Biodegradation (2011) 22:397–407
DOI 10.1007/s10532-010-9412-6

ORIGINAL PAPER

Comparison of microbial communities in pilot-scale bioreactors treating Bayer liquor organic wastes

Naomi J. McSweeney · Jason J. Plumb · Amanda L. Tilbury · Hugh J. Nyeboer · Matt E. Sumich · Anthony J. McKinnon · Peter D. Franzmann · David C. Sutton · Anna H. Kaksonen

Received: 6 May 2010 / Accepted: 26 August 2010 / Published online: 11 September 2010

Abstract Western Australian bauxite deposits are naturally associated with high amounts of humic and fulvic materials that co-digest during Bayer processing. Sodium oxalate remains soluble and can co-precipitate with aluminium hydroxide unless it is removed. Removal of sodium oxalate requires a secondary crystallisation step followed by storage. Bioreactors treating oxalate wastes have been developed as economically and environmentally viable treatment alternatives but the microbial ecology and physiology of these treatment processes are poorly understood. Analysis of samples obtained from two pilot-scale moving bed biofilm reactors (MBBRs) and one aerobic suspended growth bioreactor (ASGB) using polymerase chain reaction- denaturing gradient gel electrophoresis of 16S rRNA genes showed that members of the α-, β- and γ-*Proteobacteria* subgroups were prominent in all three processes. Despite differing operating conditions, the composition of the microbial communities in the three reactors was conserved. MBBR2 was the only configuration that showed complete degradation of oxalate from the influent and the ASGB had the highest degradation rate of all three configurations. Several strains of the genus *Halomonas* were isolated from the bioreactors and their morphology and physiology was also determined.

Keywords Bayer process · Oxalate bioreactor · 16S rRNA analysis · Microbial ecology

N. J. McSweeney · D. C. Sutton
Microbiology and Immunology, M502, School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, Nedlands, WA 6009, Australia

N. J. McSweeney (✉) · J. J. Plumb · P. D. Franzmann · A. H. Kaksonen
CSIRO Land and Water, Private Bag No 5, Floreat, WA 6913, Australia
e-mail: naomi.mcsweeney@csiro.au

A. L. Tilbury · H. J. Nyeboer · M. E. Sumich · A. J. McKinnon
Alcoa World Alumina, Technology Delivery Group, Kwinana Refinery, PO Box 161, Kwinana, WA 6966, Australia

Introduction

Aluminium is the most abundant metallic element in the Earth's crust and is extensively used in various industries due to its versatility (Driscoll and Schecher 1990; Hind et al. 1999). Bauxite is the primary ore for aluminium extraction and contains a number of aluminium containing minerals, including gibbsite ($Al(OH)_3$) and boehmite (AlO(OH)). Bauxite is refined by the Bayer process first by precipitation to produce aluminium hydroxide which is then heated to produce alumina (aluminium oxide; Al_2O_3). Alumina is then electrolysed to produce primary aluminium metal or fabricated aluminium products.

In the south-west of Western Australia, bauxite is naturally associated with large amounts of humic and fulvic materials (Hind et al. 1999). During initial digestion of bauxite with hot caustic, these materials are co-digested to produce a large range of sodium salts of organic acids including acetate, formate, malonate, succinate and oxalate (Smeulders et al. 2001). The organic acids produced by the Bayer processing of bauxite affect the quality and yield of precipitated aluminium hydroxide. Due to the cyclical nature of the Bayer process, organic acids can build up in the liquor stream. Unless removed, sodium oxalate remains saturated in the process stream and co-precipitates with aluminium hydroxide as acicular crystals which prevent alumina agglomeration. This results in excess alumina dust and caustic losses, and ultimately increases the operating costs associated with Bayer processing (Hind et al. 1999; Brown and Cole 1980). It is necessary to remove sodium oxalate from the liquor stream to avoid detrimental effects of co-precipitation with aluminium hydroxide. In some refineries, this is achieved by crystallisation of the sodium oxalate in a secondary process stream. Previous utilisation and disposal methods for the resulting solid sodium oxalate waste have proven to be expensive and require expensive control systems to prevent the emission of odorous compounds.

Bioreactor processes have been developed for use in Bayer refineries for the destruction of oxalate in a more environmentally acceptable and economically viable way (Chinloy et al. 1993; Morton et al. 1991; Thè et al. 1992; Brassinga et al. 1990). However, the microbial ecology and physiology of these oxalate-degrading bioreactors are poorly characterised.

