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ORIGINAL PAPER

Effect of nitrogen source on methanol oxidation and genetic diversity of methylotrophic mixed cultures enriched from pulp and paper mill biofilms

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Abstract Methanol-oxidizing bacteria may play an important role in the development and use of biological treatment systems for the removal of methanol from industrial effluents. Optimization of methanol degradation potential in such systems is contingent on availability of nutrients, such as nitrogen, in the most favorable form and concentration. To that end, this study examined the variation in growth, methanol degradation, and bacterial diversity of two mixed methylotrophic cultures that were provided nitrogen either as ammonium or nitrate and in three different concentrations. Methanol-degrading cultures were enriched from biofilms sampled at a pulp and paper mill and grown in liquid batch culture with methanol as the only carbon source and either ammonium or nitrate as the only added nitrogen source. Results indicate that growth and methanol removal of the mixed cultures increase directly with increased nitrogen, added in either form. However, methanol removal and bacterial diversity, as observed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR–DGGE) methods, were higher when using nitrate as the nitrogen source for enrichment and growth, rather than ammonium. Based on results described here, nitrate may potentially be a better nitrogen source when enriching or working with mixed methylotrophic cultures, and possibly more effective when used as a nutrient addition to biofilters.

Keywords Methylotroph · Nitrogen source · Methanol biofilter · Bacterial diversity

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Introduction

Methylotrophic bacteria are ubiquitous in aquatic and terrestrial environments and play an important role in the carbon cycle due to their ability to oxidize methane, methanol, and other reduced carbon substrates. Many previous studies have focused on methanotrophs, a functional group of methylotrophs able to utilize methane as their sole carbon source and to cometabolize toxic hydrocarbons. However, less attention has been given to the potential use of methylotrophs for biological control of methanol, a common pollutant in aqueous or gaseous industrial effluents. For example, the paper and allied products industry is a major contributor of methanol emissions, which are produced during wood pulping and released to the air and discharged in the mill wastewater

(Someshwar and Pinkerton 1992). In 2007, over 70% of the methanol emissions in the United States (approximately 10.8 million pounds) were released from paper and wood products companies (US. EPA, 2007). While most pulp and paper mills comply with regulations to control these methanol emissions by incinerating the methanol in thermal oxidation systems (Varma 2003), environmental and economic advantages can be achieved by using a biological treatment system that takes advantage of the natural degradation potential of the diverse bacteria classified as methylotrophs (Babbitt et al. 2009a, 2009b).

One type of biological system proposed for the pulp and paper industry is a bacterial biofilter, a stationary packed bed containing active microbial consortia capable of degrading contaminants of interest under the conditions expected to be present in the treatment train, including specific pH, temperature, and substrate concentration values (Devinny et al. 1999). When considering biofiltration of gaseous methanol from a pulp and paper mill, using an inoculum of mixed methylotrophic bacterial cultures enriched from samples obtained from the mill has been demonstrated to influence the ultimate community structure and stability of bacteria colonizing an activated carbon biofilter (Babbitt et al. 2009a). An important criterion that has not been fully resolved is the importance of the form and concentration of the nitrogen source used in enriching for methylotrophs or supporting their growth and activity in liquid culture or in biofiltration applications. The intermittent addition of nitrogen in a mineral salts mixture has been demonstrated to improve the capacity of biofilters in removing ethanol (Teran Perez et al. 2002) and toluene (Prado et al. 2002). Addition of greater concentration of nutrients appears to be most important when the biofilter is subjected to high contaminant loading rates (Gribbins and Loehr 1998). When comparing nitrogen added as either nitrate or ammonium, Yang et al. (2002) demonstrated that ammonium use resulted in higher methanol elimination capacities in a biofilter but that, at high nitrogen-to-carbon ratios, the ammonium could also inhibit methanol removal. The added nitrate did not show this inhibitory effect (Yang et al. 2002). Despite the benefit of these studies, it is still unclear how the type and concentration of nitrogen used directly affects the methanol degradation potential of mixed methylotrophic cultures.

Furthermore, extensive work has been conducted to optimize nutrients, growth factors, trace elements, and substrate concentrations for methanotrophs in batch cultures (Bowman and Sayler 1994; Park et al. 1992, 1991), but less specific attention has been focused on enrichment of the more general group of methylotrophs. For example, use of specific nutrient sources, particularly nitrate or ammonium as a nitrogen source, is not consistently reported in studies involving methylotrophs. In part, this inconsistency appears to be due to early work showing better growth of methanotrophic bacteria when using nitrate (Whittenbury et al. 1970), which has been more recently been associated with the possibility that ammonium inhibits methane oxidation in these bacteria (Boiesen et al. 1993; De Visscher and Van Cleemput 2003; Higgins et al. 1991). Therefore, some recommendations for growth of restricted or facultative methylotrophic bacteria follow those for methanotrophs (e.g., use of nitrate as nitrogen source) (Hanson 1998), whereas other studies report use of ammonium as the nitrogen source, in varied concentrations (Patt et al. 1974, El-Nawawy et al. 1990). The effect of form and concentration of nitrogen on methanol degradation potential of methylotrophs is not clearly understood, nor are the effects on population diversity and stability. Selection, enrichment, and use of an appropriate inoculum for biological treatment systems, such as bacterial biofilters, may be greatly improved with additional knowledge of optimum nitrogen requirements and concentrations. As an initial step towards this goal, this paper reports the effect of the form and concentration of nitrogen in batch enrichment cultures on the growth, methanol removal potential, and genetic diversity of mixed methylotrophic cultures enriched from biofilm samples taken from a Kraft pulp mill.

