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Biotransformation of coal linked to nitrification

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ABSTRACT

The microbial transformation of coal into methane for energy production has gathered considerable interest in the past decade. Bacteria and fungi have been shown to aerobically depolymerise and solubilise coal, the products of which can feed fermentative and methanogenic processes. Coal generally exists in subsurface environments that are low in nutrients and oxygen, which slows down the biological breakdown of the inherently recalcitrant coal. This study therefore aimed to understand how the addition of nutrients and oxygen to microbial communities influences coal degradation. Aerobic microbial cultures were set up in the laboratory and were followed over time for the production of coal-derived hydrocarbons, CO_2 and nitrate. Microbial activity in the presence of coal was evident from the increased production of CO_2 in cultures. After approximately 100 days, nitrification processes were also observed. Hydrocarbons derived from coal were below detectable limits, but apparently sufficient to sustain a community of putative hydrocarbon-degrading heterotrophs and autotrophic, nitrifying bacteria as shown by 16S rRNA-gene based community analysis. Our data suggest an interaction between nitrification and coal biodegradation processes, which has implications for engineered coal degradation through biostimulation.
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1. Introduction

The past decade has seen an increased interest around the world towards the conversion of coal to methane as an alternative source of energy to coal combustion (Faiz and Hendry, 2006; GeoScience_Australia, 2012; Papendick et al., 2011). Methane exists naturally in coal seams and has been produced in the past thermogenically by geological processes and/or biologically by metabolic activities carried out by fungi, bacteria and archaea (Strąpoć et al., 2011; Ulrich and Bower, 2008). Over time methane has accumulated in coal seams, from which it can be extracted by conventional drilling methods. While these historic reserves are limited, coal seams still harbour the potential for new methane production by stimulating and enhancing biological coal conversion and methanogenesis (Strąpoć et al., 2011). With growing demand for natural gas, this *de novo* biogenic methane production in otherwise non-exploitable coal fields, such as current or abandoned drilling wells or mines, has gained considerable interest (Opara et al., 2012).

Biogenic production of methane from coal is thought to be mediated by a multi-step anaerobic process involving complex communities of microorganisms (Strąpoć et al., 2011). In the initial step, coal is fragmented to release aliphatic and aromatic compounds, which are then fermented to yield electron donors, such as acetate or hydrogen, that are finally used by methanogenic archaea. Depending on its rank, coal contains a complex mixture of organic molecules that are slow or even recalcitrant to break down (Fakoussa and Hofrichter, 1999). In situ coal exists mostly below the surface and therefore oxygen is generally not available or limited for chemical oxidation of coal's constituent hydrocarbons or to support the growth of aerobic, coal-degrading microorganisms that could perform enzymatic oxidation (Kirk and Farrell, 1987). In addition, subsurface environments often lack adequate nutrient supply (e.g. organic or inorganic nitrogen or phosphate) to support microbial growth (Jones et al., 2010). To overcome this limitation, some success has been achieved by enhancing the anaerobic coal breakdown by the addition of nutrients (Jones et al., 2010). Treatments with strong chemical oxidants, such as potassium permanganate, have also been used for the initial break down of subbituminous coals (Huang et al., 2013); however this may be difficult to apply in the field due to detrimental side-effects of strong oxidants on subsequent microbial process (Huang et al., 2013).

The conceptual model for coal to methane conversion developed by Strąpoć et al. (2008) considers the initial attack of the coal macromolecule as the slow and rate-limiting step. In recent years, research has focused on the development of anaerobic, mixed cultures able to produce methane from coal by adding nutrients such as ammonium chloride or ammonium phosphate (Jones et al., 2010; Ulrich and Bower, 2008). However, little has been reported on the activities of mixed microbial communities under aerobic conditions. Aerobic biodegradation of coal itself has been studied for many decades, however only on single, cultured microorganisms (reviewed in Fakoussa and Hofrichter (1999); Strąpoć et al. (2011)), and this has revealed a variety of degradation mechanisms, including enzymatic attack, metal chelation (Fakoussa and Hofrichter, 1999) and surfactant production (Breckenridge and Polman, 1994; Fakoussa and Hofrichter, 1999). Some studies have applied aerobic bioreactor technologies to investigate the solubilisation of different ranks of coal, including hard coal (Hölker and Höfer, 2002;

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Igbinigie et al., 2008; Oboirien et al., 2008). However, these have involved the use of single strains, particularly fungal strains, as solubilisation agents, as opposed to mixed cultures. A study by Achten et al. (2011) used aerobic coal slurries with a soil inoculum (i.e. a mixed community) to determine the bioavailability of polyaromatic hydrocarbons adsorbed to coal. However, these authors did not explore the bioconversion of coal or the inoculum's microbial community composition. Only one study has recently reported the use of aerobic degradation of coal by a fungal strain (*Penicillium chrysogenum* MW1) as a pretreatment for the eventual production of methane from grown fungal biomass and degradation intermediates (Haider et al., 2013). In addition, the combination of oxygen and nutrient treatment of coal to biostimulate microbial hydrocarbon degradation has been claimed for commercial purposes of methane production (e.g. US patent 6,543,535). However, it is currently not known how microbial communities on coal would respond to an addition of oxygen and nutrients and how this would impact coal utilisation. In this work, we therefore established microbial cultures containing coal and supplemented with nutrients and oxygen to study the metabolic processes they perform and their microbial community composition.

2. Materials and methods

2.1. Culturing setup

Sub-bituminous coal was sourced from a freshly drilled core from the 80 m deep Lithgow seam at the abandoned Lithgow State Coal Mine in New South Wales, Australia. The coal samples were collected from the core immediately after being extracted from the ground. Samples were collected aseptically and with sterilised implements. Coal was anaerobically transported to the laboratory and pulverised with a titanium ring mill to generate a powder with an average particle size of 60 μm.

