya fischeri* isolated from the rhizosphere of the grass, *Cynodon dactylon* has been reported to degrade lignite, a low-grade coal [19–21]. A screen of the root zones of plants from a sub-bituminous coal dumpsite identified coal-active fungi belonging to Ascomycetes, Zygomycetes, Sordariomycetes and Leotiomycetes [22].

There is evidence that fungal degradation of coal waste permits its transformation into fuels or humic substances, which play important roles in carbon recycling as well as improvement of physical and chemical quality of soil structure. These invariably promote plant growth and development [21, 23–26]. Humic acid (HA) is a product of coal solubilization, and the fungi known to break it down are principally Ascomycetes and Basidiomycetes [27]. Humic acid is a heterogeneous macromolecule with a molecular weight of 10,000–100,000 Da. It is characteristically dark brown to black in color with solubility at high pH and precipitation at low pH [28–30]. On the

other hand, fulvic acid (FA) is a yellow product of depolymerization of HA. It solubilizes at any given pH and has a lower molecular weight of 1000–10,000 Da, which is lower than that of HA. It is formed by the cleavage of bonds inside the coal macromolecule [28, 30, 31].

It has been proposed that the biosolubilization and depolymerization of lignite could involve either microbial formation of alkaline substances or activities of ligninolytic enzymes or a combination of both [32]. Furthermore, the oxidative action of ligninolytic enzyme, laccase, played a key role in *N. fischeri* ECCN84 metabolizing waste low-rank coal and extracting carbon for growth and proliferation [20]. The various classes of ligninolytic enzymes reported in lignocellulosic waste degradation include laccases, lignin peroxidases, versatile peroxidase and manganese peroxidases [33].

Fungal mediated degradation of sub-bituminous coal [34–36] and the bacterial degradation of bituminous coal discards have been documented [37]. Furthermore, the previous investigation by Nsa et al., [22] focused on the identification of rhizospheric fungi from only polluted soils of an abandoned sub-bituminous coal mine site by culture techniques and microscopy. This present study has been expanded to include (1) screening of the rhizospheres of plants (weeds) growing on the coal-polluted site as well as a control site for coal-degrading fungi (2) determining the sub-bituminous coal utilization capabilities of these isolates in the laboratory (3) molecular identification of the isolates (4) potential functional attributes of the fungal isolates and (5) determining the presence of ligninolytic enzymes in the species with coal bioconversion capabilities.

2 Materials and methods

2.1 Source of soil and coal samples

Soil samples were obtained from the rhizospheres (R) of weeds growing on a coal dump site (IZ; Incident Zone) by a defunct mine, and controls were obtained from a site free of coal discards (CZ; Control Zone) [38]. The rhizospheres that were screened for fungi include *Emilia coccinea* (RIZ3, RCZ2B), *Axonopus compressus* (RIZ4 and RCZ2D), *Synedrella nodiflora* (RIZ5 and RCZ1D), *Ageratum conyzoides* (RIZ 8B, RIZ9, RIZ10 and RCZ2A), *Sida acuta* (RIZ11) and *Urena lobata* (RCZ2C).

Sub-bituminous coal samples were obtained from the defunct Ogbete mine, Udi, Local Government Area (LGA) of Enugu State, Nigeria. The coal samples were collected using hand gloves and transported on ice to the laboratory where they were pulverized using a CBR mold and rammer (4.5 kg).

2.2 Isolation and enumeration of soil fungi

2.2.1 Isolation of pure cultures

One gram of soil sourced from each of the RIZ and RCZ samples was placed into its respective bottle holding 9-ml sterile distilled water and shaken for homogenization. For each sample, tenfold serial dilutions up to 10^{-6} dilutions were made. Aliquots of 1 ml were taken from the 10^{-3} and 10^{-6} dilutions of each of the ten soil samples and plated on Sabouraud Dextrose Agar (SDA) (Rapid Labs AZ, USA) and Potato Dextrose Agar (PDA) (HiMedia Mumbai, India) using the pour plate technique. All plates were incubated at 25 °C on both SDA and PDA for 5–7 days. Plates with luxuriant growth were chosen from the mixed culture plates for screening. Individual colonies were selected after two rounds of sub-culturing on SDA plates to obtain pure cultures. Conidial suspensions were made for each isolate, and 100 µl volume was plated. The agar plates were incubated at 25 °C for 5–7 days, and the pure cultures of the isolates were stored on slants at 4 °C and -20 °C for future use.

