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ORIGINAL PAPER

Degradation of low rank coal by *Trichoderma atroviride* ES11

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Abstract A new isolate of *Trichoderma atroviride* has been shown to grow on low rank coal as the sole carbon source. *T. atroviride* ES11 degrades ~82% of particulate coal (10 g l^{-1}) over a period of 21 days with 50% reduction in 6 days. Glucose (5 g l^{-1}) as a supplemented carbon source enhanced the coal solubilisation efficiency of *T. atroviride* ES11, while 10 and 20 g l^{-1} glucose decrease coal solubilisation efficiency. Addition of nitrogen [1 g l^{-1} $(NH_4)_2SO_4$] to the medium also increased the coal solubilisation efficiency of *T. atroviride* ES11. Assay results from coal-free and coal-supplemented cultures suggested that several intracellular enzymes are possibly involved in coal depolymerisation processes some of which are constitutive (phenol hydroxylase) and others that were activated or induced in the presence of coal (2,3-dihydrobiphenyl-2,3-diol dehydrogenase, 3,4-dihydro phenanthrene-3,4-diol dehydrogenase, 1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase, 1,2-dihydro-1,2-dihydroxyanthracene dehydrogenase). GC-MS analysis of chloroform extracts obtained from coal degrading *T. atroviride* ES11 cultures showed the formation of only a limited number of specific compounds (4-hydroxyphenylethanol, 1,2-benzenediol, 2-octenoic acid), strongly suggesting that the intimate association between coal particles and fungal mycelia results in rapid and near-quantitative transfer of coal depolymerisation products into the cell.

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Introduction

Previous studies have shown that certain fungi and bacteria are able to degrade lignites and sub-bituminous coals [1–5]. However, the mechanisms of coal solubilisation are not fully understood.

Coal biodegradation is thought to involve a complex array of constituents and processes involving hydrolytic enzymes [6], oxidative enzymes [7], acidic/alkaline substances [8], chelating agents [9], and surfactants [10]. It is generally believed that oxidative enzymes are the primary factors in coal depolymerisation [11, 12]. However, the exact pathway involved in coal degradation is dependent both on the strain used and the type of coal.

Interest in coal solubilization is largely stimulated by the prospect of recovering coal derived organic intermediates [13] as chemical products for the fine chemical entities [14]. Several strategies have been developed in attempts to enhance the yield of coal biosolubilisation products, including the use of cell-free extracts [15], and the addition of chelating agents [9, 16] or alkali [8]. The generation of coal derived-organic intermediates is thought to involve one or more of the following: depolymerisation of aromatic structures which are linked by aliphatic and ether bridges [17], oxidative and decarboxylative changes to the monomers/oligomers, removal of sulfur, nitrogen and/or metals [18] and ring cleavage reactions. However, the fact that previous mechanistic studies on coal degradation have used numerous different microbial species [19] and coal types makes it difficult to establish valid comparisons.

Here we report the process and products of low rank coal solubilisation by a novel and highly active fungal strain *Trichoderma atroviride* ES11.

Materials and methods

Culture conditions

Trichoderma atroviride ES11 was isolated from soil in the Western Cape, South Africa by direct culturing on minimal salts medium agar plates containing 1% particulate low rank coal (from SASOL South Africa, 2001) as a sole C source. Minimal salts composition (per litre): 1 g $NH_4(SO_4)$, 0.52 g $MgSO_4{\cdot}7H_2O$, 5 g KH_2PO_4, 0.005 g $FeSO_4{\cdot}7H_2O$; 0.003 g $ZnSO_4{\cdot}7H_2O$, pH 5.5. Pure fungal mycelia were sub-cultured onto 3% (m/v) malt extract agar. The medium for coal solubilisation was minimal salts medium supplemented with 0.3% malt extract, pH 5.5. Ground sub-bituminous coal (~500 mm average particle size) was autoclaved separately and all other reagents were filtered (0.22 μM) and added at the time of inoculation.

