Appl Microbiol Biotechnol (2004) 65: 620–626
DOI 10.1007/s00253-004-1684-5

ENVIRONMENTAL BIOTECHNOLOGY

Sachiyo Iwashita · Timothy P. Callahan · Juan Haydu · Thomas K. Wood

Mesophilic aerobic degradation of a metal lubricant by a biological consortium

Received: 2 February 2004 / Revised: 19 May 2004 / Accepted: 11 June 2004 / Published online: 28 July 2004

Abstract The metal-forming industries require the use of greases to lubricate metal surfaces during manufacturing operations, and the residues of these lubricants must be removed prior to finishing processes to protect and improve the appearance of the final product. An aqueous, biological metal-cleaning process operating under mild conditions (pH 9, 42°C) eliminates the use of environmentally unfriendly cleaning materials such as chlorinated solvents by employing microorganisms to degrade greases and oils naturally. This process was characterized in terms of initial degradation rates of a representative metal lubricant and by phylogenetic identification of the active bacteria. The metal lubricant in a surfactant solution was degraded by a bacterial consortium, and its concentration was determined by a novel gas chromatography assay. The maximum degradation rate V_{max} and the apparent K_m were obtained as 45 mg/(day mg protein) and 24 g/l on cellular basis, and 4.6 g/(day l) and 33 g/l on a volumetric basis, respectively. Mineralization of the metal lubricant was shown by analyzing the evolved CO_2 and Cl^-, and the bacterial consortium utilized the metal lubricant as a sole carbon and energy source (μ=0.05±0.01 h^{-1} at 0.5 vol% lubricant concentration). The active bacteria in the biological metal-cleaning process were identified as *Bacillus licheniformis* for the higher lubricant concentrations (3, 5, and 7.5 vol%), *Bacillus cereus* at 1 vol%, and *Pseudomonas aeruginosa*, *Rhizobiaceae* strain M100, and *Achromobacter* sp. LMG 5431 at 0.3 vol%.

S. Iwashita · T. K. Wood (✉)
Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut,
191 Auditorium Road, U-3222,
Storrs, CT, 06269, USA
e-mail: twood@engr.uconn.edu
Tel.: +1-860-486-2483
Fax: +1-860-486-2959

T. P. Callahan · J. Haydu
BioClean USA,
Bridgeport, CT, 06607, USA

Introduction

Surface-coating industries are the source of 10% of the organics in air pollution (Kaufman 1989; Darnay 1992), and the estimated value of the metal-cleaning industry in 1994 was $450 million (Murphy 1991). With new technologies in metal coating (powder, electrocoat, water-based, and high solids), the burden of supplying a high-quality final metal product has been shifted to pretreatment, the cleaning process to remove lubricant greases and oils (Gruss 2000). Consequently, efficient metal cleaning creates a direct benefit to the entire metal-surface industry.

The most common metal cleaner is an alkaline solution, and immersing the metal pieces in a tank containing an alkaline solution is the simplest method for metal cleaning (Luetja 1994; Murphy 1991). Solvents, especially chlorinated solvents such as trichloroethylene (TCE), tetrachloroethylene (PCE), and dichloromethane (DCM), are also common metal cleaners (Luetja 1994; Murphy 1991; Mertens 2000). In both the cases, hazardous wastes are generated that create environmental concerns (Mertens 2000; Callahan et al. 2001). In addition, solvent-based cleaners cost more because they are more expensive than other cleaners and require more ventilation and adequate exhaust systems to avoid putting workers at risk (Luetja 1994; Murphy 1991; Mertens 2000). In addition, TCE and PCE are the most frequently encountered groundwater contaminants at hazardous waste sites and suspected carcinogens (McCarty 1997). As a result, alternative metal-cleaning processes are desired both for economical and environmental reasons.

A continuous, aqueous, biological metal-cleaning process (40–55°C) eliminates the use of toxic cleaners by employing microorganisms to naturally degrade grease and oils without creating hazardous waste (Callahan et al. 2001). In the water-based biological metal-cleaning process, oil and grease are utilized by the bacteria so that they do not accumulate in the solution. The aqueous solution does not have to be replaced frequently, but instead lasts much longer and becomes, basically, a zero-discharge