In this study, 16S rRNA analysis was used to characterise the microbial communities in three pilot-scale bioreactor configurations demonstrating near-complete or complete oxalate destruction from influent feeds composed of sodium oxalate slurry treated with carbonated refinery residue lake water to reduce the feed pH. Culture-dependent methods were used to isolate and identify potential key oxalate-degrading microorganisms from each configuration. Here we describe the microbial composition of pilot-scale oxalate-degrading bioreactors and identify the key microbial groups of microorganism which may contribute to the biological removal process.

Materials and methods

Bioreactor configurations and sampling

The microbial communities of three bioreactor configurations were analysed in this study. Reactor configurations differed only in operating conditions. Directional flow of oxalate containing influent and the effluent is shown in the schematic of Fig. 1. The moving bed biofilm reactors (MBBR 1 and 2) contained approximately 50% (w/v) of high-density polyethylene beads with protected surface area (300 m^2/m^3) for biofilm growth (AnoxKaldnes; Natrix model O). The aerobic suspended growth bioreactor (ASGB) was operated in the same manner as the MBBRs without the addition of the polyethylene beads for growth. Bioreactors were inoculated with activated sludge as an initial source of biomass, nutrients and growth factors. The influent of the reactors consisted of carbonated process liquor (pH 9.5) and varying concentrations of sodium oxalate (Table 1). An EDI MaxAir Diffuser (Environmental Dynamics) provided aeration to the reactors during operation at a rate of 60–65 kg/h. Flow rates and hydraulic and suspended retention times varied for the different configurations (Table 1). Reactors were considered to have developed a stable microbial community after 6–9 months of operation with no notable disruption to the biological removal of oxalate. Samples for 16S rRNA analysis were stored at −80°C prior to DNA extraction.

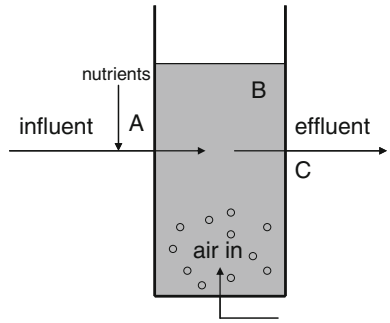

Fig. 1 Simplistic flow schematic of pilot-scale oxalate-degrading bioreactors. MBBRs were operated with 50% (w/v) polyethylene beads as a growth support whilst the ASGB configuration was not. Aeration was provided by a diffuser. Samples were taken from influent (*a*) and bead biomass (*b*) of the MBBRs and the effluent (*c*) of the MBBRs and the ASGB configurations

Table 1 Physico-chemical measurement of the bioreactors at the time of sampling for 16S rRNA analysis

	MBBR 1	MBBR 2	ASGB
Reactor volume (m^3)	3.8	3.8	3.8
Flow rate (l/h)	350	200	100
Hydraulic retention time (h)	11.4	20	35.4
Average temperature (°C)	32.5	35	38.5
Average dissolved oxygen (mg/l)	5.8	3.7	2.3
Average pH	9.6	9.6	9.6
Influent oxalate (g/l)	13.8	34.3	92
Effluent oxalate (g/l)	8.0	0	10
Percentage degradation	58%	100%	89%
Degradation rate (g/l h)	0.531	1.806	2.158

16S rRNA gene analysis

DNA extraction and PCR

Duplicate liquid samples from the influent and effluent of all reactors and beads with attached biomass from the MBBRs were collected and transported to the laboratory on ice within 2 h of collection. Reactor fines were separated from liquid samples by low speed centrifugation (3,000×*g*, 1 min). Biofilm was removed from each bead collected from the MBBRs using sterile spatulas. Total genomic DNA was extracted from liquid and biofilm samples using a phenol: chloroform: isoamyl alcohol (24:1:1; pH 8.0) extraction and sodium acetate (3 M, pH 5.2) and isopropanol precipitation as described previously (Plumb et al. 2002).