Five hundred ml flasks were inoculated with four agar plugs of active fungal mycelium. Flasks were incubated at 28 °C on a rotary shaker at 170 rpm for up to 21 days. Non-inoculated media with coal and inoculated media without coal were used as controls. Glass beads (6 mm) were added to the coal-containing media to assist in fragmentation of the fungal mycelium.

During growth experiments, aliquots were removed at regular time intervals under aseptic conditions, centrifuged and tested for coal solubilisation, specific enzyme activities and organic products. Fungal growth was quantified by washing the mycelial fractions with distilled water and drying to a constant weight at 85 °C. All determinations were performed in duplicate. Coal degradation was determined by separating fungal mycelium from coal particles and gravimetric analysis of the dried coal.

Coal

Sub-bituminous coal obtained locally was used throughout this study. Coal samples were fragmented by grinding and sieving to a particle range size of 0.2–0.5 mm and autoclaved at 120 °C for 20 min. No further pretreatment was performed.

DNA extraction

DNA extraction was performed according to Carlson et al. [20]. Mycelial fragments were lysed in microcentrifuge tubes containing three glass beads (6 mm diameter) and 100 mg of silica, after the addition of 1 ml of lysis buffer (100 mM Tris–Cl, pH 9, 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 1% PEG 8000 or 6000) and 2 μl mercaptoethanol. The tubes were processed on the Fast Prep bead beater (Bio 101, Savant) for 1 min followed by incubation at 74 °C for 30 min and centrifugation at 10,000×*g* for 10 min. The supernatants were transferred to new tubes with 800 μl of chloroform:isoamylalcohol (24:1), mixed by inversion for 2 min and then centrifuged at 10,000×*g* for 10 min. The upper phase was transferred to a clean tube and an equal volume of isopropanol added. DNA was precipitated by centrifugation at 16,000×*g* for 15 min. The pellets were rinsed with 250 μl 70% ethanol (at −20 °C), centrifuged again at 16,000×*g* for 15 min and resuspended into 50 μl of 100 mM Tris–HCl pH 8.5 containing 10 mM EDTA/RNAse (0.2 μg ml^{-1}).

Fungal ITS sequence PCR amplification

The taxonomy of strain ES11 was evaluated by phylogenetic analysis of the ITS internal region [21]. Universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) targeting the 3′ and 5′ end of the IT sequence were used for PCR. Reactions were performed in 50 μl containing 1× NEB Buffer (10 × 0.2 M Tris–HCl pH 8.8, 0.1 M KCl, 0.1 M $(NH_4)_2SO_4$, 20 mM $MgSO_4$ and 1% Triton), 1 mM dNTP, 5 μM of each primer, 0.5 μl recombinant *Taq* DNA polymerase and 1 μl template DNA. The PCR conditions were as follows: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 10 min. The confirmation of PCR products was done by agarose gel electrophoresis. Specific PCR products were purified using a GFX™ PCR kit according to the manufacturer's instructions (Amersham). Purified DNA was sequenced by fluorescent cycle sequencing (Big Dye version 3, MegaBase, Amersham) using ITS1/ITS4 primer pair. Chromatograms were analysed and edited using BioEdit version 5.0.9 [22] and the Genbank database was searched for sequence similarities using NCBI BLASTn.

Extraction of organic products

Supernatants from 11-day cultures (150 ml) were acidified with 11.6 M HCl and mixed with 100 ml of chloroform. The organic phase was evaporated to 2 ml and aliquots were analyzed by GC-MS.

Gas chromatography-mass spectrometry (GC-MS)

Extracted samples as indicated above were derivatized with *N*-methyl-*N*-(trimethylsilyl)-trifluoracetamide (MSTFA) [23] by addition of 200 μl MSTFA and 50 μl of pyridine to 300 μl of sample. The mixture was incubated for 1 h at 85 °C in a sealed glass reaction tube.