process (Callahan et al. 2001). This biological metal-cleaning process (BioClean Process, BioClean USA) uses natural bacteria in an alkaline cleaning solution (pH 8.8–9.2) to degrade metal lubricants (Eskamani et al. 2000; Callahan et al. 2001). The pH of the process is kept in a relatively narrow range because above this pH, the activity of the bacteria is diminished, while neutral pH induces significant consumption of the biodegradable surfactants contained in the alkaline cleaning solution (Callahan et al. 2001). The alkaline cleaning solution removes the lubricants from the metal surface, using surfactants, and emulsifies them. Then, the bacteria consume the emulsified greases and oils and presumably produce CO_2 (Callahan et al. 2001). The BioClean Process is environmentally safe, economical, and promising for replacing current hazardous metal cleaners used in the metal-surface industries (Callahan et al. 2001). However, the process has not been fully characterized as the active bacteria are unknown, the degradation rates have not been calculated, and the maximum allowable lubricant concentration has not been evaluated. Additionally, the firm has relied on natural bacteria present on the metal surface and lubricants to serve as the inoculum (Callahan et al. 2001).

The aims of this study were (1) to characterize the biological metal-cleaning process by evaluating the initial degradation rates of the representative metal lubricant, TUFDraw 2812RP; (2) to verify the degradation and mineralization by testing for the growth of the consortium on the metal lubricant as a sole carbon source and by analyzing evolved CO_2; and (3) to identify the active bacteria to obtain a more consistent process that may be initiated rapidly by adding appropriate strains. This work is the first quantitative and phylogenetic report for the biological metal-cleaning process.

Materials and methods

Test lubricant, chemicals, and protein analysis

TUFDraw 2812RP (Fuchs Lubricants, Harvey, Ill.) was used as the representative metal lubricant. This metal lubricant is a widely used, extreme-pressure lubricant, and it contains naphthenic petroleum (about 33%) and chloroalkanes/chloroolefins (about 30%) as major compounds and vegetable oil and others as minor compounds; it was chosen in this study to limit air-stripping losses during the biodegradation tests. The specific gravity and the flash point of this metal lubricant are 0.968 and 138°C, respectively (Anonymous 2000), and about 13% of total mass is expected to mineralize to chlorine based on the amount and the degree of chlorination of chloroalkanes (Bucher 1986). The alkaline cleaning solution and the pH control solutions were provided by BioClean USA (Bridgeport, Conn.). The alkaline cleaning solution includes several kinds of ethoxylated biodegradable surfactants and emulsifiers (the lubricant was fully emulsified) as well as some components necessary for growth of the bacteria (sodium and potassium phosphates, sodium silicate, sodium carbonate, and ammonium chloride). Methanol (MeOH), chloroform (CF), hydrochloric acid (HCl), DCM, naphthalene, *N*,*N*-dimethylformide (DMF), NaOH, phosphoric acid (85%), phenolphthalein (1%), chloramphenicol, and kanamycin were purchased from Fisher Chemical (Fair Lawn, N.J.). The protein assay kit (Sigma Chemical, St. Louis, Mo.) was used to determine the total protein for the calculation of whole-cell-specific degradation rate.

Active bacterial consortium and growth conditions

An active bacterial consortium that had been degrading miscellaneous metal lubricants from customers was obtained from BioClean USA. Initially, the consortium was cultivated in the 1-l batch reactor in our laboratory with 0.3 vol% of the representative lubricant (TUFDraw 2812RP) in the cleaning solution (pH 7) with 10% Luria-Bertani (LB) medium (Sambrook et al. 1989). The pH of the reactor was increased gradually to pH 9 over 3–6 weeks, then −80°C glycerol stocks (15 vol%) of the reactor culture were made during the period of consistent degradation (3–10 days). For the remaining experiments, the same three glycerol stocks that were made during the consistent degradation period were grown aerobically in LB medium at 37°C for 1–2 days to reach stationary phase in shake flasks (250 ml or 1 l capacity). This consortium in the LB medium was used as an inoculum (about a tenth to a fifth of the total volume) without washing to decrease the lag period. The individual, pure strains were grown in 50 mL of 0.3 vol% lubricant in alkaline cleaning solution with pH maintained at 7.

Biological degreasing reactor and initial degradation rates

An autoclavable, 2-l batch reactor (Applikon, Foster City, Calif.) was filled with the alkaline cleaning solution (1 l) and inoculated with the pre-cultures (about 200 ml) to achieve an initial optical density at 600 nm (OD_{600}) of between 1.1 and 1.6. The reactor pH was maintained manually between 8.8 and 9.2, with the pH control solutions using phosphoric/sulfuric acids and sodium hydroxide. The reactor was agitated at 300 rpm, and the temperature was maintained at 42°C, using a heated water circulator (model DC10-B3, Thermo Haake, Baden-Württemburg, Germany). Humidified air at 130 ml/min was supplied to the reactor to minimize air stripping of water.