2.3 Test for coal activity

All the strains obtained from the screen were checked for coal utilization. Coal activity of each pure isolate was tested on solid media, coal agar plate (CAP) and in liquid media, coal broth (CB).

Coal agar plate (CAP) The pure cultures of the individual fungi were inoculated on sub-bituminous coal agar plates, water agar plates and SDA plates for 5–7 days at 25 °C. The coal agar medium consisted of 20 g of pulverized coal and 15 g agar-agar per liter of distilled water [22, 39]. Water agar plates (WAP) made of 15 g of agar-agar in 1 L of distilled water served as the negative control, while SDA plates served as the positive control.

Coal broth (CB) Coal activity was also tested on a coal broth containing Minimal Salts Medium (MSM) and coal. Twenty grams of pulverized coal was added to 1 l of MSM, heated, and stirred for homogenization; 100 ml of the medium was dispensed into 250-ml conical flasks and autoclaved at 121 °C at 15 psi for 15 min. On cooling, same-age mycelia obtained from each of the 61 isolates were inoculated into CB for 7 days at 25 °C without shaking. The positive control medium was composed of MSM and glucose (Sigma-Aldrich, Taufkirchen, Germany), at a concentration of 0.056 M. The negative control medium comprised of MSM only. Selection of strains with coal utilizing capabilities was based on growth on coal agar, a tuft of mycelial mass at the base of the CB flask or by turbidity [22].

2.4 Biosolubilization assay

2.4.1 Coal pre-treatment

For the biosolubilization testing, the 17 strains that grew both on CAP and in CB were used. To remove excess phenolic compounds known to affect solubilization rates, sterile pulverized sub-bituminous coal was pre-treated with nitric acid (HNO_3) (HiMedia, Mumbai, India). One gram of coal was added to a flask containing 50 ml of 30% HNO_3 (1:50 w/v) and incubated at room temperature with shaking for 48 h [40, 41]. The pre-treated coal was rinsed with sterile distilled water (dH_2O) until a clear rinsate of $\text{pH} > 5$ was obtained, and then dried in an oven at 40 °C. The medium for the biosolubilization assay was Czapek Dox Broth containing sucrose (HiMedia, Mumbai, India) as the sole carbon source, while sodium nitrate (HiMedia, Mumbai, India) served as the sole nitrogen source [41]. The pre-treated coal was added to the Czapek Dox broth at a concentration of 2.5%.

2.4.2 Preparation and testing of fungal strains

Each fungal inoculum was prepared by rinsing 5-day old fungal mats (10 mm in diameter) from SDA plates with sterile dH_2O . Every mat was re-suspended in 2-ml sterile dH_2O , and the resulting fungal concentrate was inoculated into 100-ml Czapek Dox Broth in a 250-ml Schott bottles. Samples were incubated at 28 °C for 5 days with shaking at 150 rpm. The formation of fungal biomass was monitored visually. The pH measurements of the media were obtained, and the HA and FA concentrations in the culture supernatants were analyzed by spectrophotometry as described by Fakoussa and Frost [42]. The uninoculated flasks containing only the media served as controls. After a 5-day incubation period, either a brown-colored appearance or subsequent clarification of the broth indicated culture activity.

2.4.3 Analysis of HA standard measurements

To determine the extent of biosolubilization activity, HA production was measured. The resultant cultures from above were first filtered using a 36-µm mesh. Humic acid-like substances (HS) were extracted and analyzed using a method adapted from Janoš [30]. Humic acid in the filtrate was precipitated by reducing the pH to less than 1 using a minimal volume of concentrated HCl (32%), while the filtrate was separated and kept for FA analysis. For each culture, the pellet, containing HA, was re-suspended in 10-ml volume of 0.1 M NaOH and centrifuged using an Eppendorf 5415D desktop centrifuge, at $3220 \times g$ for 90 min at 10 °C to compact undissolved substances in a pellet. The

pellet was discarded, while the absorbance values of the supernatants were determined by spectrophotometry at a wavelength of 450 nm (A_{450}) for HA concentrations [42].

Humic-like substances in the supernatant were quantified by interpolation from standard curves for HA at (A_{450}) [42]. This was made by making five concentrations (40, 80, 120, 160, 200 mg/L) of HA standards (International Humic Substance Society, Denver, CO, USA) and plotted against their respective absorbance values. The absorbance values of the HA in the media were read, and the concentration gradient was calculated from the straight-line equation ($y = 0.0082x$) to determine the level of biosolubilization.

