Molecular microbial ecology of lignocellulose mobilisation as a carbon source in mine drainage wastewater treatment

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Abstract

The community structure of complex microbial consortia which develop in lignocellulose packed passive treatment systems for acid mine drainage remediation were investigated. An understanding of interactions between these populations is important in determining mechanisms by which such systems operate. A degrading packed bed reactor was packed with lignocellulosic material as a sole carbon source and fed continuously with simulated acid mine drainage. Samples were collected every two months at different depths of the reactor to isolate the total genomic DNA and PCR amplify section of 16S rDNA gene. PCR primers, GMSF and 907R incorporating GC clamp were used to amplify 586-bp region of 16S rDNA gene. Denaturing gradient gel electrophoresis (DGGE) indicated clearly a highly differentiated pattern of r-DNA – derived amplificates between different depths of the bioreactor. Predominant DGGE bands were further excised, reamplified, cloned and sequenced. Sequencing analysis revealed phylogenetic affiliation of specific bacterial populations in different depths of the bioreactor.

Introduction

Previous studies undertaken by Rhodes University and Pulles, Howard and de Lange Inc. (PHD) in the use of lignocellulose packed reactor in passive treatment of acid mine drainage (AMD) had shown that certain factors play an important role in their sustained operation over the long time.

Little is known, however, why under certain conditions the performance of these reactors may decline after a period of several months.

This study was part of an Innovation Fund Project in which these problems were addressed and the performance of the microbial consortia shown to play an important role in sustaining operational performance.

The novel degrading packed bed reactor was developed based on these insights and the IMPI passive treatment process has been successfully implemented by PHD.

The carbon source provided for microbiota in this study was lignocellulosic material in the form of pine chips and grass as these are easily available in the country and thus make the process economically viable. Lignocelluloses are some of the most abundant biological polymers on earth. However due to the complexity of its components and chemical structure of lignin in particular its enzymatic degradation is known to be very difficult (Bumpus, 1989; Burland and Edward, 1999; Crawford and Crawford, 1976; Crawford, 1981; Crawford et al., 1983). Except for a few lignin solubilising actinomycetes (Crawford and Crawford, 1976; Crawford, 1981; Crawford et al., 1983), lignin degradation by microorganisms is known to be mainly the domain of aerobic fungi (Achi, 1994; Blondeau, 1989; Bumpus, 1989; Dehsorter and Blondeau, 1992). Little is known about and the degradation of lignin in biosulphidogenic environment (Parek et al., 2001) however, several studies suggest that the contribution of anaerobic organisms to degradation of lignin has been underestimated and should be reevaluated (Kim et al., 1997). Recent findings point to anaerobic degradation of aromatic compounds (Burland and Edward, 1999; Meckenstock et al., 2000) We suspected that strong involvement of microorganisms belonging to different physiological groups and their collaboration may govern the degradation process and the aim of this project was to look for distinct population patterns.

Materials and methods

Sample collection

Samples were collected from a DPBR. Specimens for DNA extraction were collected directly into a sterile 50 ml Falcon tubes (Laboratory & Scientific Equipment Co.) and kept on ice or at 4°C until processed (usually within 4 days).

DNA extraction and purification

Prior to extraction samples were pelleted by centrifugation, washed once with 2 x buffer A (200 mM Tris [pH 8.0], 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate, 10 mM CaCl2) and one part 50% glycerol (Bond, P.L. Appl.Env.2000), and then resuspended in 0.5 ml of 2 x buffer A in 2 ml microcentrifuge tube. It was necessary to include this washing step as low pH of the specimen may cause the hydrolysis of DNA, and high concentration of metals could contaminate the extracted DNA and further have inhibitory effect on subsequent PCR. Polyadenilic acid (200 µg/ml), and lysozyme (3 mg/ml) were added to the suspension and incubated for 40 minutes at 37°C. Samples were subjected to four cycles of freezing in liquid nitrogen and heating for one minute at 80°C. Proteinase K (2 mg/ml) and SDS (10% wt/vol) were added to the mixture, and this was incubated overnight at 37°C. Cell lysates were extracted with phenol-chloroform-isomyl alcohol (24:24:1). Nucleic acids were precipitated with 2.5 volumes of 96% rectified ethanol overnight at -20°C, pelleted by centrifugation and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).
PCR of 16S rRNA gene