Methods

Sample collection

To study the effect of nitrogen source and concentration on methylotrophic cultures potentially used as biofilter inoculum, sampling and analysis were focused on biofilms and other biological cultures obtained directly from a pulp mill environment. These

samples were collected from a pulp and paperboard company located in the Southeast that operates a biological wastewater treatment system. Seven grab samples of biofilms were collected during June 2004 from locations believed by mill staff to be representative of methanol-degrading consortia or having a high number of bacteria present. Samples were stored on ice in sterilized Teflon collection vessels until they could be processed in the lab or stored over a longer term at 4°C. Initial culture-dependent growth and isolation techniques demonstrated that two of the seven samples may be good candidates for inocula in a methanol treatment system, based on their rapid growth rates, methanol degradation rates, and morphologically diverse, culturable communities (determined by identification of visibly distinct colonies on agar plates; data not shown). These samples, "SA" and "SB," are described as follows. The SA biofilm was obtained directly from the vent tubes of a pure oxygen activated sludge "UNOX" (Union Carbide Oxidation) reactor, where mill staff estimated the conditions to include temperatures between 32 and 36°C, methanol concentrations between 1,000 and 5,000 mg/l, and nitrogen as ammonium in concentrations between 20 and 140 mg/l (ammonium is added to the reactor intermittently to improve performance). The SB biofilm was collected from the return activated sludge system, with conditions reported by mill staff to include ambient outdoor temperatures (26–30°C), and low methanol (<10–100 mg/l) concentrations.

Enrichment for methylotrophic bacteria

Homogenized subsamples (10 ml each) from SA and SB were first mixed with 90 ml of sterile phosphate-buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; pH 7.3) for 1 h on a rotary shaker at 30°C at 250 rpm. This mixture was used to inoculate batch cultures in both modified nitrate mineral salts (NMS) and ammonium mineral salts (AMS) media, containing 0.2% methanol (vol/vol) as recommended by Hanson (1998). The basal medium (without nitrogen) contained, on a g/l basis: $MgSO_4*7H_2O$, 1.0; $CaCl_2$, 0.2; KH_2PO_4, 0.026; Na_2HPO_4, 0.033. Trace elements were added, on a mg/l basis: $FeSO_4*7H_2O$, 0.5; $ZnSO_4*H_2O$, 0.4; EDTA disodium salt, 0.25; $CoCl_2*6H_2O$, 0.05; $MnCl_2*4H_2O$, 0.02; H_3BO_3, 0.015; $NiCl_2*6H_2O$, 0.01; $Na_2MoO_4*4H_2O$, 0.005; and FeEDTA, 0.0038. Vitamins were added, on a mg/l basis: biotin, 0.02; folic acid, 0.02; thiamin*HCl, 0.05; calcium pantothenate, 0.05; riboflavin, 0.05; nicotinamide, 0.05; and B12, 0.001. The nitrogen source was added to the medium as nitrate (1.0 g/l KNO_3) or ammonium (0.5 g/l NH_4Cl). All chemicals used were obtained from Fisher Scientific (Pittsburgh, PA, USA) or Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity available. The cultures were maintained in a 1:10 volume ratio of inoculum to medium to a total volume of 55 ml in 250 ml Erlenmeyer flasks at 30°C on a rotary shaker at 250 rpm. Initial enrichment cultures were incubated for one month, while subsequent transfers to fresh medium were made twice, with a two-week period between transfers.

Comparison of growth and methanol degradation using two types of nitrogen source

To compare methanol degradation by batch mixed methylotrophic cultures with two potential nitrogen sources, a factorial (3^2) design was used (Ott and Longnecker 2001). This design included either nitrate added as KNO_3 at levels of 0, 1.0, and 2.0 g/l or ammonium added as NH_4Cl at levels of 0, 0.5, and 1.0 g/l (these levels correspond to 0, 0.13, and 0.26 g N/l), and methanol added at 10, 100, and 1000 mg/l in the liquid phase. These nitrogen levels reflect common ranges that may be used in batch or biofilter applications (Yang et al. 2002; Gribbins and Loehr 1998). Concentrations of 0 g N/l were also included to assess whether the cultures could degrade methanol with only soluble cell nitrogen or atmospheric N_2 present, as such a condition might be expected if nutrients become exhausted in a biofilter or batch culture.