2.4.4 Depolymerization test

Fulvic acid standard measurements were analyzed to determine depolymerization activity. For each sample, the saved filtrate from the biosolubilization assay was allowed to stand for 1 h before centrifugation under the same conditions as conducted for HA. The supernatant was decanted, and analyzed by spectrophotometry at a wavelength of 280 nm for FA. The FA analysis was done by interpolation from standard curves for fulvic acid [42, 43].

2.5 Molecular identification of fungal isolates

2.5.1 Fungal DNA isolation

Genomic DNA was extracted from the fungal isolates using Zymo Research Quick-DNA Fungal/Bacterial Miniprep™ extraction kit (CA, USA) according to the manufacturer's instructions.

2.5.2 Amplification of internal transcribed spacer (ITS) regions of fungal isolates

Polymerase chain reaction (PCR) of the extracted genomic DNA from the 61 isolates was done in a Bio-Rad T100™ PCR thermocycler (Bio-Rad, Hercules, CA, USA). Each 25- μ l reaction mix consisted of 12.5 μ l of One Taq® 2 $\times$ Master Mix with Standard Buffer (New England BioLabs, MA, USA), 50 ng/ μ l of DNA template, 0.2 μ M each of forward (ITS1-F: 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4-R: 5'-TCC TCC GCT TAT TGA TAT GC-3') primers [44] and sterile nuclease-free water. Reaction mixture without DNA template was used as the negative control. The PCR conditions were as follows: initial denaturation step, 94 °C for 30 s, 35 cycles of 30 s denaturation at 94 °C, annealing at 55 °C for 60 s, elongation at 68 °C for 60 s, a final elongation step at 68 °C for 5 min. The Quick-Load Purple 1 kb DNA ladder (New England BioLabs, MA, USA) and the amplicons were loaded on a 1% ethidium bromide agarose gel that was run at 60 V for 1 h with

Bio-Rad® PowerPac™ Basic Power Supply and visualized with Bio-Rad Gel Doc™ EZ Imager (Bio-Rad, Hercules, CA, USA).

2.5.3 DNA sequencing and phylogenetic analyses of isolates

The amplicons were sequenced at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. The sequences were checked for quality and assembled using BioEdit (version 7.6.2.1) Sequence Alignment Editor [45]. The consensus sequence obtained for each was compared to the GenBank nucleotide data library using the Basic Local Alignment Search Tool, BLAST software [46] at the National Centre for Biotechnology Information (NCBI (<http://www.ncbi.nlm.nih.gov>)). Sequence homology was used for preliminary identification of the isolates, using the BLAST. Mothur software pipeline was used to cluster sequences of the 17 isolates (that were active on coal) into operational taxonomic units (OTUs) at sequence similarity of $\geq 97\%$. Phylogenetic reconstruction of the sequences was done by multiple sequence alignments of the OTU sequences with closely related sequences in the Genbank as well as an outgroup sequence of *Gigaspora margarita*. The sequence alignment was done on MAFFT [47] and aligned multiple sequences were edited with evolutionary history among them inferred using the Neighbor-Joining method [48]. The phylogenetic relationship established was constructed in MEGA X [49] using maximum composite likelihood model [50] with 1000 bootstrap replications [51].

2.5.4 Identification of laccase gene

Amplification of the laccase gene was done using primer sets LacF (5'-CAYTggCAYggNTTY TTYCA-3'), LacR (5'-TgRAA RTCDATRTgRCARTg-3') [52, 53]. Genomic DNA extracted from each fungus with positive coal activity served as a template in the PCR. The reaction mix was made of 2 μ l of template DNA, 0.5 μ l LacF Primer (10 μ M), 0.5 μ l LacR Primer (10 μ M), 12.5 μ l of One Taq 2 $\times$ Master Mix and 9.5 μ l sterile distilled water in 25 μ l total volume. PCR mix without DNA was used as a negative control for each PCR run. The PCR run conditions in the thermal cycler (Bio-Rad T100™) were initial denaturation of 94 °C for 30 s, 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 60 s), extension (68 °C for 60 s) and then by a final extension of 68 °C for 5 min. PCR products were run on a 1% agarose gel and viewed on the Bio-Rad Gel Imager.