Silylated samples were analyzed directly by GC-MS, using a Finnigan-Matt GCQ equipped with a 30 m × 0.25 mm × 0.25 µm J&W DB-5MS capillary columns. The following oven program was used: temperature was maintained at 60 °C for 4 min, raised to 270 °C at 20 °C/min, maintained for 3 min and then further raised to 310 °C at 15 °C/min for 20.17 min. The He carrier gas (research grade 99.95% purity) was used at a flow rate of 1 ml/min. A sample volume of 5 µl was injected. The injector and the transfer lines were maintained at 275 °C. The Mass Selective Detector was operated in the Electron Ionization mode according to the manufacturer's recommendations in the full scan range of 40 to 950 *m/z*.

Enzyme assays

All enzymes assays on intracellular extracts were performed in duplicate at room temperature. Where error bars are not shown, the standard error of duplicates was smaller than the size of the symbol.

Phenol hydroxylase

Phenyl hydroxylase activity was assayed according to the method of Gurujeyalakshmi and Oriel [24]. The assay mixture (1 ml) contained 50 mM potassium phosphate buffer pH 7.2, 1.0 mM NADH and 100 µM phenol. 100 µl of cell extract was added to the solution to initiate the reaction. The solution was incubated for 15 min at room temperature and the reaction stopped by the addition of 12 µl of 2% 4-aminoantipyrine (in 0.25 M $NaHCO_3$) and 40 µl of 2% potassium ferricyanide (in 0.25 M $NaHCO_3$). After 15 min incubation, the absorbance at 510 nm was recorded against non-enzymic control samples. One unit of enzyme activity was defined as 1 µmol of phenol converted per min.

Catechol-2,3-dioxygenase and catechol-1,2-oxygenase

Both assays were performed according to the method of Ali et al. [25]. Reaction mixtures (1 ml) in 50 mM potassium phosphate buffer pH 7.2 contained 1 µmol catechol and 100 µl of cell extract. The increase in absorbance at 375 nm, caused by the formation of the reaction product 2-hydroxymuconic semialdehyde ($\varepsilon_M = 4.4 \times 10^4$ mol^{-1} 1 cm), was monitored. For catechol-1,2-oxygenase the formation of *cis*-muconic acid was monitored at 260 nm ($\varepsilon_M = 1.75 \times 10^3$ mol^{-1} 1 cm).

9-Fluorenol dehydrogenase

Enzyme activity was assayed according to the method of Trenz et al. [26]. The solution contained 50 mM potassium phosphate buffer pH 7.2, 0.5 mM 9-fluorenol and 2.7 mM NAD^+ in a final volume of 1 ml. The reaction was initiated with the addition of 100 µl of cell extract and absorbance monitored at 240 nm. One unit of activity was defined as the amount of enzyme required to reduce 1.0 µmol of NAD^+ per min.

2,3-dihydrobiphenyl-2,3-diol dehydrogenase, 3,4-dihydro phenanthrene-3,4-diol dehydrogenase, 1,2-dihydro-1, 2-dihydroxynaphthalene dehydrogenase and 1,2-dihydro-1,2-dihydroxyanthracene dehydrogenase

These assays were performed according to the method of Patel and Gibson [27] by measuring absorbance increases at 340 nm. Reaction mixtures contained 50 mM potassium phosphate buffer pH 7.2, 60 µM biphenyl, 100 µM phenanthrene, 100 µM naphthalene or 100 µM anthracene respectively, and 2.7 mM NAD^+ in a final volume of 1 ml. Reactions were initiated with the addition of 100 µl of cell extract. One enzyme unit was defined as the amount of enzyme required to reduce 1.0 µmol of NAD^+ per min.

Results and discussion

PCR amplification of purified ES11 genomic DNA using ITS primers resulted in the expected 600 bp product. BLAST analysis of amplicon sequence showed high homology values [99% (530 bp)] with the sequence from *T. atroviride* T221 (AY585878).