Polymerase chain reaction (PCR)

16S rRNA genes were amplified using polymerase chain reaction (PCR) using the HotStar Taq PCR kit with bacterial 27F forward primer and universal 1492R reverse primer (Table 2) with the following thermal cycling program: 15 min at 94°C; 35 cycles of 95°C for 1 min, 48°C for 1 min and 72°C for 2 min; 10 min at 72°C. Primers for PCR were used at a concentration of 25 μM. PCR products were purified using the UltraClean PCR Purification kit (MoBio Laboratories). These products were used as templates for a second amplification with bacterial 357F-GC forward primer and bacterial 907R reverse primer (Table 2) with the following thermal cycling program: 95°C for 15 min; 94°C for 1 min, 63°C for 1 min and 72°C for 2 min. The annealing temperature of 63°C was decreased by 1°C every second cycle until it reached 53°C, followed by a final extension step at 72°C for 10 min.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the DCode Universal Mutation Detection System (BIORAD) as per the

Table 2 Primers used for PCR amplification and sequencing of 16S rRNA genes

Primer[a]	Sequence (5′ to 3′)	Target	Reference
27F[b]	GAGTTTGATCCTGGCTCAG	*Bacteria*	Lane (1991)
357F	CCTACGGGAGGCAGCAG	*Bacteria*	Muyzer et al. (1996)
357F-GC[c]	CCTACGGGAGGCAGCAG	*Bacteria*	Muyzer et al. (1996)
530F[d]	GTGCCAGC**M**GCCGCGG	Universal	Lane (1991)
518R[d]	G**W**ATTACCGCGGC**K**GCTG	Universal	Muyzer et al. (1996)
907R[b]	GTGCTCCCCCGCCAATTCCT	Universal	Lane (1991)
1492R[b,e]	ACGG**dI**TACCTTGTTACGACTT	Prokaryotes	Lane (1991)

[a] The number corresponds to the *E. coli* position to which the 3′ end of the primer anneals. F and R correspond to forward and reverse primers, respectively

[b] Modified from the original primer

[c] GC is a 40 nucleotide GC-rich sequence attached to the 5′ end of the primer. The GC sequence is 5′-CGCCCGCCGC GCGCGGCGGGCGGGGCGGGGGCACGGGGGG

[d] Primer mixed base codes (Geneworks): K (GT); M: (AC); W (AT); Y (CT)

[e] dI denotes a deoxyinosine modification

manufacturer's instructions on a 7% (w/v) polyacrylamide gel (40% acrylamide/bis solution, 37.5:1) with a gradient of 30–80% or 40–60% (100% denaturant: 7 M urea, 40% deionised formamide). Gels were run at 150 V (60°C) for at least 5 h. Bands were excised from the gels with sterile scalpel blades and DNA was eluted into RNase/DNase- free water. DNA fragments were reamplified with primers 357F and 907R (Table 2) and purified with the UltraClean PCR Purification kit. Sequencing of the 16S rRNA gene variable regions was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with bacterial reverse primer 907R (1 pM). Products were purified using the AmPure purification system (Agencourt Biosciences Corporation) and sequenced by electrophoresis at the LotteryWest Biomedical Facility (Royal Perth Hospital, Western Australia). DNA sequences were analysed with the Genbank database available at http://www.ncbi.nlm.nih.gov using BLAST (Basic Local Alignment Search Tool) analysis to provide the identity of the sequence (Altschul et al. 1990).

Isolations

Isolates were obtained by serial decimal dilution in liquid culture and the streak plate method at 35°C (without shaking) in a modified haloalkaline oxalate medium (HAO mod) (g/l: NaCl, 15; sodium oxalate, 5; sodium citrate, 3; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 1; urea: ammonium: nitrate solution (Flexi-N), 0.668 ml; $(NH_4)_3PO_4$, 0.0016; pH 9.5) based on moderately haloalkaline *Halomonas* medium (DSMZ 1034). The highest dilutions from each liquid sample exhibiting growth and individual colonies were subcultured three consecutive times to ensure purity.

Microscopy, physiology and phylogeny of isolated strains

Bioreactor samples and isolates were examined by phase contrast and bright field microscopy with Gram and Sudan B Black staining for determination of morphology. Cardinal growth temperatures (T_{MIN}, T_{OPT} and T_{MAX}) of representative isolates were determined using a temperature gradient incubator (TerraTec) as described previously (Hawkes et al. 2006; Ratkowsky et al. 1983). Growth was measured as optical density (OD) at 540 nm. Initial and end-point sodium oxalate and sodium citrate concentrations were measured by gas chromatography and total organic carbon analysis respectively. DNA was extracted and the 16S rRNA genes were amplified as previously described. Near full-length 16S rRNA genes were sequenced with the following bacterial primers (1 pM): 27F; 357F; 530F; 519R; 907R and 1492R (Table 2). 16S rRNA gene sequence alignments were constructed using the ClustalW alignment tool in the Mega4 software (v. 4.0.2) (Tamura et al. 2007) with 23 reference sequences obtained from the GenBank database available at http://www.ncbi.nlm.nih.gov (Altschul et al. 1990). A phylogenetic tree of the isolates was constructed using the distance matrix and neighbour joining method with Jukes and Cantor single parameter correction method with the Mega4 software.