The initial degradation rates of the metal lubricant were determined by sampling the cultures (less than 3.5 ml/day) from the 1-l batch reactors for 4–6 days. For each lubricant concentration (0.3–15 vol%), a new reactor experiment was performed. The initial degradation rates were calculated by extrapolating the linear regression line to day 0, using daily degradation rates (1–4 days). Separate experiments were performed to obtain the stripping rates

(2–4 days) in sterile reactors (400 μg/ml of chloramphenicol and 200 μg/ml of kanamycin added). The sterility of the reactors was checked by plating daily on LB plates.

Metal-lubricant assay

The concentrations of metal lubricant were determined via gas chromatography (GC). One-milliliter samples were extracted with 3.75 ml MeOH and CF (2:1 v/v), using the method of Kates (1986), to obtain the lubricant in the presence of a large amount of cell metabolites. The samples were centrifuged at 4,960 *g* for 4 min (25°C), and the cell pellets were extracted again with 4.75 ml of a MeOH:CF:HCl (0.01%) aqueous solution (2:1:0.8 v/v). After centrifugation, the combined organic supernatants were mixed with CF (2.5 ml) and distilled water (2.5 ml), and then centrifuged at 4,960 *g* for 4 min (25°C). The lower CF-rich phase was pipetted into a new glass tube with a glass capillary pipette and dried under nitrogen gas (37°C). The remaining metal lubricant was dissolved in 1.8 ml DCM, and 1 μl was injected into a GC equipped with a flame-ionization detector (5890 Series II, Hewlett Packard, Palo Alto, Calif.), with 1% (w/v) naphthalene stock solution in DMF as an internal standard. A Hewlett Packard-1 capillary column (polydimethylsiloxane, 5 m, 0.35 mm × 2.65 μm film thickness) with a temperature gradient from 70°C to 280°C at a rate of 30°C/min was used to separate the peaks. For low concentrations (0.03 vol% and 0.1 vol%), a temperature gradient from 70°C to 280°C at a rate of 25°C/min was used. The initial and final hold times were 1 min, and the injector and the detector temperatures were set at 290°C and 300°C, respectively. The concentrations were determined by a comparison of peak heights to a standard curve. A spiked control sample (0.15 vol% lubricant added directly to DCM) indicated the extraction efficiency was 85%.

Metal lubricant conversion to CO_2

The method for the evolved CO_2 analysis followed International Standard ISO 9439 (Anonymous 1999), with some modifications. Ten milliliters of the cell suspension (OD_{600} of 0.5) in the alkaline cleaning solution were prepared with the metal lubricant (0.03 and 0.1 vol %) in a 60-ml glass serum vial covered with a Teflon-coated septum and an aluminum crimp seal. The inverted vials were shaken at 42°C at 250 rpm on a shaker (HS 250 basic, IKA Works, Wilmington, N.C.). After 1 day or 3 days, the activity was quenched by injecting 75 μl NaOH (7 M), using a liquid-tight syringe to increase the pH above 12 and to trap the evolved CO_2 as carbonate in the liquid phase prior to opening the vials; the vials were shaken for at least 1 h at 200 rpm on the shaker (Battersby 1997). Part of the cell suspension (7.5 ml) was transferred to another 19-ml glass serum vial that was connected to a 60-ml plastic syringe (Becton Dickinson, Franklin Lakes, N.J.), and 75 μl phosphoric acid (85%) was injected using a liquid-tight syringe to decrease the pH to less than 3. After acidification, the distribution of CO_2 between gas and liquid phases was 1.0 (Battersby 1997), which enabled taking almost all the CO_2 in the liquid phase to the plastic syringes, using the vacuum created by pulling the plunger slowly with occasional shaking. A total of 60 ml of gas was taken from the serum vials, filling a part of two or four 60-ml plastic syringes, depending on the concentration of metal lubricant. The same amount of atmospheric air was gradually added to the serum vial from the other side, using another 60-ml plastic syringe. The amount of CO_2 in the atmospheric air was negligible compared to CO_2 from the cells. The gas introduced to the 60-ml plastic syringes was contacted with a total of 50 ml or 144 ml $Ba(OH)_2$ aqueous solution (12.5 mM), depending on the concentrations of metal lubricant. Through this process, CO_2 in the syringes became $BaCO_3$ while the syringes that contained both the gas and $Ba(OH)_2$ were shaken for at least 1 h at 200 rpm on the shaker. Titration with HCl (5 mM) for the $Ba(OH)_2$ solution determined the amount of CO_2, using phenolphthalein as an indicator. The titration was stopped at pH 8.3 to minimize the error induced by using only the color change of phenolphthalein (Anonymous 1998). Mineralization was calculated by subtracting the amount of CO_2 from the negative control vials that contained only cell suspensions (no metal lubricant), which indicated the amount of CO_2 from cell metabolism of the alkaline cleaning-solution components or the residual LB from the inoculum. For determining the concentrations of the metal lubricant and protein, 1- and 0.7-ml samples were taken for the GC and the protein analyses, respectively. Two independent experiments for each concentration were performed for all the analyses.