3 Results and discussion

3.1 Determination of fungal isolates

The mixed cultures of the initial fungal screen of the rhizosphere samples on PDA (Fig. S1) yielded a total of 61 unique isolates (34 and 27 from RIZ and RCZ respectively) (Table 1; Fig. S2). They were identified as belonging to the genera *Aspergillus*, *Trichoderma*, *Fusarium*, *Mucor*, *Simplicillium*, *Penicillium*, *Purpureocillium*, *Plectosphaerella* and *Cunninghamella*. They were obtained from the screening of twelve weed-rhizosphere soil samples from a coal dump site and an unpolluted control zone. All the species identified in this study have been previously isolated from soil, namely *Purpureocillium lilacinum* [54], *Plectosphaerella cucumerina* [55], *Fusarium oxysporum*, *Fusarium fujikuroi* [56], *Mucor circinelloides*, *Cunninghamella bertholletiae* [57, 58], *Aspergillus aculeatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium citrinum*, *Penicillium daleae* [59–61] *Simplicillium subtropicum*, *Trichoderma asperellum* and *Trichoderma koningiopsis* [62, 63].

Aspergillus flavus and *Trichoderma asperellum* were the most versatile having been recovered from four and three different rhizospheres, respectively. Among the cultivable fungi across the rhizospheres, *Aspergillus* was the dominant genus followed by *Trichoderma*. The most frequently occurring species identified from different rhizospheres were *A. flavus*, *A. niger* and *P. griseofulvum*, while there were single occurrences of *S. subtropicum* and *T. reesei* from the rhizospheres of *A. compressus*. and *S. nodiflora*, respectively.

Furthermore, the presence of *T. koningiopsis*, *C. bertholletiae* and *S. subtropicum* in the rhizospheres of *E. coccinea*, *A. conyzoides* and *A. compressus*, respectively, from the coal dumpsite is supported by a previous report in which a coal-degrading fungus, *N. fischeri*, was isolated from the root zone of *Cynodon dactylon* [39]. Moreover, it is plausible that the presence of these organisms may relate to the survival of these plants in the coal-polluted environment. This is in tandem with various studies that confirm that a correlation exists between root microflora and plants' capabilities for remediation [32, 39, 64].

The records on fungal degradation of sub-bituminous coal are inadequate in comparison with the numerous reports of the other low ranked coal, lignite. Biodegradation of coal by fungal species, first reported by Fakoussa [65] and subsequently by Cohen and Gabriele [23], led to a series of studies that demonstrated the ability of a range of microbial species that are able to catalyze this process [19, 23, 66–69]. Particularly, there have been no accompanying reports to the studies on the *Penicillium*

simplicissimum-mediated solubilization of Nigerian sub-bituminous coal [34, 35].

3.2 Coal activity assay

Seventeen isolates, namely RCZ1D.4, RCZ2A.1, RCZ2B.2, RCZ2C.2, RCZ2D.2, RCZ2D.5, RIZ3.1, RIZ4.2, RIZ4.4, RIZ5.3, RIZ9.3, RIZ10.2, RCZ2B.1B, RCZ2B.2B, RCZ2B.3B, RIZ4.1B and RIZ4.2B showed good growth on CAP, whereas scanty or no growth was observed on the control WAP (Fig. 1). Out of these 17 isolates, seven had the most mycelial growth in the liquid medium and were selected on the inference that more biomass should indicate the suitability of the fungus biodegradation studies (Fig. 2). They include RCZ2B.2, RCZ2B.2B, RCZ2D.2, RIZ3.1, RIZ4.2, RIZ5.3 and RIZ9.3.

The efficient growth rate of the seven fungal species, *Aspergillus tubingensis*, *Mucor circinelloides*, *Cunninghamella bertholletiae*, *Simplicillium subtropicum*, *Penicillium daleae*, *Trichoderma koningiopsis* and *Purpureocillium lilacinum*, on both CAP and CB could be explained by their capabilities to utilize coal as a nutrient source for their survival in the absence of other simple carbon sources. Some of the organisms from this study identified to be capable of bioconversion of coal and its derivatives have been frequently reported as being indigenous to the coal environment. They include organisms of the genera *Penicillium*, *Mucor*, *Aspergillus*, *Cunninghamella* and *Trichoderma* [22, 70–75].