Community 16S rRNA genes were amplified by PCR in 25 µl reaction containing approximately 30 ng of purified DNA per µl, 1 x PCR buffer, 200 µM concentration of each of the four deoxynucleoside triphosphates, 2.5 mM MgCl2, 0.04 U of High Fidelity PCR System Taq (Roche Biochemicals), 350 mM reverse and forward primers per microliter. Both primers GM5 F (5’ - acct acg gga ggc age ag - 3’) and 907 R (5’ - cgc cgg cgc cgc cgc ggc cgc gtc cgc cgc cgc cgc gcc ggc gcc gtc aat tcc gag tgt - 3’) incorporating GC clamp were obtained from IDT, USA. Amplification was performed on Hybaid PCR Sprint thermocycler using touchdown PCR procedure with initial denaturation at 95°C followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 68°C decreasing every 4 cycles by 2°C for 45 s, extension at 72°C and completed with final extension at 72°C for 5 minutes. PCR product was purified using QIAquick PCR purification columns (Qiagen). These were quantified on a 0.8% agarose gel.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was done according to previously described protocol (Muyzer, 1993). The PCR products were separated on denaturing gradient from 40% (6% [wt/vol] acrylamide-bisacrylamide [37.5:1], 8% formamide, 1.4 M urea, 2% glycerol) to 50% (6% [wt/vol] acrylamide-bisacrylamide [35.5:1], 20% formamide, 3.5 M urea, 2% glycerol). The electrophoresis was performed in an electrophoresis cell (Sigma-Aldrich) with 1 x TAE buffer (Tris-acetate-EDTA) at 60°C and 150 V for 4.5 hours. DNA was stained with CyberGreen and visualised as described before (Muyzer, 1993). Bands were excised with sterilised scalpel blade transferred to 200 µl Tris-EDTA buffer. The DNA was extracted, reamplified and cloned.

Cloning

DNA extracted from DGGE bands was reamplified with GM5 F and 907 R primers. The motilities of the resulting product were checked by DGGE as described before in order to ensure the pure product. Cloning was performed by using pGemT-easy vector (Promega USA) in accordance with the manufacturer instruction. The cells were transformed into JM109 High Efficiency Competent Cells (Promega, USA). Restriction digest using EcoR1 was performed in order to confirm the insert. Plasmids were extracted using QIA miniprep from Qiagen.

Sequencing

Plasmids were sequenced using BigDye Terminator 3 sequencing kit (Applied Biosystems) with 100 to 200 ng of template DNA according to the manufacturer instruction. For initial analysis, partial sequence was obtained using primer GM5 F. Extended sequences were obtained by using universal sequencing primers T7 and SP6 (Promega USA). The extension products were purified using DNA Clean & Concentrator columns (Zymo Research, USA). DNA sequence was determined on an automated ABI 3100 Prism Genetic Analyzer at Rhodes University, Grahamstown, South Africa.

Phylogenetic analysis

Clones from the various DGGE bands spread over sample position and time were sequenced. The 16S sequences obtained from these clones in one direction were aligned using Clustal X and classified into groupings based on their sequence similarity. Representative sample clones were sequenced in both directions. The latter sequences were aligned with each other and a range of other 16S sequences from the GenBank database including those most closely related to each sequence on Blast analysis. The aligned sequences were analyzed phylogenetically using Neighbor Joining algorithm from Clustal X using two Archeabacterial 16S rRNA sequences as the outgroup.

Results and discussion

DGGE Analysis

To analyse the microbial population for the presence of specific bacteria, the DGGE separation patterns were stained with cyan green and excised for the elution of DNA. The eluted DNA was sequenced.

The DGGE revealed different pattern in distances in migration indicating different species present within single amplificate. This was confirmed by sequencing analysis, results of which are presented in Table 2. However, the number of fragments, which could be visualised on DGGE gel, may in some cases, underestimate the actual diversity of a microbial population and should be considered as a lower limit of an estimation of the total number of bacterial species present.