The last transfer of the enrichment cultures was made to a 2400 ml flask, in which 500 ml of the culture was grown to ¾ log phase, harvested by centrifugation in a J2-HS Beckman floor model centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA), then twice washed with PBS and recentrifuged to remove residual nitrogen and methanol. Cells were resuspended in the appropriate mineral salts medium with no added nitrogen to obtain an optical density of 0.1 at 600 nm. For each combination of nitrogen and carbon concentrations, a master mixture of cells and

medium in a 1:10 ratio was prepared. Liquid methanol was added to the mixture to the desired concentration, and 4 ml from each master mixture was aliquoted into 20 ml glass vials and sealed with crimp top Teflon-lined septa. Three replicate cultures were prepared for each of the nine nitrogen and methanol combinations. Control vials were prepared with killed cells and with no cells, to account for any methanol that might be removed by physical adsorption to the cells or volatilized during the handling and analysis process. The cultures were incubated for 48 h on a rotary shaker at 250 rpm and 30°C. Every 4–6 h during this incubation, growth was assayed by optical density using a spectrophotometer at 600 nm. At 48 h, the cells were pelleted using the floor centrifuge, and 2 ml of the liquid supernatant were collected and analyzed for final methanol concentration. Aqueous methanol concentrations were analyzed by GC/FID using a Clarus 500 (PerkinElmer, Wellesley, MA, USA), with helium at 31.3 psig as the carrier gas, and hydrogen and air at 45 ml/min and 450 ml/min, respectively, as combustion gases. Cyclohexanol was used as the internal standard.

Diversity of microbial populations in cultures enriched with different nitrogen sources

To determine the genetic diversity of the bacterial populations enriched from both samples with both nitrogen sources, denaturing gradient gel electrophoresis (DGGE) was performed using the polymerase chain reaction (PCR)-amplified DNA extracted from the enriched cultures and from the original biofilm samples. Genomic DNA was extracted from enriched cultures using UltraClean Microbial DNA kits (MO BIO Laboratories, Carlsbad, CA, USA) and the accompanying protocol for DNA extraction and purification from microbial samples. In addition, DNA was extracted from the original SA and SB biofilm samples using UltraClean Soil DNA kits (MO BIO Laboratories, Carlsbad, CA, USA) and the accompanying protocol for DNA extraction.

The polymerase chain reaction (PCR) was used to amplify specific DNA sequences found in expected methylotrophic (methanol-oxidizing) populations. In all known gram-negative methylotrophic bacteria, methanol oxidation is catalyzed by the enzyme methanol dehydrogenase (MDH), the large subunit of which is encoded by the highly conserved functional gene mxaF (Barta and Hanson, 1993; McDonald and Murrell, 1997). Therefore, mxaF-specific primers f1003 (5′-3′ GCC CGC CGC GCC CCG CGC CCG TCC CGC CGC CCC CGC CCG GCG GCA CCA ACT GGG GCT GGT), which includes a 39-bp GC-clamp at the 5′ end, and r1561 (5′–3′ GGG CAG CAT GAA GGG CTC CC) were used to detect methylotrophs as described by McDonald and Murrell (1997) and McDonald et al. (1995). A universal primer set for 16S rRNA was used to compare results to the functional gene. The 16S rRNA sequences were amplified using primers f27 (5′–3′ CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GAG AGT TTG ATC MTG GCT CAG), which includes a 40-bp GC clamp at the 5′ end, and r534 (5′–3′ ATT ACC GCG GCT GCT GC).

Initial PCR and DGGE conditions were based on Henckel et al. (1999), Fjellbirkeland et al. (2001), McDonald et al. (1995), and McDonald and Murrell (1997), but optimized for this specific system and primer set. The PCR reaction mixture was prepared in 0.2 m thin-walled PCR tubes and contained 1X $MgCl_2$-free PCR buffer, 1.5 mM $MgCl_2$, 100 uM of each dNTP,1U *Taq* polymerase (all from Invitrogen, Carlsbad, CA, USA), 0.5 µM of each primer (Integrated DNA Technologies, Inc, Coralville, IA, USA.), 1–2 µl of template DNA (50–100 ng), and sterile water to a final volume of 50 µl. Amplifications with mxaF primers were carried out using a Mastercycler Personal 5332 thermocycler (Eppendorf North America, Westbury, NY, USA) with the block preheated to 92°C, using a reaction program of initial denaturation at 92°C for 3 min, a total of 30 cycles of denaturation (30 s at 92°C), annealing using a touchdown program (30 s per cycle from 60 to 50°C at −0.5°/cycle for the first 20 cycles and 50°C for the last 10 cycles), and extension (45 s at 72°C), and a final extension at 72°C for 4 mins. The same reaction setup was used for the 16 s rRNA primers, but with an annealing touchdown temperature profile of the first 10 cycles from 55 to 50°C at −0.5°/cycle and the last 20 cycles at 50°C. The touchdown program was used because it increased yield and number of bands observed on subsequent DGGE gels, compared to a set annealing temperature. PCR products were verified on a 1.2% agarose gel, photographed, and their yield estimated using ImageJ software (Rasband, 2006) calibrated with a low DNA

marker (50–2,000 bp, BioNexus, Inc, Oakland, CA, USA.).

DNA separation using DGGE

DNA fragments were separated using denaturing gradient gel electrophoresis (DGGE) with a 16 × 16 cm, 1 mm thick gel containing 6% acrylamide, 1X TAE, and a linear gradient of 35–65% denaturant (100% denaturant is equivalent to 7 M urea and 40% formamide), cast for 90 min. Approximately 500 ng of PCR product was mixed with 10-20 µl of 2X gel loading dye (70% glycerol, 0.05% Bromophenol Blue, 2 mM EDTA), loaded on the gel, and electrophoresed at 60 degrees C for 5 h at 150 V in 1X TAE, using a DCode Universal Mutation Detection System Model 475 Gradient Delivery System (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained with 50 µg/ml ethidium bromide in 1X TAE for 15 min and destained in 1X TAE for 10 min. Bands were visualized and photographed using a Fisher Biotech Model 88A variable UV intensity Transilluminator and DCode DocIt software system (Bio-Rad Laboratories, Hercules, CA, USA).