Metal lubricant conversion to Cl^-

The inorganic Cl^- generated from the mineralization of the chloroalkanes/olefins in the metal lubricant was measured spectrophotometrically as described previously (Canada et al. 2002). Thrice-washed cell suspensions in 15 ml M9 Cl^--free medium (Luu et al. 1995) were contacted (OD_{600} of 0.5) with the metal lubricant (1 vol% or 3 vol%) for 1–2 days, with shaking at 42°C. The metal lubricant in the samples (1 ml) was extracted with 5 ml of MeOH:CF (1:2 v/v), followed by centrifugation (4,960 *g* for 4 min at 25°C). NaCl is expected to distribute both to upper and lower phases (Folch et al. 1957), and this was accounted for with a calibration curve generated from known Cl^- samples extracted in the presence of 1 vol% lubricant. Two independent experiments for each concentration were performed.

Phylogenetic identification of the active bacteria

Samples were taken from 1-l batch reactors when the metal lubricant (0.3–7.5 vol%) was degraded actively, and then streaked on LB agar plates to observe individual

colonies; 19 colonies with different phenotypes were picked and purified on LB agar plates. Microscopic observation indicated that most of the cells in the consortium colonized LB agar plates, presumably because the pre-cultures were prepared with LB medium. Bacterial genomic DNA was extracted from each putative species, following the method of Rodriguez and Tait (1983), except no protease was used. Polymerase chain reaction (PCR) was performed with 200 ng of the extracted genomic DNA, as described previously (Shim et al. 2000), using the primers (the positions on *Escherichia coil* K12 are indicated) HK12 (5′-GAGTTTGATCCTGGCTCAG, positions 9–27) and JCR14 (5′-ACGGGCGGTGTGTAC, positions 1,392–1,406), HK12 and HK13 (5′-TACC TTG TTA CGA CTT, positions 1492–1507) or JCR15 (5′-GCCAGCAGCCGCGGTA, positions 517–532) and JCR14. The PCR products were purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia, Calif.) then used as templates for sequencing. A dye terminator cycle sequencing protocol (Canada et al. 2002) and Vector NTI (InforMax, Bethesda, Md.) were used to obtain the sequences of 16S rRNA genes. The obtained sequences for the active bacteria were compared to those of GenBank as well as to the Michigan State University Ribosomal Databank (Maidak et al. 2001).

Specific growth rate on the metal lubricant and LB

Growth on the metal lubricant (0.1 vol% and 0.5 vol%) by the consortium was determined with ATCC 1554 mineral salts medium at 42°C (no other carbon source initial pH 7). Fifty milliliters were inoculated with the three separate LB-medium overnight cultures (30 μl each) prepared from the −80°C glycerol stocks of the consortium, as described previously. The overnight cultures were washed once with ATCC 1554 mineral salts medium to remove residual LB. It was necessary to plate the cultures for monitoring growth because the turbidity induced by the metal lubricant affected the optical density. Growth was monitored by plating the cultures on LB plates for 5 days, but the specific growth rate was obtained using the viable cell numbers on the first day. The maximum specific growth rates of the pure cultures obtained from the original consortium were also determined in LB medium at 37°C and 42°C. The initial absorbance was always less than 0.03, and the specific growth rate was determined from the data with the absorbance between 0.05 and 0.70.

Results

Growth on the metal lubricant, evolved CO_2 analysis, and extent of degradation

The metal lubricant may be used as a sole carbon and energy source by this consortium since the viable cell numbers increased for 2 days for both 0.1 vol% lubricant (cell number increased from $70\pm40\times10^5$ cells/ml to $360\pm90\times10^5$ cells/ml) and 0.5 vol% lubricant ($70\pm40\times10^5$ cells/ml to $750\pm150\times10^5$ cells/ml), while the viable cell number without the metal lubricant remained the same. The specific growth rates were $0.033\pm0.001\ h^{-1}$ and 0.051 ± 0.01^{-1} for 0.1 vol% and 0.5 vol% of the metal lubricant, respectively.