Previous studies have shown that *T. atroviride* can solubilise coal [6, 28]. We have demonstrated that isolate ES11 of this species was capable of degrading low rank coal in liquid culture at up to 100 g l^{-1} loading (data not shown). In optimising the process of coal solubilization, we modified the basal medium by addition of various C and N sources. Addition of glucose at 5 g l^{-1} as a supplementary carbon source enhanced both the rate and degree of coal degradation (Figs. 1, 2). However at 10 and 20 g l^{-1} glucose, the efficiency of coal solubilisation was decreased (Fig. 1). This result is contrary to the findings of Gokcay et al. [29] who reported that a high concentration of glucose (10 and 20 g l^{-1}) was necessary for coal solubilisation. Time course experiments under different culture conditions showed that *T. atroviride* ES11 degraded ~82% coal (10 g l^{-1}) in malt extract glucose media over a period of 21 days, with 50% reduction after 6 days of incubation. Coal solubilisation efficiency was decreased when coal was used as a sole carbon source (~33%) (Fig. 2). When coal was added only after 3 days of incubation on glucose (in order to induce enzymes that might be repressed in the presence of glucose), only ~40% coal solubilisation was observed (data not shown). Neither galactose nor xylose (at 5 g l^{-1}) was as effective as glucose in stimulating coal

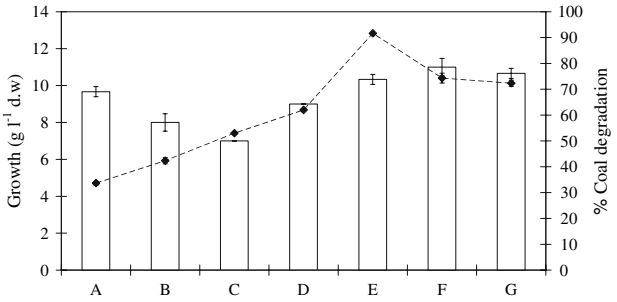

Fig. 1 Effect of glucose concentration in *T. atroviride* ES 11 cultures on coal degradation (*filled diamond*) % coal degradation, and biomass yield (*bars*) g dry wt l^{-1}. Media contained 10 g l^{-1} coal supplemented with glucose (g l^{-1}): (*A*) 0, (*B*) 0.5, (*C*) 1.0, (*D*) 3.0, (*E*) 5.0, (*F*) 10, (*G*) 20

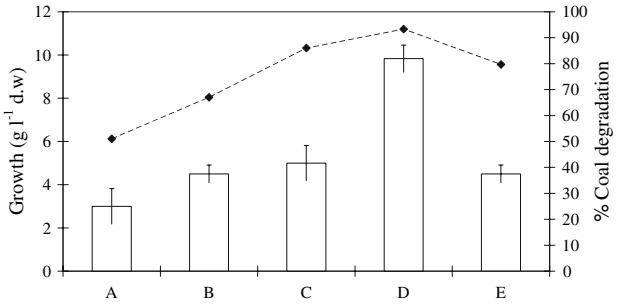

Fig. 3 Effect of nitrogen concentration (as g l^{-1} ammonium sulphate) on growth (*bars*) g l^{-1} dry weight and coal degradation (*filled diamond*), % coal degradation in *T. atroviride* ES11 cultures. (*A*) 0, (*B*) 0.1, (*C*) 0.5, (*D*) 1.0, (*E*) 1.5