Phylogenetic analysis

Figure 2 shows a N-J analysis of the 16S rRNA sequences. It is clear that one group of bacteria predominate in the first third of the column for the whole of the bioremediation process. These organisms are Gram +ve low G+C eubacteria related to the Clostridia according to the phylogenetic analysis. Although part of the main Clostridium branch containing as closest species Clostridium thiosulfatireducans, a proteolytic, thiosulfate and sulfur-reducing bacterium and Clostridium peptidovorans, and also a peptide fermenting organism from an anaerobic digestor, they group with a range of as yet uncultivated species obtained from environmental samples. The latter include AJ488074, part of a consortium in-
volved in dechlorination of chlorobenzene, AB062820, a termite
gut bacterium, AF371834, a pig gut bacterium, AJ229251, a
polysaccharide degrading bacterium from anoxic soil and
AF129865/Ay275916, both from lignocellulose anaerobic digestors.
Initially, also present in the first third of the column are a variety of
Spirochaetales most closely related to Treponema spp. The closest,
AF275918, is an uncultivated eubacteria from an anaerobic
digestor; while Treponema vincentii and uncultivated eubacteria
AY995981 are oral strains. Finally, AJ289178 is an strain from an
anaerobic corneal ulcer. However, these Treponema strains are not
detected at later stages in the bioremediation process.

In the second third of the column, one group of related
organism is present throughout the bioremediation process. These
are strains closely related to Bacteriodes and Cytophaga. However,
this group is quite diverse and although it groups together on a
signal branch of the phylogenetic analysis in Fig. 1, it can be
subdivided into two subgroups. One subgroup, containing A, F and
H, groups with Bacteroides has AF157056 from murine gut biota
as closest. The major group containing Bacteroides fragilis,
Bacteroides uniformis and Bacteroides sterconis all form part of the same
Bacteroides branch as A, F and H. In contrast, K is most closely
related to Cytophaga fermentens as well as two uncultivated

Figure 2
N-J analysis of the
16S rRNA
sequences
Conclusions

It appears that the mobilisation of the complex carbon source in the sequential pattern observed here must be sustained in order to maintain the operational performance of the DPBR. It has been shown that the initial fermentative groups play the important role in poising the redox of the system. The supplementation of this group has been developed in the IMPI process with long-term application of the process showing promising results.

The results of the sequencing analysis, demonstrate clear pattern of hierarchy within microbial community, with simple organisms present at the top of the reactor and gradually developing more specialised species of phenol degraders followed by cellulose degrading organisms.

Molecular biology methods used in this study clearly illustrate rich diversity of the microorganisms in the degrading packed bed reactor. Distribution of the microbiota in the DPBR is a convincing indication of distinct nutritional niches that microorganisms establish in the environment in order to proceed with biodegradation. Clear competition and synthyrophy between the community structure leads to the sequence of events that dictates chain of chemical reactions resulting in degradation of lignocelluloses material within bioreactor.

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References

ACHI OK (1994) Growth and coal-solubilizing activity of Penicillium simplicissimum on coal related aromatic compounds. Biores. Technol. 48 (1) 53-57.
BLONDEAU R (1989) Biodegradation of natural and synthetic humic acids by white-rot fungus Phanerochaete chrysosporium. Appl. Environ. Microbiol. 22 1282-1285.
BUMPUS JA (1989) Biodegradation of polycyclic aromatic hydrocarbons by Phanerochaete chrysosporium. Appl. Environ. Microbiol. 55 (1) 154-158.
BURLAND SM and EDWARD E (1999) Anaerobic benzene biodegradation linked to nitrate reduction. Appl. Environ. Microbiol. 65 5529-533.
CRAWFORD DL and CRAWFORD RL (1976) Microbial degradation of lignocellulose: The lignin component. Appl. Environ. Microbiol. 31 714-717.
CRAWFORD RL (1981) Lignin Biodegradation and Transformation. Wiley-Interscience, New York.
CRAWFORD DL et al. (1983) Lignin degradation by Streptomyces viridosporus: Isolation and characterization of a new polymeric lignin degradation Intermediate. Appl. Environ. Microbiol. 45 895-904. DEHORTER R and BLONDEAU R (1992) Extracellular enzyme activities during humic acid degradation by the white rot fungi Phanerochaete chrysosporium and Trametes versicolor. FEMS Microbiol. Lett. 109 117-123.
KIM SK et al. (1997) Biodegradation of recalcitrant organic matter under sulphate reducing and methanogenic conditions in the landfill column reactors. Water Sci. Res. 36 91-98.
MECKENSTOCK RU et al. (2000) Anaerobic naphthalene degradation by a sulphate reducing enrichment culture. Appl. Environ. Microbiol. 66 249-256.
MANZ W et al. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: Problems and solutions. System. Appl. Microbiol. 15 593-600.
MUYZER W et al. (1993) Profiling of complex microbial population by denaturation gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59 695-700.
PAREK S et al. (2001) Degradation of lignin and lignin model compounds under sulphate reducing conditions. Water Sci. Technol. 44 351-358.