Biological degradation of the metal lubricant led to complete mineralization since after 1 day of contact, 6–7% of lubricant was converted to evolved CO_2 (Table 1). Lubricant degradation was corroborated by independent GC analysis (6–7%). Similar results were obtained after 3 days (Table 1). Although the protein concentration indicated some increase in cell mass, the alkaline cleaning solution also provides a carbon source, and it is not possible to distinguish which carbon source, the alkaline cleaning solution or metal lubricant, was used for the increase of the cell protein.

Based on the degradation measured with the CO_2 and GC analyses, the yield coefficient (g CO_2 produced/g oil degraded) was calculated. The amounts of evolved CO_2 solely from the metal-lubricant degradation were obtained by subtracting that evolved from the vials without the metal lubricant, but which contained the alkaline cleaning solution. The yield coefficient was 2–3 as shown in Table 1. The GC analysis of the vials without the consortium proved that no abiotic degradation occurred.

Chloroalkanes/olefins mineralization to Cl^-

At 1 vol% and 3 vol% lubricant concentrations, 37 ± 8.1 μM and 92 ± 4.1 μM inorganic Cl^- was generated from the mineralization of the metal lubricant after 2 days (18 ± 7.9 μM Cl^- and 80 ± 0.9 μM Cl^- were detected, respectively, after 1 day). Although chloroalkanes are classified as non-biodegradable (Wischnak et al. 1998), these compounds in this metal lubricant were clearly degraded to Cl^- by our consortium. For one negative control, *E. coil* TG1/pBS(Kan) (Canada et al. 2002)

Table 1 Metal-lubricant degradation and CO_2 produced by the consortium in 60-ml closed vials

Lubricant concentration (vol%)	Contact period (day)	Biodegradation[a] (%)	CO_2 production (%)	Protein concentration (μg/ml)	Yield coefficient (g CO_2/g oil)
0.03	1	6±4	5.7±0.2	58±7.0	2.8±0.1
	3	16±1.4	10±2	78±10	1.7±0.4
0.1	1	7±3	6.4±0.1	59.1±0.6	2.52±0.03
	3	10.2±0.9	10±2	71.6±0.7	2.6±0.4

[a]Metal-lubricant degradation via gas chromatography

contacted with 1 vol% of the metal lubricant produced less than 10 μM Cl^- after 2 days. Considering that the minimum detectable concentration with this method is 8 μM (Ryoo et al. 2000), the Cl^- signals obtained in this study were higher than background (chloride reported above controls grown without lubricant).

Kinetics of initial degradation of the metal lubricant

The initial volumetric and specific degradation rates (metal lubricant was fully emulsified) increased monotonically for the metal-lubricant concentration for concentrations up to approximately 7 vol% then decreased significantly (Fig. 1). It appears that large concentrations (greater than 7 vol%) of the metal lubricant are toxic. Based on these initial rates, V_{max} was 45 mg/(day mg protein) and K_m was 24 g/l on a cellular basis and 4.6 g/(day l) and 33 g/l, respectively, on a volumetric basis. Concentration-dependant air stripping was observed during the sterile reactor stripping tests (Fig. 1), and the stripping amounts were subtracted prior to calculating V_{max} and apparent K_m.

Identification of the active bacteria and specific growth rates on LB medium

Seven bacteria purified from the consortium were identified using 16S rRNA gene sequencing (788–1,392 bp, Table 2). The GenBank accession numbers are shown for these seven isolates, and the *B. cereus* and P. aeruginosa strains have been deposited at ATCC (AT CC BAA-1005 and BAA-1006, respectively). Although there is no fixed rule to differentiate species based on 16S rRNA gene sequencing, it has been suggested that two species are the same if they have fewer than 5–15 base differences (Fox et al. 1992; Turenne et al. 2001). Accordingly, the species that showed 98% similarity (genera *Rhizobiaceae* and *Ochrobactrum*) to the sequences in GenBank are probably distinct species (17 bp and 28 bp differences, respectively).

All five individual bacteria purified at 0.3 vol% lubricant degraded the lubricant in 250-ml shake flasks after 2 days of contact based on GC analysis (pH 7). The best strain, *Pseudomonas aeruginosa*, degraded 20% more than the original consortium, and the least degradation was seen with *Rhizobiaceae* strain M100 (17% less than the original consortium). These results demonstrated that no species showed degradation in a dominant fashion. In addition, none of five species survived at pH 9 when grown independently, so the consortium was beneficial for surviving at the higher pH. *P. aeruginosa* AL98 is reported to be a potent degrader of natural rubber (Linos et al. 2000). *Achromobacter xylosoxidans* is reported to have been recovered in the septum samples from cystic fibrosis patients (Liu et al. 2002), and it did not seem to be relevant to biodegradation. However, this species is reported as a possible contaminant of deionized water (Reverdy et al. 1984); probably the non-sterile distilled water used to prepare the alkaline cleaning solution provided this species.