Nucleotide sequence accession numbers

16S rRNA sequences for the isolates were submitted to GenBank using the online BankIt software (http://www.ncbi.nlm.nih.gov/BankIt/) under the accession numbers GU228472-GU228486.

Results

Bioreactor performance

At the time of sampling for 16S rRNA gene analysis, MBBR1, MBBR2 and ASGB were degrading 58, 100 and 89% of the sodium oxalate contained within the influent feeds, respectively. The degradation rate of oxalate for MBBR1, MBBR2 and ASGB was 0.531, 1.806 and 2.158 g/l h, respectively. The reactors were considered to have stable microbial communities after operating continuously for 6–9 months without any major disruptions to the process. MBBR1, MBBR2 and ASGB had hydraulic retention times of 11.4, 20 and 35.4 h and oxalate feed concentrations of 13.8, 34.3 and 92 g/l, respectively. The average temperatures in MBBR1, MBBR2 and ASGB were 32.5, 35 and 37°C, respectively. The dissolved oxygen concentrations in MBBR1, MBBR2 and ASGB were 5.8, 3.7 and 2.3 mg/l, respectively. The average pH in each reactor stayed consistent at approximately 9.6.

Microbial community composition of the bioreactors

DGGE fingerprints were obtained by analysing the bacterial DNA extracted from influent and bead samples from MBBR1 and MBBR2 and effluent samples from MBBR1, MBBR2 and ASGB (Fig. 1). Bands were excised from one of the two duplicate DNA extracts from each bioreactor sample. The bands showing the same relative migration distances were considered to have originated from same species. The assignment of bands into species was performed by sequence analysis of the eluted DNA from the selected bands numbered 1–36 in the DGGE fingerprints (Fig. 2). Bands which failed to give quality sequences are not listed in this Table.

DGGE analysis of DNA obtained from MBBR1, MBBR2 and ASGB showed that the microbial populations in the three reactor configurations were similar and that the overall diversity within the reactors was low (Table 3). Species closely related to *Halomonas (H.) nitritophilus* (99% similarity) and *H. salina* (99% similarity) were detected in the influent of MBBR1 and in the effluent and bead biofilm of MBBR1 and MBBR2. A band representing *γ-Proteobacterium* was also detected in the ASGB configuration (95% similarity). Species related to *Azoracus (Az.) denitificans* (95% similarity) and *Az. tolulyticus* (95% similarity) were detected in the effluent of MBBR2 and ASGB. Bands with closest DNA similarity matching to *Paracoccus kocurri* (96% similarity) were also detected in the bead biofilm from MBBR1. Species closely related to *Rhodobaca (Rca.) barguzinensis*, *Rca. bogoriensis* and *Roseinatronobacter monius* (all with 99% similarity) were detected in bead biofilm and in the effluent of MBBR1 and the bead biofilm in MBBR2. The sequences from these bands could not differentiate between the three species. Other members belonging to the α subgroup of *Proteobacteria* and related to *Devosia (Dva.) yakushimanenisis* and *Dva. subaequoris* were also detected in the effluent of MBB1 and the bead biofilm in MBBR2 (<93% similarity).

