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Pulp mill wastewater sediment reveals novel methanogenic and cellulolytic populations

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ABSTRACT

Pulp mill wastewater generated from wheat straw is characterized as high alkalinity and very high COD pollution load. A naturally developed microbial community in a pulp mill wastewater storage pool that had been disused were investigated in this study. Owing to natural evaporation and a huge amount of lignocellulose's deposition, the wastewater sediment contains high concentrations of organic matters and sodium ions, but low concentrations of chloride and carbonate. The microbiota inhabiting especially anaerobic community, including methanogenic arhcaea and cellulolytic species, was studied. All archaeal sequences fall into 2 clusters of family *Halobacteriaceae* and methanogenic archaeon in the phylum *Euryarchaeota*. In the methanogenic community, phylogenetic analysis of methyl coenzyme M reductase A (*mcrA*) genes targeted to novel species in genus *Methanoculleus* or novel genus of order Methanomicrobiales. The predominance of Methanomicrobiales suggests that methanogenesis in this system might be driven by the hydrogenotrophic pathway. As the important primary fermenter for methane production, the cellulolytic community of enzyme GHF48 was found to be dominated by narrower breadth of novel clostridial cellulase genes. Novel anoxic functional members in such extreme sediment provide the possibility of enhancing the efficiency of anoxic treatment of saline and alkaline wastewaters, as well as benefiting to the biomass transformation and biofuel production processes.

1. Introduction

In recent years, Archaea has been studied extensively in microbial ecology research and, as a result, significant changes in our knowledge and great discoveries have taken place. Especially, for the fundamental importance in cycling of carbon in many ecosystems, diversity investigations of methanogenic Archaea are not only across several natural habitats such as rice paddy soils (Lueders et al., 2001), aquatic sediments (Conrad et al., 2010), wetland (Zhang et al., 2008), and hypersaline mud volcanoes (Lazar et al., 2011), but also involved in some renewable biomass anaerobic digestion systems including agricultural biogas plants, and landfills (Luton et al., 2002; Nettmann et al., 2010). In addition, as a functional members in anaerobic environments digesters, methanogens have a key function in anaerobic engineered process of treating various domestic, agricultural, and industrial wastes, especially in utilizing renewable materials for methane production. In such anaerobic environments, mineralization of complex organic matters occurs through the collaboration of a variety of microorganisms. Primary fermenters, such as cellulose degraders, are important members in breaking down the complex molecules and fermenting the hydrolysis products (Springer et al., 1995). Therefore, the microbial communities in anaerobic environments that utilize lignocellulose as the primary carbon source are also of great value for anaerobic engineered process.

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Though many researches focus on methanogenic species in various environments, the phylogenetic analysis of methanogenic Archaea in saline and alkaline environments is poorly understood, for the survival difficulty of methanogen in these extreme environments (Antony et al., 2012). Especially, alkaline and saline environments created artificially are more harsh habitats for methanogen existence (Kotsyurbenko et al., 2004). It is well known that these artificial environments have restricted alpha diversity compared to natural type, typically limited to *Bacillus* or the related genera that survive by producing endospores (Grant, 2003). To our knowledge, few reports have referred to prokaryotic diversity in these artificial biotopes. As we have described previously, the pulp mill wastewater generated from alkaline extraction technology possesses extreme properties, including a high pH (pH 11–13) value and chemical oxygen demand ($COD_{cr} > 100{,}000$ mg l^{-1}). It is difficult to treat this type of wastewater (called as black liquor), and few ideal treatment technologies have been found to date (Yang et al., 2008). The extreme nature of these types of environment results in a low survival of microorganisms, although our previous investigation of a black liquor storage pool in a pulp mill demonstrated a naturally developed microbial community, and various *Bacillus* and *Clostridium* species were isolated from such wastewater (Yang et al., 2010).

The above storage pool for black liquor was disused after 6 years of operation. One year later, due to the natural evaporation, only sludge remained and the salt content increased greatly. The methane content was measured as a higher value than the background concentration (2.560 mg m^{-3} VS 0.979 mg m^{-3}). In addition, the concentration of hydrogen sulfide above the storage pool is five times higher than the background concentration (0.033 mg m^{-3} VS 0.006 mg m^{-3}). Large amounts of lignin, hemicelluloses, and cellulose were deposited at the bottom of the pool, and the height of the sediment reached 1.4 m. Therefore, the sediment was characterized as saline (high concentration of sodium ion), alkaline, and lignocellulose matter-rich.