DGGE image analysis and diversity measurements

The digitized gel images were analyzed using ImageJ (Rasband 2006). The background was subtracted using a rolling ball radius of 50. Bands in each lane were automatically detected and plotted. Peak area and relative intensity of each band was measured, and bands contributing less than 1% to the total intensity within one lane were omitted from subsequent analysis.

Diversity in each sample was estimated using measurements of species richness (S), diversity (H), and evenness (E). S was determined by counting the bands in each lane, with the assumption that a single species would migrate to each unique location. Shannon's H (Hayek and Buzas 1996) was used as a diversity index.

$$H = -\sum_i p_i \ln(p_i)$$

where p_i is the relative intensity of the ith band compared to the total intensity of all bands in that lane. E was calculated from Pielou's evenness (Hayek and Buzas 1996).

$$E = \frac{H}{\ln(S)}.$$

DNA sequencing and phylogenetic analysis

To further characterize the bacteria under both nitrogen use profiles, selected bands from the mxaF and 16 s rRNA DGGE gels were excised for sequencing. Bands were chosen from DNA that showed the highest intensity when visualized on the UV transilluminator and were excised using a sterile pipet tip and scalpel. The gel fragments were eluted overnight at 30 degrees C at 250 rpm in 30 µl of an elution buffer containing 10 mM Tris–Cl (pH 7.5), 50 mM NaCl, and 1 mM EDTA (pH 8.0) (Chory and Pollard 1999). Gel fragments were removed, and DNA was precipitated from the liquid by adding 50 µL of 95% cold ethanol, chilling 30 min at −40°C, and pelleting the DNA by centrifuging 10 min at 10,000 × g. After pouring off the ethanol supernatant, the pellet was dried at 40°C for 4–5 h and resuspended in 30 µl of TE buffer (Chory and Pollard 1999). This template was reamplified using the same methods as described previously and checked on a DGGE gel for purity and for migration to the same gradient position as in the original sample. Sequencing was performed at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) using the fluorescent dideoxy terminator method of cycle sequencing on either a Perkin Elmer Applied Biosystems Division (PE/ABD) 373A or 377 automated DNA sequencer, following ABD protocols, with consensus sequences generated using the Sequencher Software from Gene Codes. Sequences of partial mxaF and 16 s rRNA gene fragments have been deposited in the GenBank database. Fragments M1, M3, and M6 were identical to bands sequenced from biofilter samples (Babbitt et al. 2009a), which have previously been submitted under accession numbers EU099402, EU099404, and EU099407, respectively. For this study, mxaF fragments M2, M4, and M5 were submitted under accession numbers EU138867, EU138868, and EU 138869, respectively; and 16 s rRNA fragments U1–U7 were submitted under accession numbers EU138870–EU138876, respectively.

Published sequences with high similarity to sample sequences were obtained by performing a

nucleotide-nucleotide BLAST (NCBI) search. The 10 most similar sequences of known species with *E* scores lower than 1*E*-20 were chosen for each sample, with duplicates removed. Sequences were aligned using ClustalW, with default gap penalties, and manual inspection and refinement of alignments. A phylogenetic tree was constructed using the Neighbor Joining method and bootstrapped with 1,000 replicates. Because all known γ-proteobacteria clustered into a distinct branch, this group was selected as the out-group. All phylogenetic and molecular evolution analyses were conducted using MEGA version 3.1 (Kumar et al. 2004).

Results and discussion

Comparison of growth and methanol degradation

The two pulp mill biofilm samples (SA and SB) enriched using different nitrogen sources were compared on the basis of methanol degradation under different methanol and nitrogen concentrations. Both samples, regardless of nitrogen source or concentration, showed 100% methanol removal after 48 h when incubated with initial methanol concentrations of 10 and 100 mg/l. Differences among the cultures only became apparent when methanol was introduced at concentrations of 1,000 mg/l. The percent of methanol removed, based on an initial 1,000 mg/l concentration, is shown in Fig. 1. For all cultures, the percent of methanol removed from the liquid phase increases with increasing nitrogen concentration. In addition, for all cultures assessed in medium with added nitrogen, a higher methanol removal was achieved when nitrate served as the nitrogen source. In fact, the SB culture enriched in NMS medium with the highest concentration of nitrate (0.26 g N/l) showed 100% removal. On the other hand, after transfer to medium with no added nitrogen, both SA cultures showed significantly higher methanol removal than SB, regardless of the original enrichment N-source.