Morphological, physiological, and phylogenetic analysis of isolates

Morphology

Microorganisms contained within all bioreactor samples were morphologically similar and mostly

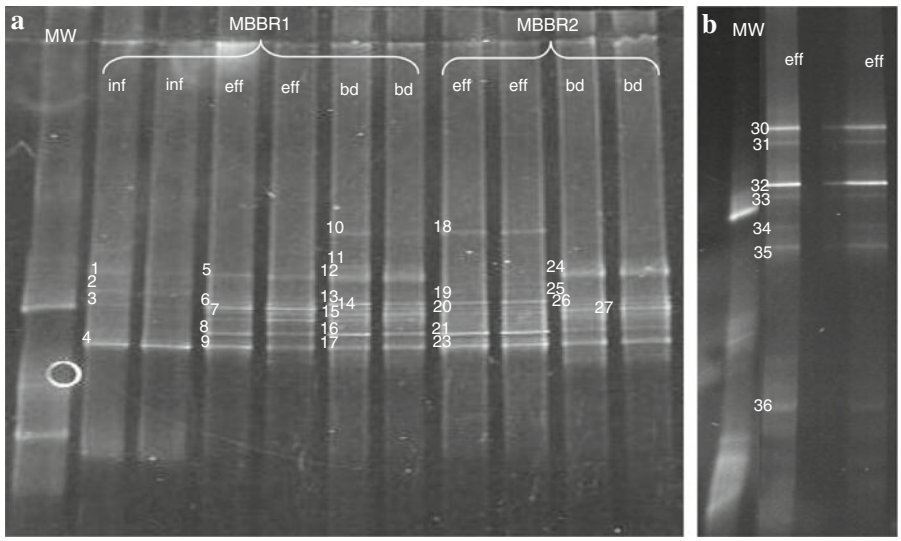

Fig. 2 Denaturing gradient gel electrophoresis (30–80%) of the 16S rRNA genes obtained from DNA extracts from MBBR1 and MBBR2 (**a**) and ASGB (**b**). Samples from each reactor configuration were run in duplicate and are labelled with the location of where the sample was taken: influent (inf); effluent (eff); bead (bd). Bands that were excised are numbered 1–36. Closest relatives were determined using BLAST analysis on the GenBank database and are listed in Table 3 (MW: molecular weight marker)

Table 3 Closest relatives to 16S rRNA variable region sequences from bioreactor samples as detected by 16S rRNA PCR-DGGE analysis

Band(s)[a]	Closest relative	*Proteobacteria* subgroup	% Similarity[b]	Accession no[c]	Reference
1	Uncultured *Marinomonas* sp. clone F3C93	*Gamma*	92	AY794175	Unpublished
2, 3, 4, 17	*Halomonas nitritophilus* isolate WST3	*Gamma*	96–99	DQ289066	Unpublished
5	*Devosia yakushimanenis*	*Alpha*	91	AB361068	Unpublished
6, 7, 14, 15, 25, 26, 27	*Rhodobaca barguzinensis* strain VKM B-2406	*Alpha*	99	EF554833	Boldareva et al. (2008)
	Rhodobaca bogoriensis strain LBB2		99	AF384205	Unpublished
	Roseinatronobacter monicus strain ROS 10		99	DQ659237	Boldareva et al. (2007)
8, 9, 23	*Halomonas salina* isolate LLM	*Gamma*	99	DQ333297	Joshi et al. (2008)
13	*Paracoccus kocurri* strain JCM 7684	*Beta*	96	D32241	Katayama et al. (1995)
16	*Azoarcus denitrificans* tol-4	*Beta*	97	L33694	Zhou et al. (1995)
18, 19, 20, 21, 31, 32, 34, 35, 36	*Azoarcus tolulyticus* strain 4FB10	*Beta*	97	AF229876	Song et al. (2000)
24	*Devosia subaequoris* type strain HST3-14	*Alpha*	93	AM293857	Lee (2007)
30	Gamma *proteobacterium* M2-24D	*Gamma*	95	AY730243	Unpublished

[a] Bands are numbered as indicated in Fig. 2. Bands that did not produce a readable sequence are not listed

[b] Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank database

[c] Accession number of the closest relative found by BLAST analysis

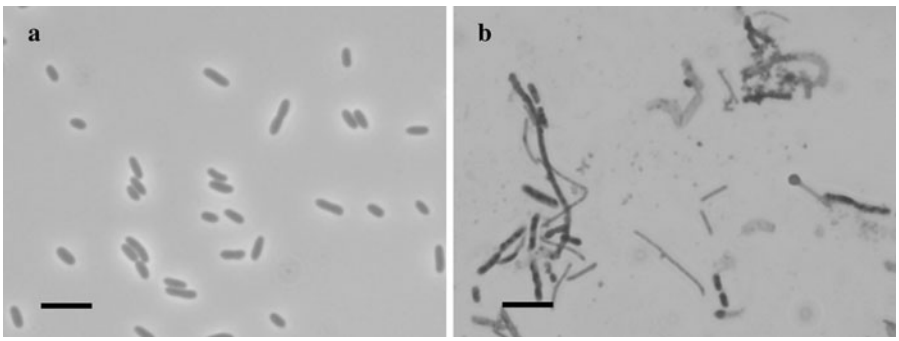

Fig. 3 Phase contrast micrograph showing the morphology (**a**) and bright field micrograph of the Sudan B Black staining showing the polyhydroxybutyrate particles (**b**) of representative strain HE0.1.1 obtained from MBBR1. *Scale bar* 5 μm