Only a few kinds of colonies were observed for the higher concentrations (1–7.5 vol%): *Bacillus cereus*, *P. aeruginosa*, and *B. licheniformis*. The dominant species were *B. cereus* at 1 vol% of metal lubricant and *B. licheniformis* at 3–7.5 vol% of metal lubricants. *B. licheniformis* strain M1-1 and *B. licheniformis* DSM 13 both had 100% identical 16S rRNA sequences to the *B. licheniformis* isolated in this study, but neither species of *B. licheniformis* has been linked to biodegradation previously. The *B. licheniformis* strain isolated in this study grew rapidly at 37°C (1.69±0.00 h^{-1} in LB) and even faster at 42°C (2.12±0.01 h^{-1} in LB) and degraded the metal lubricant. This is comparable to the growth of fast-growing *E. coli* TG1 with a plasmid for the degradation of TCE at 37°C (1.56±0.05 h^{-1} in LB) (Canada et al. 2002). This novel, fast-growing *B. licheniformis* is currently being studied further for the enhanced biological

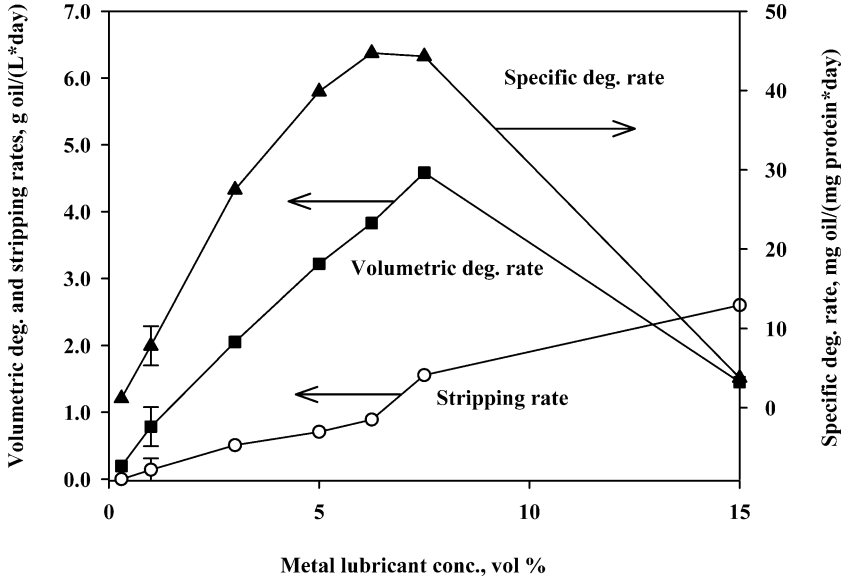

Fig. 1 Volumetric and specific initial degradation rates and stripping rates at various metal-lubricant concentrations with the batch biological metal-cleaning reactor. The volumetric and specific initial degradation rates are in excess of the stripping losses. The *error bars* represent standard errors of the average from two independent experiments. Each initial degradation rate was obtained using multiple sampling points over the 4 days

Table 2 Identification of bacterial species associated with the metal-lubricant degradation

Metal-lubricant concentration (vol %)	Colony designation	Percentage of population[a] (%)	Closest identified relative[b] (% sequence similarity)	GenBank accession no. of closest match	GenBank accession no. (this study)	Size of sequence (bp)
1	I	86	*Bacillus cereus* (100)	AF290548	AY631056	1,372
3–7.5	V	97	*Bacillus licheniformis* (100)	X68416	AY631057	1,360
0.3–7.5	1	20	*Pseudomonas aeruginosa* (100)	AY162138	AY631058	1,392
0.3	6, 16	40	*Rhizobiaceae* strain M100 (99)	AF345860	AY631059	788, 1,305
0.3	11	1–15	*Achromobacter xylosoxidans*(100)	AF411021	AY631060	1,338
0.3	19	5	*Ochrobactrum intermedium* (98)	AJ242582	AY631061	1,293
0.3	20	45	*Achromobacter* sp. LMG 5431 (100)	AF227159	AY631062	830

[a]The percentage of population was determined by estimating the populations of the colonies on Luria-Bertani-medium plates inoculated from cultures from 1-l batch reactors while the metal lubricant (0.3–7.5 vol%) was actively degraded. Since some colonies were obtained from several experiments at the same concentration, the percentage of population may exceed 100%.
[b]Identified by the nucleotide-nucleotide BLAST (blastn) in the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov).

metal-degreasing process at higher temperatures. The active bacteria identified include two bacterial species that are known surfactant-producing bacteria, *P. aeruginosa* and *B. licheniformis* (Banat et al. 2000; Cybulski et al. 2003).