A slightly different trend was observed when comparing growth rate with an initial 1,000 mg/l methanol concentration and varied nitrogen sources and concentrations (Fig. 2). These results showed that the mixed cultures grew almost equally as fast from zero to 48 h with either ammonium or nitrate present at the high (0.26 g N/l) or medium (0.13 g N/l)

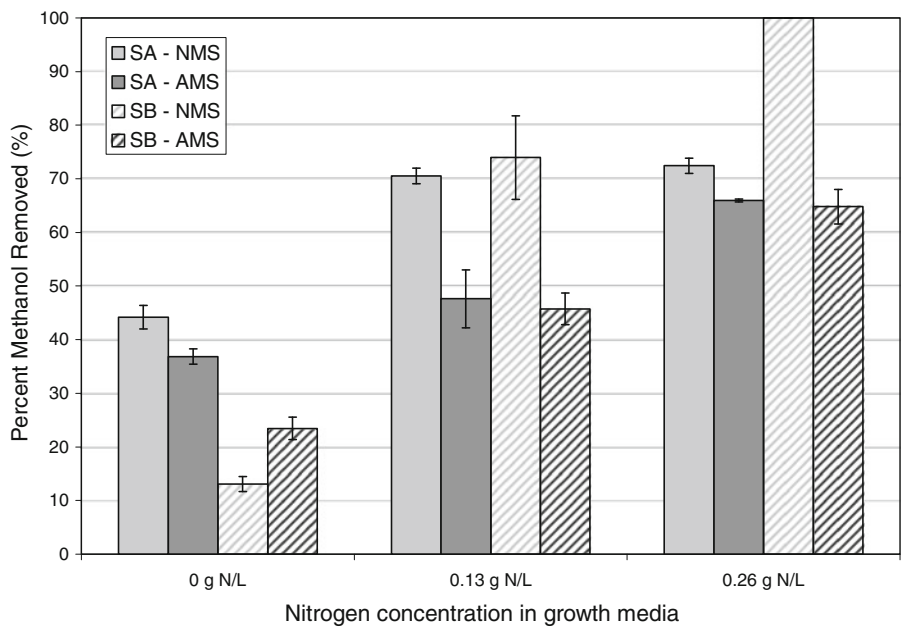

Fig. 1 Comparison of methanol removal by SA and SB cultures in both AMS and NMS medium with an initial methanol concentration of 1,000 mg/l. *Bars* represent standard error from samples in triplicate

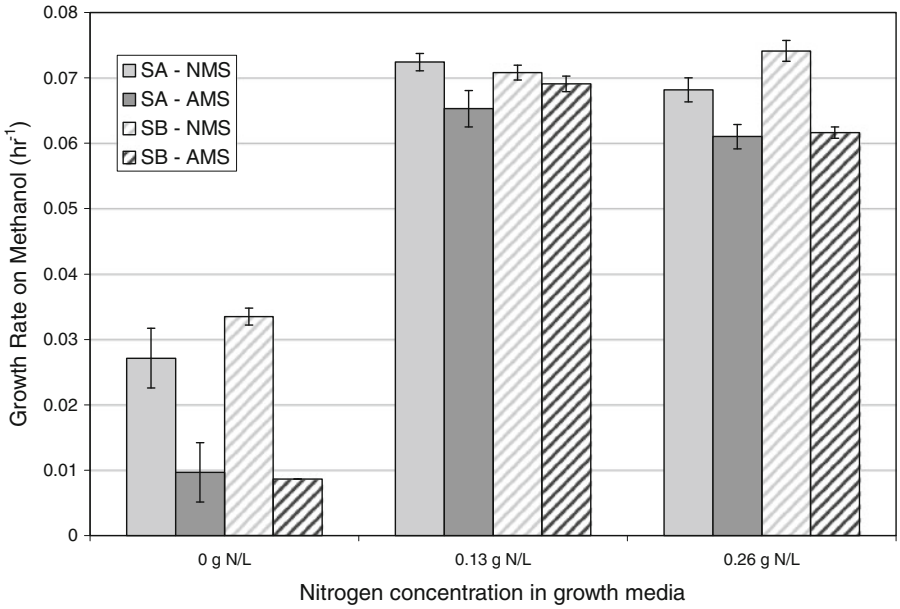

Fig. 2 Comparison of batch growth rates in SA and SB cultures in both AMS and NMS medium with an initial methanol concentration of 1,000 mg/l. *Bars* represent standard error from samples in triplicate

concentrations tested. Growth rate slightly increased when NMS was used, but the trends were not as dramatic as when comparing methanol removal. However, the growth rate was significantly lower when the cultures were grown with no added nitrogen in either form (Fig. 2).

Diversity of microbial populations in cultures enriched with different nitrogen sources

To expand growth and activity comparisons to the community level of the enriched samples, DGGE was used to separate DNA fragments amplified for methylotrophs and all bacteria from the original biofilm samples and their enrichments, as shown in Fig. 3. Quantitative estimates of diversity, based on banding patterns in the DGGE gels, are provided in Table 1. Results obtained for methylotrophs and universal bacteria were not compared directly, because different primer sets can amplify entirely different populations; however, both sets of results were used for determining trends in the population changes for the nitrogen sources used. The results in Table 1 show consistent trends among the different enrichment and molecular methods, except for the methylotrophs enriched from SA, where regardless of which nitrogen source was used, the diversity of this type of bacteria dropped to zero, with potentially only one dominant methylotrophic species present. When considering methylotrophs in SB and all bacteria in SA, species richness, diversity, and evenness generally showed a smaller decrease from the quantities observed in the original biofilm culture to the levels shown in the enriched culture. In all of these cases, diversity metrics were greater for the mixed cultures enriched using nitrate, as compared to ammonium, as the nitrogen source. This result could potentially correspond to the observation that cultures enriched in nitrate also showed higher methanol removal.