consisted of Gram negative and PHB accumulating bacillus. Fifteen pure strains were isolated from the MBBR1, MBBR2 and ASGB configurations with HAO medium. The isolates were examined with phase contrast and bright field microscopy to determine cellular morphology. All strains were Gram negative, motile bacilli, 3–5 μm in length and 0.5–1 μm in width (Fig. 3a). All strains stained positively for the presence of polyhydroxybutyrate (PHB) when stained with Sudan B Black (Fig. 3b).

Physiology

The optimum temperatures (T_{OPT}) for representative strains HB0.1.1 (Fig. 4) and HE0.1.1 (Fig. 5) were 44.2 and 32.4°C respectively. The citrate concentration had decreased by approximately 82% and the oxalate concentration had decreased by approximately 32% (data not shown) in pure cultures growing on a medium that contained both oxalate and citrate.

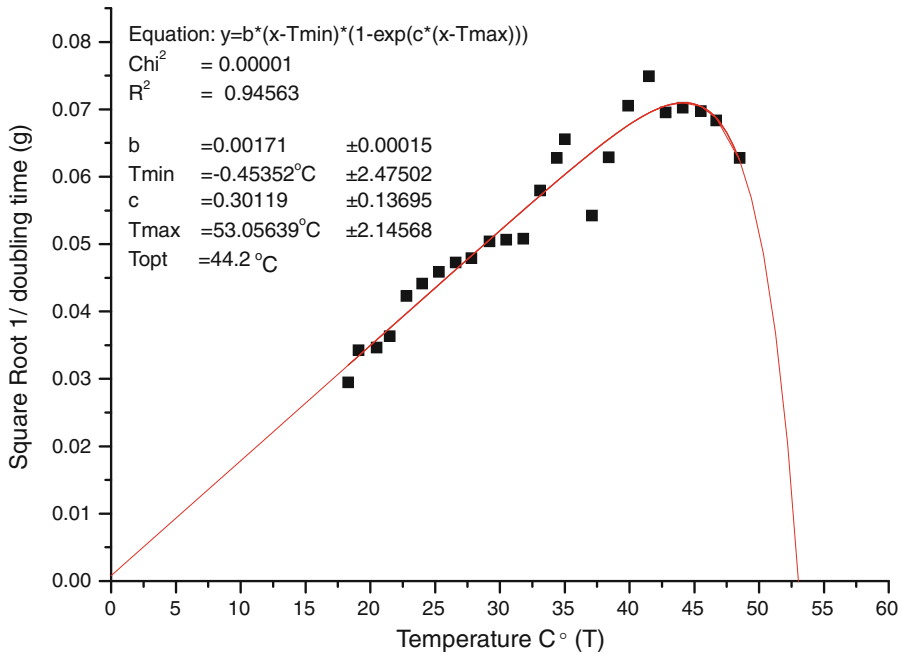

Fig. 4 Optimum temperature of growth for representative strain HB.0.1.1 as determined by temperature studies using the Ratkowsky method (Ratkowsky et al. 1983)