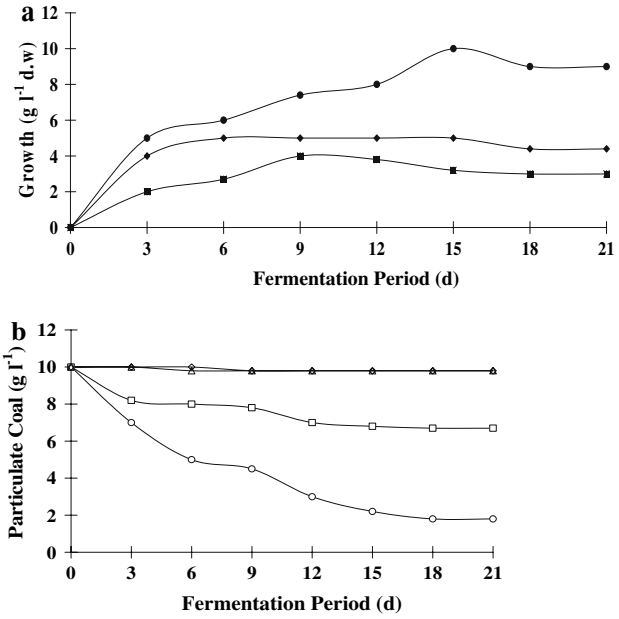

Fig. 2 Biomass yield and coal degradation for *T. atroviride* ES11 shake flask experiments. Fungal mycelia were grown at 28°C in 3% malt extract medium until exponential growth was achieved. This culture was used to inoculate malt extract medium containing coal (10 g l^{-1}; *filled box*) or coal plus glucose (5 g l^{-1}; *filled circle*). In the biomass yield control flask fungal mycelia were grown in medium supplemented with glucose (*open diamond*). Controls for coal degradation analysis consisted of growth medium containing coal (*open triangle*) or coal plus glucose (*open box*), incubated without mycelium inoculation. Biomass yield **a** and coal degradation **b** were measured over a period of 21 days. Samples were taken after every three days

solubilisation (68 and 64% respectively: cf. 93% for glucose, under otherwise identical conditions).

Several studies have demonstrated that high nitrogen and pH levels in growth media enhance coal solubilisation in *T. atroviride* and *Fusarium oxysporum* [28, 30]. Solubilisation is typically attributed to the action of alkaline metabolites produced under these conditions [8]. Low rank coal has a low N content (1.74%) [31] and cultures growing on high concentrations of low rank coal might be expected to become N limited. Conversely, ligninolytic enzymes are optimally induced under low nitrogen levels [5]. The present study demonstrated (Fig. 3) that supplementation with inorganic nitrogen up to 1 g l^{-1} resulted in significant increases in both fungal growth and coal solubilisation.

The enzymology of coal solubilisation is not well understood. Various extracellular enzyme activities, including esterases and oxidases, have been frequently implicated in the depolymerisation process [32, 33]. However, when supernatants from active coal-grown cultures were added to fresh particulate coal (at 5 g l^{-1}), only 10% reduction in coal dry weight was observed after an incubation of 6 days (data not shown). In parallel experiments using autoclaved culture supernatants, no reduction in coal mass was observed, suggesting that extracellular enzymes might be involved in partial coal solubilisation but that the presence of a mycelial mass is a requirement for effective coal solubilisation.

We also monitored the activities of a range of intracellular and extracellular enzymes that are implicated in depolymerisation processes, including oxidative depolymerisation, aromatic ring activation and ring cleavage [34, 35]. Assay results from coal-free and coal-supplemented cultures (Fig. 4a–g) suggested that the intracellular enzymes tested fell into three separate response groups: (1) those that were apparently constitutive (phenol hydroxylase), (2) those where the activity was reduced in the presence of coal (catechol-1,2-dioxygenase; fluorenol dehydrogenase) and (3) those that were activated or induced in the presence of coal (2,3-dihydrobiphenyl-2,3-diol dehydrogenase, 3,4-dihydro phenanthrene-3,4-diol dehydrogenase, 1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase, 1,2-dihydro-1,2-dihydroxyanthracene dehydrogenase). No catechol-2, 3-dioxygenase activity was detectable in any culture.

Most of the enzymes in group (3) use aromatic compounds as substrates, oxidizing both non-phenolic and phenolic rings [36]. The apparent induction of these activities is consistent with the hypothesis that they play an active role in the intracellular degradation of coal-derived aromatic compounds.