The ability to enrich potential biofilter inocula cultures to maximize growth and diversity may be a beneficial tool for improving degradation potential and reliability in biofiltration systems. Some studies have shown correlations between degradation potential and diversity or density of microbial populations colonizing a biofilter (Khammar et al. 2005). Friedrich et al. (2002) related efficient degradation potential observed in a full-scale industrial biofilter to high microbial diversity and the presence of pre-adapted microorganisms that allowed the system to respond to variable operating conditions. Others have observed increasing bacterial diversity in biofilters,

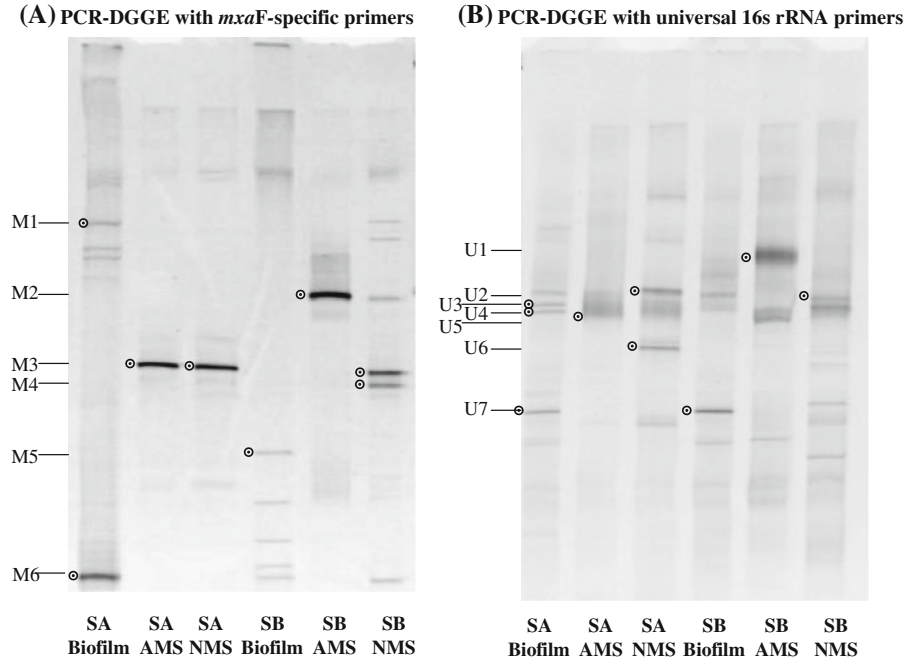

Fig. 3 DGGE analysis of mxaF and 16 s rRNA gene sequences. *Bands* marked with a circle were excised and sequenced, and all marked bands on a vertical gradient labeled by M (methylotrophs) or U (universal) were confirmed as identical in sequence

Table 1 Bacterial species, diversity, and evenness for SA and SB cultures in AMS or NMS media

Sample	Methylotrophs (mxaF)			Universal (16 s rRNA)		
	S	H	E	S	H	E
SA	4	1.04	0.75	9	2.02	0.92
SA-AMS	1	0	0	3	1.06	0.96
SA-NMS	1	0	0	7	1.74	0.89
SB	5	1.56	0.97	10	2.09	0.91
SB-AMS	2	0.34	0.49	8	1.48	0.71
SB-NMS	6	1.39	0.78	9	1.90	0.87

Species richness is indicated by (*S*), diversity is indicated by (*H*), and evenness is indicated by (*E*). *AMS* ammonium mineral salts, *NMS* nitrate mineral salts

corresponding with acclimatization (Hu et al. 1999) or increased pollutant concentration (Borin et al. 2006). With these findings in mind, one implication of results discussed herein may be that nitrate is a better nitrogen source for use in enriching and maintaining robust mixed methylotrophic cultures for use in biofilters or other applications. Further study in this area can elucidate mechanisms by which the nitrogen source may influence selection of hardy strains during enrichment or lead to microbial consortia with robust tolerance to elevated contaminant levels.

Phylogenetic analysis of dominant species

The genetic comparisons among the cultures were expanded by selecting dominant species within each culture and determining their mxa-F or 16 s rRNA sequence and phylogenetic relationship to other closely related known bacteria. All excised and sequenced bands are denoted in Figs. 3a, b as indicated by circles placed adjacent to sequenced bands. The phylogenic relationships among the species dominating the cultures in these experiments and known bacteria are shown in Figs. 4 and 5.

Figure 4 shows the distribution of selected and recovered dominant bands that were produced by amplifying the functional gene for methanol dehydrogenase. Bands from the six samples show similarity to sequences found in the alpha-, beta-, and gamma-proteobacteria. Dominant bands from the original SA biofilm species are labeled as M1, which appears closely related to beta-proteobacteria *Methylophilus methylotrophus*, a ribulose monophosphate (RuMP) cycle restricted facultative methylotroph; and M6, which shows the greatest genetic similarity to a dominant species from the SB biofilm (M5), and both are grouped with other alpha-proteobacteria in the order *Rhizobiales*.