Given the special characteristics of the sediment, the saline and alkaline, lignocellulose-based sediment community is of great interest to science and of great values for wastewater treatment and bioenergy production. The present study is undertaken to investigate the Archaea diversities, and more importantly, to explore the potential of anaerobic community in this environment. The combination of enrichment strategy and 16S rRNA gene clone library was used to characterize the key functional genes of methyl coenzyme M reductase A (*mcrA*) in methanogen, as well as the cellulolytic community of enzyme GHF48 (GHF48) in cellulose-utilizing species.

2. Materials and methods

2.1. Sample collection and nucleic acid extraction

Three samples were collected at just below the sediment surface, at depths of 0.5 m and 1 m, respectively. They were collected in autumn 2009, in sterile bottles and stored at −80 °C until analysis. The chemical parameters of the sample were shown in Table 1, including pH value measured by an Orion model 828 pH meter, salinity measured by a salinity refractometer (Atago Inc., Japan), and inorganic ions determined by atomic absorption spectrophotometer (PE Inc., America). Genomic DNA was extracted using a previously described procedure and purified with a Wizard DNA Clean-up system (Promega) (Yang et al., 2010). The methane content above the storage pool was analyzed by gas chromatograph (Shimadzu GC-2010) with a thermal conductivity detector. Nitrogen was used as a carrier gas at a flow rate of 0.3 ml min^{-1}. Hydrogen sulfide content was analyzed by the Methylene blue method (Cline, 1969).

2.2. Amplification and phylogenetic analyses

All the primers used in present study are listed in Table 2. Using the primer pair of 27F/1492R, the following conditions were used for bacterial 16S rRNA genes amplification: one cycle of 94 °C for 3 min, 20 cycles of 94 °C for 15 s, 58 °C for 60 s 58 °C for 50 s, and 72 °C for 2 min, and then an extension step for 5 min at 72 °C. Primers of 21F/958R, 109F/915R, and 46F/1017R were used for archaeal 16S rRNA genes amplification in the following procedure: one cycle of 94 °C for 3 min, 30 cycles of 94 °C for 15 s, 50–60 °C for 50 s, and 72 °C for 2 min, and then an extension step for 5 min at 72 °C. Negative controls to which no template DNA was added were included. The PCR products were concentrated and purified with a Qiagen II Extraction kit (Qiagen Corp., Germany), ligated into the pGM-19T vector (Promega), and transformed into *Escherichia coli* DH5α competent cells. Clones were randomly selected, and approximate 600 bp of sequence was obtained for each using the primers 27F and 21F, respectively. The resulting sequence data were checked for the presence of PCR-amplified chimeric sequences with the check_chimera program (Cole et al., 2005). A total of 124 clones for bacteria and 41 clones for Archaea were selected for phylogenetic analysis. Clones with a sequence identity (≥99%) were defined as operational taxonomy units (OTUs). The correct sequences were compared to the 16S rRNA gene sequences in the GenBank nucleotide library by a BLASTN search (http://www.ncbi.nlm.nih.gov/). One representative

Table 1 – Physicochemical characteristics of sediment samples. A represents sample at 1 m depth, B represents sample at 0.5 m depth, and C represents sample near the surface.

Parameter	Sample		
	A (g L^{-1})	B Ca^{2+} (mg L^{-1})	C Mg^{2+} (mg L^{-1})
pH	9.1	9.1	8.9
Salinity (%)	2.5	2.3	2.0
Conductivity (mS cm^{-1})	121	119	109
CO_3^{2-} (mM)	0.10	0.11	0.12
HCO_3^- (mM)	0.5	0.5	0.9
SO_4^{2-} (mM)	22.9	35.4	49.0
Cl^- (mM)	42.3	42.1	39.4
Na^+ (mM)	1040	980	960
K^+ (mM)	56.4	53.9	51.3
Ca^{2+} (mM)	3.5	3.3	2.8
Mg^{2+} (mM)	1.5	1.4	1.1
Fe^{2+} (mM)	53.6	53.6	51.8