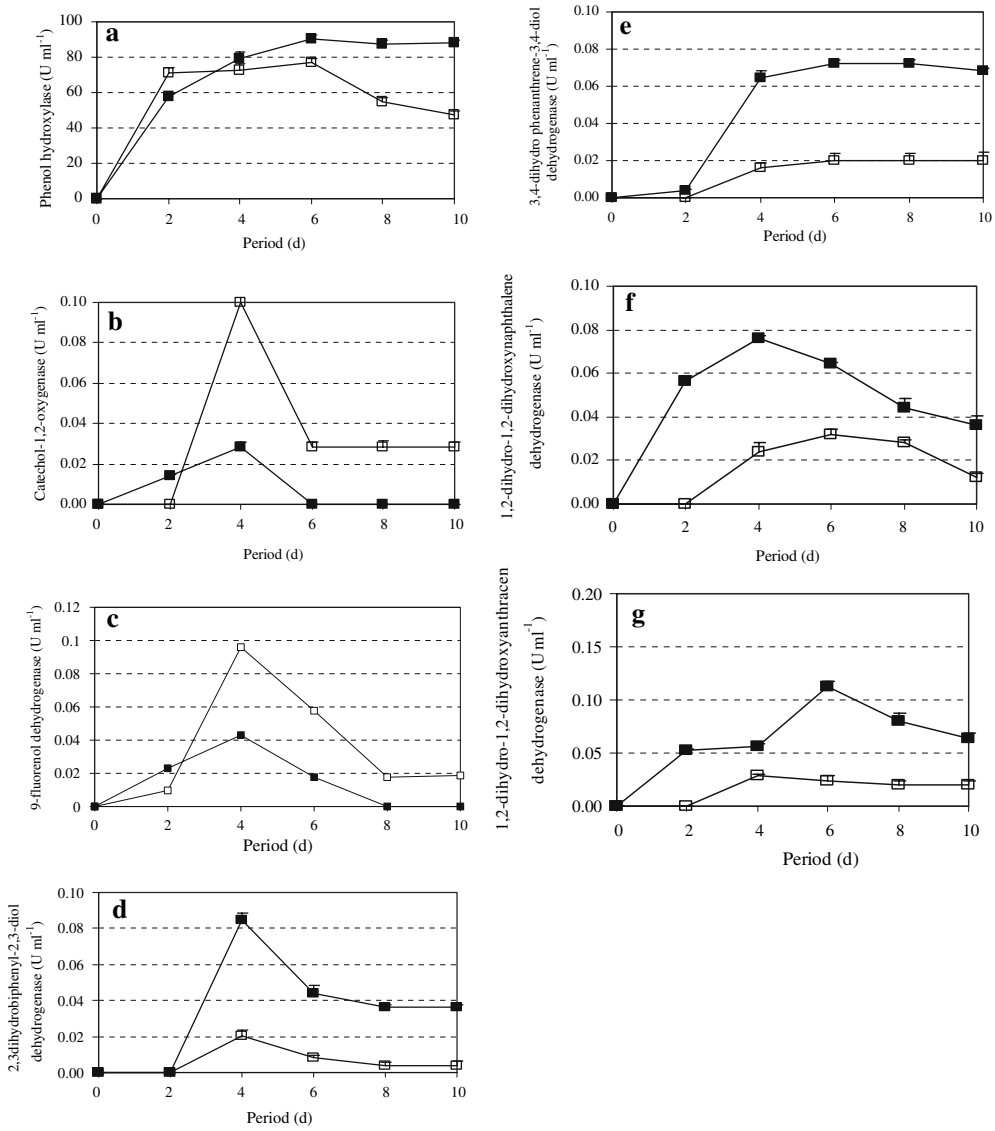

Fig. 4 Intracellular enzyme activities in coal-supplemented (*filled box*) and coal free (*open box*) *T. atroviride* ES 11 cultures: **a** phenol hydroxylase, **b** catechol-1,2-oxygenase, **c** 9-fluorenol dehydrogenase, **d** 2,3-dihydrobiphenyl-2,3-diol dehydrogenase, **e** 3,4-dihydro phenanthrene-3,4-diol dehydrogenase, **f** 1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase, **g** 1,2-dihydro-1,2-dihydroxyanthracene dehydrogenase