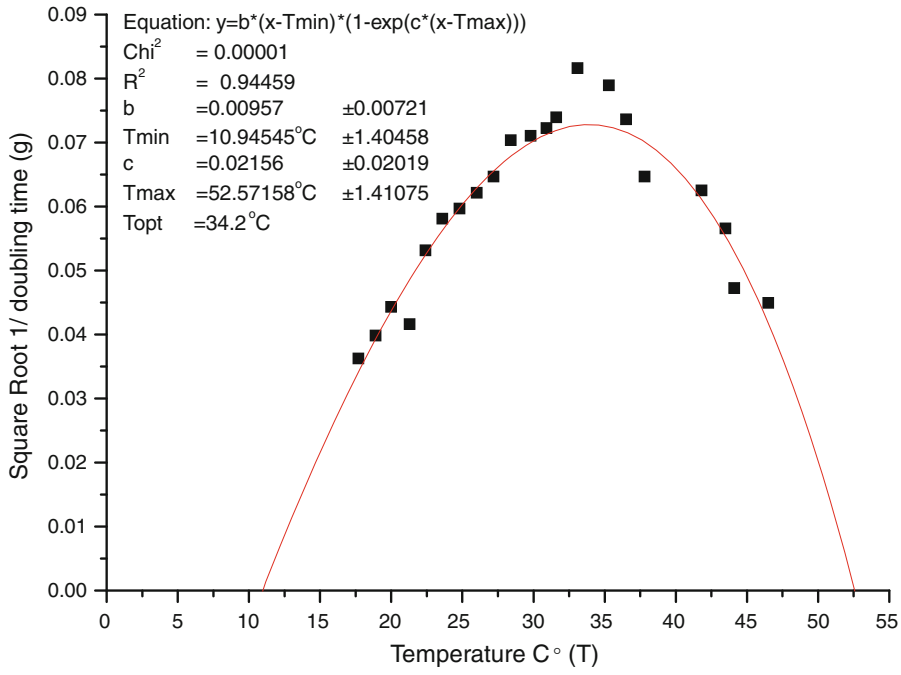

Fig. 5 Optimum temperature of growth for representative strain HE.0.1.1 as determined by temperature studies using the Ratkowsky method (Ratkowsky et al. 1983)

Phylogeny

Almost complete 16S rRNA gene sequences (approximately 1500 nucleotides) from isolates were obtained following PCR amplification and sequencing. BLAST analysis using the GenBank database indicated that all isolates belonged to the genus *Halomonas*. A phylogenetic tree constructed using Mega4 confirmed that the isolates clustered with a few members of this genus (Fig. 6). Isolates HB0.1.3, HI10.1, HB01, HE0br, HE01, HI0.1.1, HB10.1 isolated from MBBR2 influent, effluent and bead