Interestingly, enriching SA in AMS or NMS produced similar mxaF profiles that appear to be

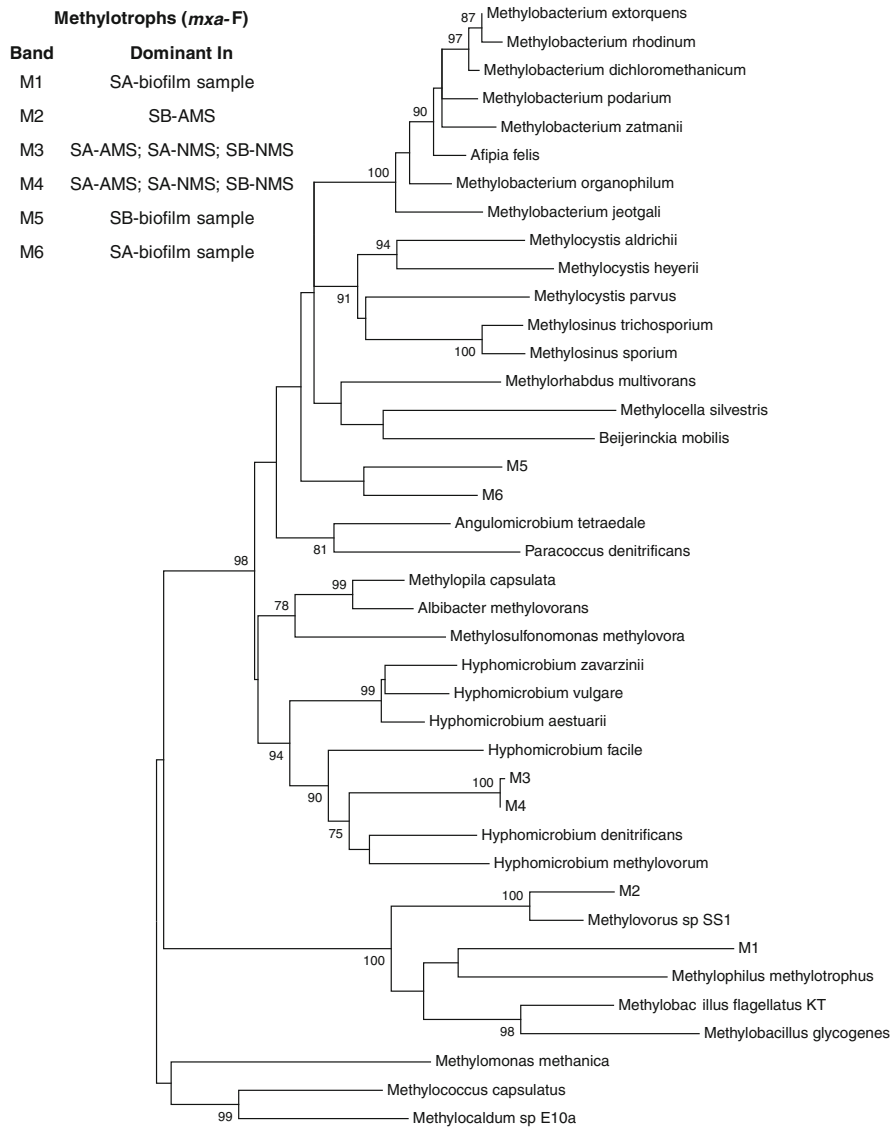

Fig. 4 Phylogenetic reconstruction of known methylotrophic bacteria and unknown culture strains using mxaF gene sequences (Bootstrap values represent 1,000 replicates, and values greater than 75% are shown)

dominated by a single methylotroph, band M3, which is not visible in the original culture, possibly because its DNA was present in too small a concentration to be amplified sufficiently for visualization in the DGGE gel. Band M3 showed high sequence similarity to one cluster within the genus *Hyphomicrobium*, bacteria also classified as non-N_2 fixing, restrictive facultative methylotrophs, but which use the serine pathway and are members of the alpha-Proteobacteria (Lidstrom 2006; Rainey et al. 1998). The community shift observed when enriching SB in AMS or NMS was not as consistent as with SA. M2, a strong band from enrichments of SB in AMS, appeared to be closely related to a species within the genera of *Methylovorus*, an RuMP pathway, restrictive facultative methylotrophs classified as beta-Proteobacteria (Doronina et al. 2005). However, M3 was also present for SB enriched in NMS, as was band M4, which has a highly similar mxaF sequence. In these cases, it may be that the ultimate genetic similarity of strains colonizing enriched cultures was influenced less by the form of nitrogen in the enriching media and more by the presence of resilient *Hyphomicrobium* strains in the parent culture or by the inability of other initial strains in SA and SB to grow in the selected mineral salts media.