The induction of dehydrogenase enzymes reported here indicates that the coal structure was depolymerised by metabolic processes corresponding to the degradation pathways typically acting on polyaromatic hydrocarbon (PAH) compounds. The metabolism of polyaromatic hydrocarbon-related compounds is generally initiated by catechol dioxygenase activity, converting the subtrates to *cis*-dihydrodiols, followed by re-aromatisation catalysed by a series of dehydrogenases [37]. In the coal degradation system, catechol-1,2-dioxygenase would be capable of the primary hydroxylation/de-aromatisation step. The evidence of induction of a set of polyaromatic dehydrogenases supports the hypothesis that the coal—catechol dioxygenase products would be further metabolized by re-aromatisation and ring cleavage, yielding simpler compounds suitable for further metabolic processing as nutrient carbon.

GC-MS analysis of chloroform extracts obtained from coal degrading *T. atroviride* ES11 cultures revealed a limited number of compounds. All samples were derivatized using MSTFA, which results in TMS-ethers and esters with typical mass ion fragment at M-15 corresponding to the loss of a $-CH_3$ group from the molar ion, together with other characteristic signals for MSTFA derivatization [*m/z* 73 $[(CH_3)_3Si]^+$, *m/z* 75 $[OH = Si(CH_3)_2]^+$ and *m/z* 147 $[(CH_3)_2Si = O\text{-}Si(CH_3)_3]^+$ [38]]. The *m/z* values for the base peaks measured in the mass spectra for all the compounds are shown in Table 1. The compounds putatively identified by mass spectrometry (4-hydroxyphenylethanol,

Table 1 Characteristic fragment ions of MSTFA derivatives from cultures of *T. atroviride* ES 11

GC retention time (min)	Fragment ion (*m/z*)	Putative compound[a]
11:27	73 (100%), 179, 201, 282 (M^+)	4-Hydroxyphenylethanol
23:17	73 (100%), 107, 169, 254 (M^+)	1,2-Benzenediol
32:43	73, 109, 131, 161, 199 (100%), 279, 301 (M^+)	2-Octenoic acid

All compounds shown above were found to be present in coal-supplemented cultures, but absent from coal-free control cultures

[a] Identified as TMS-derivatives

1,2-benzenediol, 2-octenoic acid) are of structural classes expected from coal depolymerisation and secondary oxidative reactions.

A notable difference between these results and those reported by other researchers [13] is the limited diversity of aromatic compounds. We attribute this to the physical association of the coal particles with the fungal mycelium that prevents accumulation of coal-derived depolymerisation products in the culture medium. We suggest that the products of extracellular depolymerisation/solubilisation of the coal matrix are absorbed and metabolized by the fungal mycelium without significant release to the bulk medium. This is consistent with the observed induction of intracellular aromatic ring cleavage enzymes such as phenanthrene dehydrogenase and naphthalene dehydrogenase and the effective growth of *T. atroviride* on low rank coal as a sole C source. Although a full carbon mass balance has not yet been established, it is evident that the primary fate of coal is biomass and CO_2.

It is also evident that the engineering of this strain such that coal derived organic compounds are accumulated in the bulk medium may not be trivial. Firstly, it is not clear as to whether coal-derived aromatic polymers are degraded to monomers prior to uptake. The low titres of monomeric coal derivatives in the bulk medium suggests that oligomeric structures are rapidly translocated into the cell to be further metabolised in the intracellular environment. The extracellular accumulation of monomeric aromatics might therefore require both a reduction in the rate of intracellular secondary metabolic processes, and/or the re-export of the coal-derived organic monomers.

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