Surface soil containing small coal pieces found in a coke oven at the abandoned oil shale processing plant in Newnes (New South Wales, Australia) was used as a microbial inoculum. This soil sample was chosen as inoculum as it likely contained aerobic microorganisms that had been in contact with coal. All samples were taken under permit EK7543 issued by the Department of Industry and Investment of the New South Wales State Government.

Microbial cells were collected from the soil as follows: three grams of soil was mixed with 50 ml modified M9 medium (0.171 g/l Na_2HPO_4, 1.372 g/l NaH_2PO_4–$2H_2O$, 0.5 g/l NaCl, 2 g/l NH_4Cl, 0.241 g/l anhydrous $MgSO_4$ and 0.011 g/l $CaCl_2$), vortexed for 5 min and centrifuged at 50 ×*g* for 4 min. The supernatant containing the dislodged cells was transferred to a collection bottle. The pelleted material was resuspended in 10 ml fresh medium, vortexed and centrifuged as above and the supernatant was collected. The pellet was once more resuspended in 10 ml fresh medium and sonicated first for 30 s and then 2 min at 100 W in an ultrasonic bath (Unisonics Australia) with removal and pooling of the supernatant between ultrasonic treatments into the same collection tube. The supernatants of the two vortex/centrifugation treatments and the two sonication treatments were combined, and 10 ml was inoculated into 160 ml glass serum bottles containing 90 ml of modified M9 medium supplemented with 1% trace elements solution (Kirk et al., 1986) and 20 mg/ml pulverised coal.

The following cultures were set up: 1) coal, but no inoculum, to understand the response of microorganisms indigenous to the coal, 2) coal and inoculum, to understand, if aerobic soil microorganisms can facilitate coal degradation 3) no coal, but inoculum, to measure metabolic activities from the inoculum that are independent of coal and 4) autoclaved cultures with coal and medium, to define abiotic processes. Cultures were set up in triplicate. All serum bottles were sealed and crimped to allow for the measurement of gases in the headspace. The headspace (60 ml) contained 40 ml of air and 20 ml of pure oxygen filtered through a 0.22 μm syringe filter, that is approximately 47.3% oxygen. Cultures were incubated statically in the dark at 22 °C.

2.2. CO_2, nitrate and pH measurements

To monitor microbial respiration, the CO_2 concentration of the cultures' headspace was measured over time using a Shimadzu GC-2010 Plus gas chromatograph (GC) fitted with a thermal conductivity detector (TCD) and 320 μm × 30 m HP-plotQ column (J&W Agilent technologies, Victoria, Australia). Headspace gas samples (100 μl) were manually injected with a split ratio of 10 and helium as the carrier gas (flow rate: 10 ml/min). The injector temperature was set at 250 °C, the column was set at an isotherm of 60 °C for 5 min and the TCD was at 150 °C. Standards were prepared by injection of known amounts of CO_2 in the headspace of serum bottles containing the same amount of medium as the cultures.

At each sampling time point, two millilitres of culture was removed to measure nitrate concentration and pH. Nitrate was measured using an IntelliCAL with an ISENO3181 Nitrate Ion Selective Electrode probe attached to an HQ440d bench top metre (Hach Pacific, Australia). The instrument was calibrated with standard nitrate solutions from the manufacturer. The pH was measured using a pH probe (TPS, Australia) and pH adjustments of the cultures were done by titration with 1 M NaOH when the pH dropped below 6.0.

2.3. Nitrification inhibition

Six sub-cultures were prepared as described above using 10% inoculum from active cultures containing coal and soil inoculum. Controls included a set of three autoclaved cultures containing coal and medium and three cultures with no inoculum, but coal and medium only. The serum bottles were sealed and crimped and the headspace was injected with 20 ml pure oxygen filtered through a 0.22 μm syringe filter. Incubation was static and in the dark at 22 °C. After observing nitrate accumulation in active cultures, allylthiourea (86 μM), a nitrification inhibitor, was added at days 42 and 84 to three of the six replicate cultures. Allylthiourea acts by chelating copper from the active site of the enzyme ammonium monooxygenase (Ginestet et al., 1998).

2.4. Hydrocarbon content measurements

To assess the release of aromatic hydrocarbons, the supernatant of each culture was collected by centrifugation. Absorbance over the wavelengths of 195 to 500 nm was performed using a Cary 100 Bio UV/VIS spectrophotometer (Varian Australia). Gallic acid (25 mg/l), a phenolic compound with absorption at 215 and 260 nm, was used as a reference.

The content of hydroxylated and non-hydroxylated hydrocarbons in the supernatant was measured by GC with a flame ionisation detector (FID) at days 70 and 126 of the incubation. The culture supernatant (3 ml) was filtered through a 0.45 μm filter and acidified with 1 M HCl. This aqueous solution was extracted three times with 5% ethanol in ethylacetate and dried by passing through a column of anhydrous sodium sulphate (~1 g). The extract was concentrated using a centrifugal evaporator (Savant Speedvac, Thermo Fisher Scientific, Australia) for 1 h. The residue was resuspended with 20 μl of acetonitrile and 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane using a glass syringe to generate trimethylsilyl derivatives of hydroxylated or carboxylated compounds. Samples were vortexed for a few seconds and then heated at 70 °C for 1 h. The derivatised residue was reconstituted in ethyl acetate (~1 ml) prior to analysis by GC. The analysis was carried out with an Agilent 7890A GC equipped with an FID detector and a DB5 column (60 m × 0.32 mm × 320 μm). The inlet was set in split-less mode and heated at 250 °C; the carrier gas was He (3 ml/min). The oven temperature programme was as follows: 150 °C for 0.5 min followed by a ramp of

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