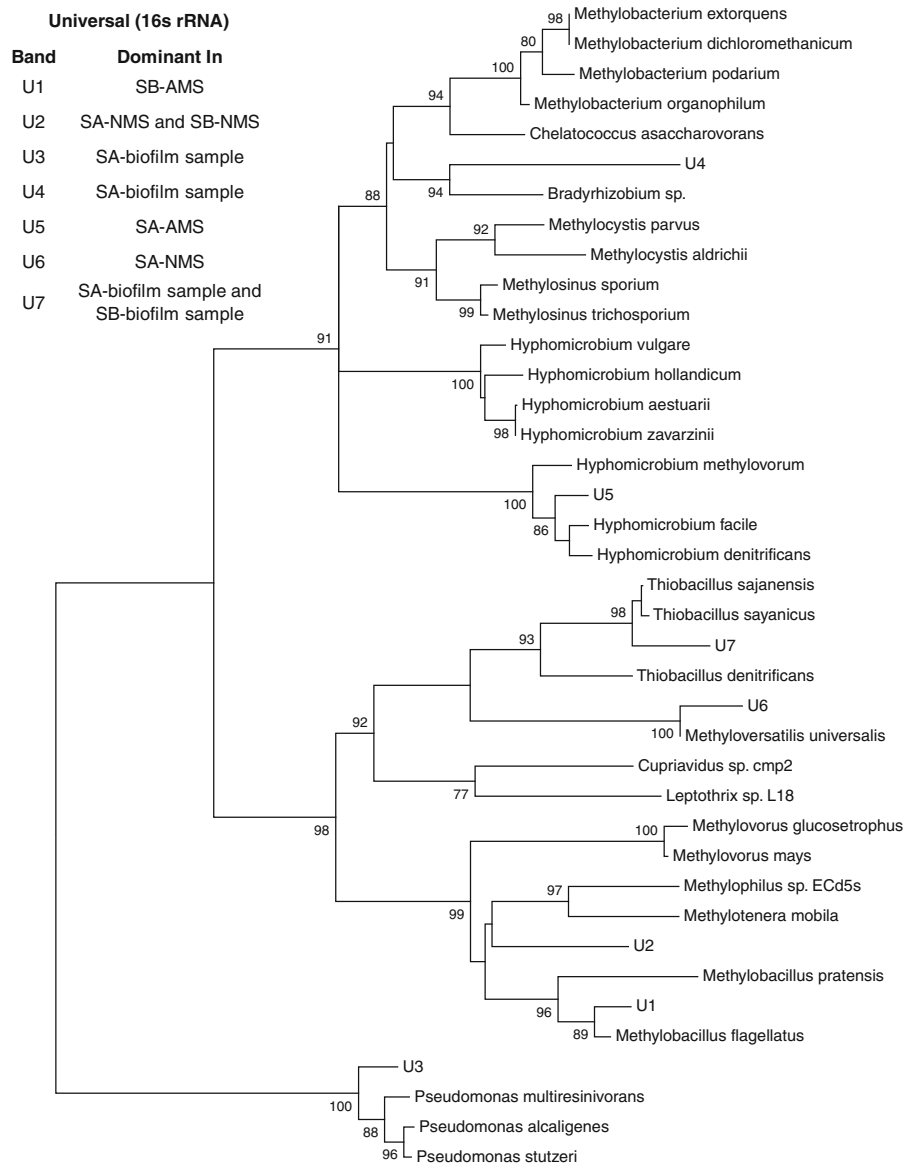

Fig. 5 Phylogenetic reconstruction of known bacteria and unknown culture strains using 16 s rRNA gene sequences (Bootstrap values represent 1,000 replicates, and values greater than 75% are shown)

As shown in Fig. 5, dominant bands obtained from 16 s rRNA of the samples also showed wide distribution among the alpha-, beta-, and gamma-proteobacteria and no consistent community structure under either enrichment profile. The original SA biofilm sample had three sequenced dominant bands: U3, U4, and U7. Band U3 was most similar to three *Pseudomonas* species (gamma-Proteobacteria); band U4 was grouped with a *Bradyrhizobium* species (alpha-Proteobacteria); and band U7 was highly similar to several *Thiobacillus* species (sulfur oxidizing chemolithoautotrophic beta-Proteobacteria). On enrichment in AMS, one dominant band was shown, U5, which was grouped with one cluster of the *Hyphomicrobium*, a similar result to what was observed when using mxaF primers. Although U5 also appeared to be present in the SA NMS enrichment, that was not confirmed by sequencing. However, the dominant SA NMS bands were U2 and U6, which were most similar in 16 s rRNA sequence to methylotrophs found in the beta-Proteobacteria, including serine cycle methylotrophs in the family

Rhodocyclaceae and RuMP cycle methylotrophs in the family *Methylophilaceae*, respectively. The original SB biofilm sample was dominated by one band, U7, which was also found in SA and described above. SB enriched in AMS produced an intense band, U1, with a 16 s rRNA sequence similar to several *Methylobacillus* species. SB enriched in NMS produced multiple strong bands, although only one was able to be recovered and sequenced, U2, which was also found in SA-NMS and shown to be similar to methylotrophic beta-Proteobacteria.

Conclusions

Results from this study illustrate that the type and concentration of nitrogen source used when enriching mixed methylotrophic cultures from samples removed from a pulp mill influences the growth and methanol degradation ability of these bacteria. Generally, cultures enriched with nitrate showed faster growth and higher methanol removal than those enriched with ammonium. Higher concentrations of nitrogen in either form also resulted in greater methanol removal in all cases. The form of nitrogen used also affected the diversity and community structure of the methylotrophic populations present in each of the final cultures. Although the enrichment process did decrease the overall diversity from the original samples, the cultures grown with nitrate as nitrogen source resulted in a higher level of species number, diversity, and evenness than those grown with ammonium. Based on the results reported herein, nitrate may potentially be a better nitrogen source when enriching or working with methylotrophic cultures from pulp and paper mills, and should be investigated further as a nutrient addition to biofilters for more effective methanol control from waste effluents.

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