3.3 Evidence for humic acid and fulvic acid production

For the coal activity testing, the control flask (without inoculum) had a pH of 5.69, while the media for isolates RCZ2B.2, RIZ5.3, RIZ3.1, RCZ2D.2, RIZ9.3, RIZ 4.2 and RCZ2B.2B had pH values of 6.09, 5.69, 5.35, 5.20, 5.11, 4.84 and 4.50, respectively. For HA concentrations, RCZ2B.2B was the highest (118.9 mg/l) followed by RCZ2B.2 (43.9 mg/l) and RIZ5.3 (5.85 mg/l), RIZ3.1 (5.49 mg/l), RCZ2D.2 (5.00 mg/l), RIZ4.2 (3.29 mg/l) and RIZ9.3 (2.80 mg/l). Fulvic acid analyses showed high concentration values for RIZ9.3 (67.03 mg/l), RIZ4.2 (45.95 mg/l), RCZ2D.2 (42.70 mg/l) and RIZ3.1 (42.43 mg/l), but low values for RIZ5.3 (5.95 mg/l), RCZ2B.2 (5.68 mg/l) and RCZ2B.2B (2.97 mg/l) (Figs. 3 and 4).

Essentially, the chemical pretreatment of the sub-bituminous coal before the application of the fungal inoculum improved the solubilization rate of the HA obtained. A related study by Sabar et al. [76] also conducted both chemical and fungal treatment on sub-bituminous coal to obtain a good yield of HA. In this study, the coal activity of the biosolubilizers (*M. circinelloides* and *A. tubingensis*) and depolymerizers (*C. bertholletiae*, *S. subtropicum*,

Table 1 Fungal species isolated and GenBank accession numbers for their partial ITS sequences

S/N	Sample ID	Lab stock number	Probable ID	Source	GenBank accession No
1	RCZ1D.1	IYN17	<i>Aspergillus flavus</i>	<i>Synedrella nodiflora</i>	MW260075
2	RCZ1D.2	IYN18	<i>Trichoderma asperellum</i>		MW260076
3	RCZ1D.3	IYN19	<i>Trichoderma asperellum</i>		MW260077
4	RCZ1D.4	IYN20	<i>Trichoderma asperellum</i>		MW260078
5	RCZ2A.1	IYN21	<i>Trichoderma asperellum</i>	<i>Ageratum conyzoides</i>	MW260079
6	RCZ2A.2	IYN22	<i>Trichoderma asperellum</i>		MW260080
7	RCZ2A.3	IYN23	<i>Trichoderma asperellum</i>		MW260081
8	RCZ2A.4	IYN24	<i>Trichoderma asperellum</i>		MW260082
9	RCZ2B.1	IYN25	<i>Fusarium oxysporum</i>	<i>Emilia coccinea</i>	MW260083
10	RCZ2B.1B	IYN2	<i>Aspergillus flavus</i>		MW260071
11	RCZ2B.1S	IYN6	<i>Aspergillus flavus</i>		MW250191
12	RCZ2B.2	IYN26	<i>Aspergillus tubingensis</i>		MW260084
13	RCZ2B.2B	IYN13	<i>Mucor circinelloides</i>		MT421897 [85]
14	RCZ2B.2S	IYN7	<i>Aspergillus aculeatus</i>		MW250192
15	RCZ2B.3	IYN27	<i>Mucor circinelloides</i>		MW260085
16	RCZ2B.3B	IYN3	<i>Aspergillus flavus</i>		MW260072
17	RCZ2B.4	IYN28	<i>Fusarium oxysporum</i>	<i>Urena lobata</i>	MW260086
18	RCZ2C.1	IYN29	<i>Aspergillus flavus</i>		MW260087
19	RCZ2C.2	IYN30	<i>Aspergillus oryzae</i>		MW260088
20	RCZ2C.3	IYN31	<i>Aspergillus oryzae</i>		MW260089
21	RCZ2C.4	IYN32	<i>Fusarium sp.</i>	<i>Axonopus compressus</i>	MW260090
22	RCZ2D.1	IYN33	<i>Penicillium sp.</i>		MW260091
23	RCZ2D.2	IYN34	<i>Penicillium daleae</i>		MW260092
24	RCZ2D.3	IYN35	<i>Penicillium citrinum</i>		MW260093
25	RCZ2D.4	IYN36	<i>Penicillium sp.</i>		MW260094
26	RCZ2D.5	IYN37	<i>Aspergillus sp.</i>		MW260095
27	RCZ2D.S	IYN8	<i>Aspergillus oryzae</i>		MW250193
28	RIZ3.1	IYN38	<i>Trichoderma koningiopsis</i>	<i>Emilia coccinea</i>	MW260096
29	RIZ3.2	IYN39	<i>Aspergillus sp.</i>		MW260097
30	RIZ3.3	IYN40	<i>Trichoderma koningiopsis</i>		MW260098
31	RIZ3.4	IYN41	<i>Penicillium griseofulvum</i>		MW260099
32	RIZ3.5	IYN42	<i>Aspergillus sp.</i>	<i>Axonopus compressus</i>	MW260100
33	RIZ3.6	IYN43	<i>Aspergillus niger</i>		MW260101
34	RIZ4.1	IYN44	<i>Penicillium griseofulvum</i>		MW260102
35	RIZ4.1B	IYN4	<i>Aspergillus aculeatus</i>		MW260073
36	RIZ4.2	IYN45	<i>Simplicillium subtropicum</i>		MW260103
37	RIZ4.2B	IYN5	<i>Aspergillus flavus</i>		MW260074
38	RIZ4.3	IYN46	<i>Penicillium griseofulvum</i>		MW260104
39	RIZ4.4	IYN47	<i>Purpureocillium lilacinum</i>		MW260105
40	RIZ4.5	IYN48	<i>Purpureocillium lilacinum</i>	<i>Synedrella nodiflora</i>	MW260106
41	RIZ5.1	IYN49	<i>Aspergillus niger</i>		MW260107
42	RIZ5.2	IYN50	<i>Fusarium fujikuroi</i>		MW260108
43	RIZ5.3	IYN51	<i>Purpureocillium lilacinum</i>		MW260109
45	RIZ5.5	IYN15	<i>Trichoderma reesei</i>	<i>Synedrella nodiflora</i>	MT421898 [85]
46	RIZ8B.S	IYN9	<i>Fusarium oxysporum</i>	<i>Ageratum conyzoides</i>	MW250194
47	RIZ9.1	IYN53	<i>Aspergillus niger</i>	<i>Ageratum conyzoides</i>	MW260111
48	RIZ9.2	IYN54	<i>Aspergillus niger</i>		MW260112
49	RIZ9.3	IYN55	<i>Cunninghamella bertholletiae</i>		MW260113
50	RIZ9.4	IYN56	<i>Cunninghamella bertholletiae</i>		MW260114
51	RIZ9.S	IYN10	<i>Aspergillus niger</i>		MW250195