Discussion

This paper shows clearly that the metal lubricant may be biodegraded based on evolved CO_2, growth on the metal lubricant as a sole carbon and energy source, and generation of Cl^-. The paper also reports the kinetic parameters for the metal-lubricant degradation, and it identifies seven bacterial species that are responsible for the degradation. Relatively fast degradation rates were observed without a lag period, and high yield coefficients were obtained, which means that most of the metal lubricant was probably not used for a growth-associated product but used for energy spilling-associated products and converted to CO_2 (Liu and Tay 2000). This was also seen in the relatively small increase of cell number before reaching a plateau. The yield coefficients obtained in this study (1.7–2.8 g/g) were more than three times higher than those reported for the degradation of petroleum hydrocarbons in aged soil (0.61 g/g) (García-Rivero et al. 2002). If one assumes the formula weight of the representative metal lubricant is about 421 (Bucher 1986; Ronningsen et al. 1989; Liley 1997), the apparent K_m value obtained in this study (24 g/l) would be about 5.7×10^{-2} M. Considering that most enzyme substrates have a value of apparent K_m between 10^{-2} M and 10^{-5} M (Bailey and Ollis 1986), the apparent K_m for this metal lubricant would be classified as low affinity, which would indicate that a high substrate concentration is required to saturate the enzyme. Because of the chlorine, an expected formula weight of the representative metal lubricant is larger than those of normal hydrocarbons.

Identification of the active bacteria was pursued so that a more consistent process would be obtained by adding the appropriate strains to initiate biological degradation in real metal-surface industries. Only a few species were seen for the higher concentrations of lubricant (greater than 1.0 vol %), and *B. licheniformis* was practically the only species at 3–7.5 vol% of the metal lubricant. This trend suggests that if it is desirable to keep bacterial diversity so that a wider variety of lubricants may be degraded, perhaps a biofilm is desirable to retain diverse species. As shown here for bacteria (Fig. 1) and as seen by others for coral (Mercurio et al. 2004), metal lubricants may be toxic at high concentrations, so their concentration as well as any biosurfactant may be important to obtain consistent metal-lubricant degradation over long periods. Biofilm reactors also have the advantage of shielding bacteria from high concentrations; hence, we are currently creating a biofilm reactor for metal-lubricant degradation.

Acknowledgements This project is supported by BioClean USA and by the Connecticut Innovations Yankee Ingenuity Technology Program.

References

Anonymous (1998) Acidity. In: Franson MAH (ed) Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC, pp 2.24–2.26

Anonymous (1999) Water quality—Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium—Carbon dioxide evolution test. International standard ISO 9439. International Organization for Standardization, Geneva, Switzerland

Anonymous (2000) The material and safety data sheet. Fuchs Lubricants, Harvey

Bailey JE, Ollis DF (1986). The kinetics of enzyme-catalyzed reactions. Biochemical engineering fundamentals. McGraw-Hill, New York, pp 86–156

Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495–508

Battersby NS (1997) The ISO headspace CO_2 biodegradation test. Chemosphere 34:1813–1822

Bucher JR (1986) Toxicology and carcinogenesis studies of chlorinated paraffins (C23, 43% Chlorine) (CAS no. 108171-27-3) in F344/N rats and B6C3F_1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser 305:1–202

Callahan TP, Norman M, Hajdu J (2001). Field experience with an integrated biological degreasing system. AESF week 2001—AESF/EPA conference for environmental excellence, Orlando, Fla.

Canada KA, Iwashita S, Shim H, Wood TK (2002) Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and trichloroethylene degradation. J Bacteriol 184:344–349

Cybulski Z, Dziurla E, Kaczorek E, Olszanowski A (2003) The influence of emulsifiers on hydrocarbon biodegradation by Pseudomonadaceae and Bacillaceae strains. Spill Sci Technol B 8:503–507

Darnay AJ (1992) Statistical record of the environment. Gale Research, Detroit

Eskamani G, Brown D, Daniels A (2000). Evaluation of BioClean USA, LLC, biological degreasing system for the recycling alkaline cleaners: environmental technology verification report. Concurrent Technologies Corporation

Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509

Fox GE, Wisotzkey JD, Jurtshuk PJ (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42:166–170

García-Rivero M, Saucedo-Castaneda G, Hoyos SFD, Gutiérrez-Rojas M (2002) Mass transfer and hydrocarbon biodegradation of aged soil in slurry phase. Biotechnol Prog 18:728–733

Gruss B (2000) Pretreatment. In: Murphy M (ed) Metal finishing, vol 98. Elsevier, New York, pp 17–25

Kates M (1986) Lipid extraction procedure. In: Burdon RH, van Knippenberg PH (eds) Techniques of lipidology, vol 3. Elsevier, Amsterdam, p 106

Kaufman W (1989) Air pollution and forests: an update. Am For 95:36–44

Liley PE (1997) Section 2: physical and chemical data. In: Perry RH, Green DW, Maloney JO (eds) Perry's chemical engineer's handbook. McGraw-Hill, New York, p 119

Linos A, Reichelt R, Keller U, Steinbüchel A (2000) A gram-negative bacterium, identified as *Pseudomonas aeruginosa* AL98, is a potent degrader of natural rubber and synthetic *cis*-1,4-polyisoprene. FEMS Microbiol Lett 182:155–161

Liu Y, Tay JH (2000) A kinetic model for energy spilling-associated product formation in substrate-sufficient continuous culture. J Appl Microbiol 88:663–668

Liu L, Coenye T, Burns JL, Whitby PW, Stull TL, LiPuma JJ (2002) Ribosomal DNA-directed PCR for identification of *Achromobacter* (*Alcaligenes*) *xylosoxidans* recovered from sputum samples from cystic fibrosis patients. J Clin Microbiol 40:1210–1213

Luetja RE (1994) Surface cleaning. In: Cotell CM, Sprague JA, Smidt JFA (eds) ASM handbook, vol 5. ASM International, Materials Park, pp 3–78

Luu PP, Yung CW, Sun AK, Wood TK (1995) Monitoring trichloroethylene mineralization by *Pseudomonas cepacia* G4 PR1. Appl Microbiol Biotechnol 44:259–264

Maidak BL, Cole JR, Lilburn TG, Parker CTJ, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001) The RDP-II (Ribosomal database project). Nucleic Acids Res 29:173–174

McCarty PL (1997) Breathing with chlorinated solvents. Science 276:1521–1522

Mercurio P, Negri AP, Burns KA, Heyward AJ (2004) The ecotoxicology of vegetable versus mineral based lubricating oils. 3. Coral fertilization and adult corals. Environ Pollut 129:183–194

Mertens JA (2000) Vapor degreasing with chlorinated solvents. In: Murphy M (ed) Metal finishing, vol 98. Elsevier, New York, pp 43–51

Murphy DP (1991) Metal surface treatment: cleaning. In: Howe-Grant M (ed) Kirk-Othmer encyclopedia of chemical technology, vol 16. Wiley, New York, pp 432–436

Reverdy ME, Freney J, Fleurette J, Coulet M, Surgot M, Marmet D, Ploton C (1984) Nosocomial colonization and infection by *Achromobacter xylosoxidans*. J Clin Microbiol 19:140–143

Rodriguez RL, Tait RC (1983) DNA isolation. Recombinant DNA techniques: an introduction. Benjamin/Cummings, Menlo Park, pp 37–44

Ronningsen HP, Skjevrak I, Osjord E (1989) Characterization of North Sea petroleum fractions: hydrocarbon group types, density, and molecular weight. Energy Fuel 3:744–755

Ryoo D, Shim H, Canada K, Barbieri P, Wood TK (2000) Aerobic degradation of tetrachloroethylene by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1. Nat Biotechnol 18:775–778

Sambrook J, Fritsch EF, Maniatis T (1989) Appendix A: Bacterial medium, antibiotics, and bacterial strains. In: Nolan C (ed) Molecular cloning: a laboratory manual, vol 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp A.2–A.12

Shim H, Chauhan S, Ryoo D, Bowers K, Thomas SM, Canada KA, Burken JG, Wood TK (2000) Rhizosphere competitiveness of trichloroethylene-degrading, poplar-colonizing recombinant bacteria. Appl Environ Microbiol 66:4673–4678

Turenne CY, Tschetter L, Wolfe J, Kabani A (2001) Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* sp. J Clin Microbiol 39:3637–3648

Wischnak C, Löffler FE, Li J, Urbance JW, Müller R (1998) *Pseudomonas* sp. strain 273, an aerobic α,ω-dichloroalkane-degrading bacterium. Appl Environ Microbiol 64:3507–3511