Table 1 (continued)

S/N	Sample ID	Lab stock number	Probable ID	Source	GenBank accession No
52	RIZ10.1	IYN57	<i>Fusarium sp.</i>	<i>Ageratum conyzoides</i>	MW260115
53	RIZ10.2	IYN58	<i>Fusarium oxysporum</i>		MW260116
54	RIZ10.3	IYN59	<i>Penicillium griseofulvum</i>		MW260117
55	RIZ10.4	IYN60	<i>Fusarium oxysporum</i>		MW260118
56	RIZ10.5	IYN61	<i>Fusarium solani-melongenae</i>		MW260119
57	RIZ10.6	IYN62	<i>Fusarium fujikuroi</i>		MW260120
58	RIZ11.1S	IYN11	<i>Aspergillus flavus</i>	<i>Sida acuta</i>	MW250196
59	RIZ11.2S	IYN12	<i>Aspergillus niger</i>		MW250197
60	RIZ11.3S	IYN14	<i>Trichoderma asperellum</i>		MW250198
61	RIZ11.4S	IYN16	<i>Aspergillus sp.</i>		MW250199

Fig. 1 Representative plates of fungal activity on coal agar plates (CAP) (left) and water agar plates (WAP) (right) and the plant rhizospheres from which they were isolated **A** RIZ4.2 (*Axonopus compressus*). **B** RIZ3.1 (*Emilia coccinea*). **C** RIZ5.3 (*Synedrella nodiflora*). **D**

RIZ9.3 (*Ageratum conyzoides*). **E** RCZ2D.2 (*Axonopus compressus*). **F** RCZ2B.2 from *Emilia coccinea*. **G** RCZ2B.2B (*Emilia coccinea*). **H** Uninoculated coal agar plate (CAP) and water agar plate (WAP)

P. daleae and *T. koningiopsis*) signified the potential of these organisms for bioconversion of coal to value-added products. If HA is depolymerized to FA, a reverse order in concentration is expected to confirm bioconversion capabilities of the strains. In our investigation, a corresponding increase in FA concentration was noted in the media with a decreased HA concentration in *C. bertholletiae* (HA = 2.80 mg/l, FA = 67.03 mg/l), *S. subtropicum*

(HA = 3.29 mg/l, FA = 45.95 mg/l), *P. daleae* (HA = 5.00 mg/l, FA = 42.70 mg/l) and *T. koningiopsis* (HA = 5.49 mg/l, FA = 42.43 mg/l). On the other hand, *P. lilacinum* recorded low amounts of both HA as well as FA and could not convert the bulk of the coal as evident by the deep black coloration of the medium. *P. daleae* was the only depolymerizer isolated from the control zone, while all other strains with a higher concentration of FA were isolated from the

Fig. 2 Fungal activity in glucose broth (positive control), sub-bituminous coal medium and minimal salts medium (MSM) (negative control). **A** RIZ4.2, **B** RIZ3.1, **C** RIZ5.3, **D** RIZ9.3, **E** RCZ2D.2, **F** RCZ2B.2, **G** RCZ2B.2B

Fig. 3 Biosolubilization activity of seven most active strains. **A** RIZ4.2 (*Axonopus compressus*). **B** RIZ3.1 (*Emilia coccinea*). **C** RIZ5.3 (*Synedrella nodiflora*). **D** RIZ9.3 from (*Ageratum conyzoides*). **E** RCZ2D.2 (*Axonopus compressus*). **F** RCZ2B.2 (*Emilia coccinea*). **G** RCZ2B.2B (*Emilia coccinea*). **H** Control

coal dump site. The best solubilizer, *Mucor circinelloides*, was also isolated from the control zone.

The pH values of the controls were unchanged after solubilization. The pH of the medium inoculated with *Aspergillus tubingensis* increased, and this is most likely due to the secretion of alkaline substances, a requirement for the effective solubilization of coal and its derivatives [77,

Fig. 4 Amounts of humic acid and fulvic acid produced by the seven candidate coal degraders due to biosolubilization and depolymerization, respectively

78]. There was a decrease in the pH of all other inoculated media with a maximum reduction in pH from 5.69 to 4.50 in *M. circinelloides*. In the other flasks with a decreased pH, other mechanisms apart from increasing alkalinity might be responsible for the coal solubilization by these microbes [68]. One of which is that the increased acidity in the broth could be attributed to the formation of acidic metabolites during the conversion process. In line with the above findings, Quigley et al. [79] reported a direct relationship between alkaline solubility and biosolubilization of lignite, whereby the pH of the alkaline buffer was found to have decreased from its original value due

to the formation of acidic compounds during coal solubilization. In another related study on the solubilization of Neyveli lignite by *Aspergillus fumigatus* and *Fusarium udum* into HA, Tripathi et al. [74] documented a reduction in pH from 7.5 to 6.0; an increase in acidity in the medium had the maximum yield of HA. It has been reported that coal solubilization precedes depolymerization of the released HA to FA [80].

3.4 Molecular identification, phylogenetic and taxonomic analyses of isolates

An amplicon size of 500–600 bp representing the ITS 1 and 2 regions of the fungal isolates was observed (Fig. S3). The isolates identified in this study either belong to the Ascomycetes or Zygomycetes, and their corresponding ITS sequences have been deposited in NCBI GenBank under various accession numbers MW260071–MW260120, MW250191, MT421897 and MT421898 (Table 1). The isolates used for biotransformation experiments were determined to be RCZ2B.2 (*Aspergillus tubingensis*), RCZ2B.2B/IYN 13 (*Mucor circinelloides*), RCZ2D.2 (*Penicillium daleae*), RIZ3.1 (*Trichoderma koningiopsis*), RIZ4.2 (*Simplicillium subtropicum*), RIZ5.3 (*Purpureocillium lilacinum*) and RIZ9.3 (*Cunninghamella bertholletiae*) (Table 1).

The phylogenetic relationship among the coal solubilizers was plotted, and the isolates with coal utilization attributes were members of Ascomycota and Zygomycota and each formed a clade of its own (Fig. 5, Table 1).

3.4.1 Amplification of laccase gene

Laccase gene was amplified in all the depolymerizers *Trichoderma koningiopsis* (RIZ3.1), *Penicillium daleae* (RCZ2D.2), *Simplicillium subtropicum* (RIZ4.2) and *Purpureocillium lilacinum* (RIZ5.3) except *Cunninghamella bertholletiae* (RIZ9.3). Conversely, laccase gene was not amplified in the solubilizers, *Aspergillus tubingensis* and *Mucor circinelloides*.

The amplification of laccase gene in *T. koningiopsis*, *P. daleae* and *S. subtropicum* indicates that its presence in these strains is suggestive of a role for laccase in their depolymerization of humic acid. It was therefore not surprising that strains with higher FA and lower HA were found to have laccase, hinting that the enzyme might be

responsible for catalyzing the depolymerization of HA to FA. The absence of laccase gene in *C. bertholletiae* (RIZ9.3) was expected as laccase activity has not been found in the species [81, 82]. From our findings, it appears that the depolymerization observed is not solely linked to laccase activity. *C. bertholletiae* might be employing another oxidative/ligninolytic enzyme other than laccase for its depolymerization activity. Similarly, the absence of laccase in the two efficient biosolubilizers from this investigation, *A. tubingensis* and *M. circinelloides*, could imply the exploitation of another ligninase or that a lignin-modifying enzyme might not be required for the process.

4 Conclusion

The culture methods of isolation of the fungi from soils in the root zone of the plants employed in this study are limited. Essentially, they provided a peek into the types of microorganisms in the rhizospheres of these plants but appreciably, an immense number of fungi would have been missed during cultivation. To obtain a comprehensive overview of the fungi present and how they relate in driving the metabolic activities, it would be necessary to investigate the full identities of the members of the fungal communities in this environment and their structure in the rhizosphere soils using the omics approaches. It will also be important to understand other enzyme mechanisms, in addition to laccase that aid these identified fungi from the Ascomycetes and Zygomycetes in their catalysis of coal biotransformation.

The following sub-bituminous coal biodegraders *Aspergillus tubingensis*, *Mucor circinelloides*, *Simplicillium subtropicum*, *Penicillium daleae*, *Trichoderma koningiopsis* and *Cunninghamella bertholletiae* were identified in this study. These isolates can be assembled to form a biocatalytic consortium for degradation of sub-bituminous coal to value-added products in the environment as well as in agro-industrial projects. In general, both humic acid and fulvic acid have agricultural roles and medicinal uses. Therefore, sub-bituminous coal could serve as a valuable raw material for the extraction of humic acid for the production of value-added products like ceramic dispersants [83] and as a soil conditioner [84].

Fig. 5 Neighbor-joining phylogenetic tree of ten representative OTUs of the isolates with coal activity and their relatives. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. This analysis involved 61 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 154 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Acknowledgements We thank Dr. Nwanneka Marie Akinyemi and Mr. Ayodeji Adebola Odunsi of the Department of Microbiology, University of Lagos, for their participation in the studies. We also express our immense gratitude to the Agricultural Research Council (ARC)—Soil, Climate and Water, South Africa, for unhindered access to laboratory facilities during this research as well as to the members of its Environmental Microbiology and Biotechnology Research Group for all the assistance provided.

Authors' contributions Conceptualization was done by Imade Yolanda Nsa and Eric Egbe Igbinigie; Methodology was done by Imade Y. Nsa, Busayo Tosin Akinyemi, Maryam Bello-Akinosho, Eric Egbe Igbinigie and Rasheed Adegbola Adeleke; Formal analysis and investigation were carried out by Imade Yolanda Nsa, Busayo Tosin Akinyemi, Maryam Bello-Akinosho, Simon Nnamdi Ezechukwu, Tomisin Bridget Bayode, Eric Egbe Igbinigie and Rasheed Adegbola Adeleke; Writing—original draft preparation were done by Imade Yolanda Nsa, Busayo Tosin Akinyemi, Maryam Bello-Akinosho, Simon Nnamdi Ezechukwu, Tomisin Bridget Bayode, Eric Egbe Igbinigie and Rasheed Adegbola Adeleke; Writing—review and editing were done by Imade Yolanda Nsa, Maryam Bello-Akinosho, Eric Egbe Igbinigie and Rasheed Adegbola Adeleke; Funding acquisition was done by Imade Y. Nsa and Rasheed Adeleke; Resources were done by Imade Y. Nsa, Busayo Tosin Akinyemi, Maryam Bello-Akinosho, Simon Nnamdi Ezechukwu, Tomisin Bridget Bayode, Eric Egbe Igbinigie and Rasheed Adeleke; Supervision was done by Imade Yolanda Nsa, and Rasheed Adeleke.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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