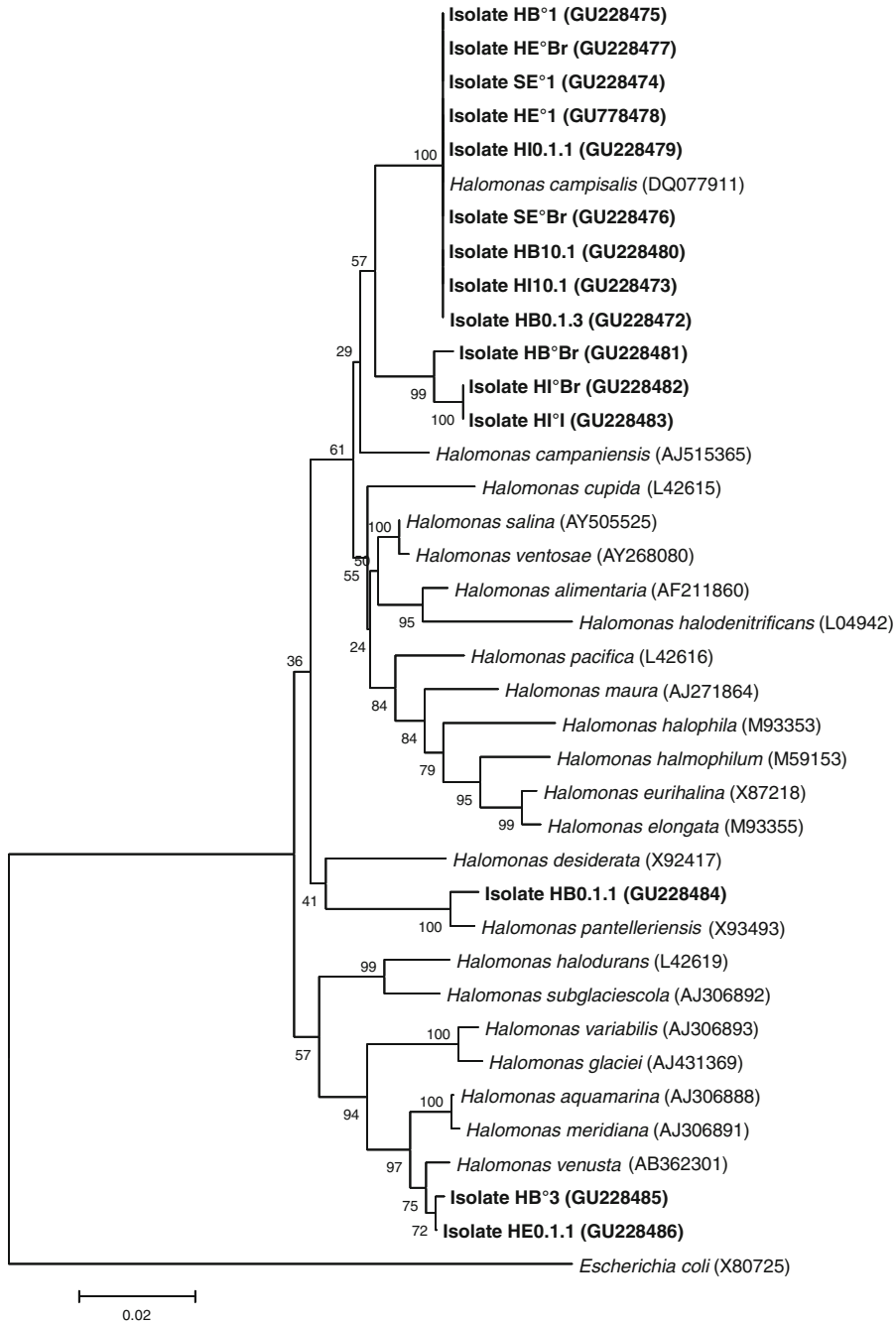

Fig. 6 Phylogenetic tree of isolates and members of the genus *Halomonas* based on 16S rRNA gene sequence analysis. The scale shows 0.02 nucleotide substitutions per gene. Bootstrap values were determined from 1000 resamplings. Isolates from MBBR2 were labelled with the prefix HI, HE and HB and isolates from ASGB were labelled with the prefix SE. Isolates are indicated in *bold font* and NCBI GenBank accession numbers are given in *parentheses*

biofilm and SE01 and SE0br isolated from ASGB effluent were most closely related to *Halomonas campisalis* strain LL6 (99%) which was isolated from a salt plain of Alkali Lake, Washington State (USA) (Joshi et al. 2008). Strains HB0br, HI0br and HI01 isolated from MBBR2 influent and bead biofilm were

also most closely related to this strain of *Halomonas campisalis*, though the 16S rRNA similarity was slightly lower (98–99%). Strain HB01.1 isolated from MBBR2 bead biofilm was most closely related (99%) to a strain of *Halomonas pantelleriensis* isolated from the sand of an alkaline lake in Pantelleria Island, Italy (Romano et al. 1996). Strains HB03 and HE0.1.1 isolated from MBBR2 effluent and bead biofilm, were most similar (100%) to an uncultured strain of *Halomonas venusta* isolated from a dialysis unit treating people for kidney disease [unpublished]. Based on the evolutionary distances of these strains from their nearest described relatives some of the isolates may represent novel species or sub-species within the genus *Halomonas*. The morphology of all isolated strains matched the described morphology of their closest relatives (Arahal and Ventosa 2006; Mormile et al. 1999; Romano et al. 1996; Joshi et al. 2008).

Discussion

16S rRNA gene analysis was used to profile the microbial communities within three pilot-scale oxalate-degrading bioreactors. A comparison of the degradation rate of each configuration showed that the ASGB was degrading more oxalate at a faster rate than both MBBR1 and 2. ASGB also had a significantly longer HRT than the MBBRs. Despite the differences observed in the operating conditions of the three pilot-scale configurations, PCR-DGGE fingerprints showed that the microbial communities in each of the bioreactors were similar and that the microbial diversity in each of the bioreactors was low. All microorganisms detected belong to the α, γ and β subgroups of *Proteobacteria*. The closest relatives were described as moderately halophilic, alkaliphilic bacilli.

Representatives belonging to the *γ-Proteobacteria* subgroup were detected in the influent, effluent and bead of MBBR1, the effluent and bead of MBBR2 and the effluent of ASGB. "*Halomonas nitritophilus*" (96–99% similarity) [unpublished] and *Halomonas salina* (99% similarity) (Caton et al. 2004) were the closest relatives to the microorganisms detected in the MBBR1 and MBBR2 bioreactor populations. A match to an uncultured *γ-Proteobacterium* clone was also detected in the ASGB configuration but with only 95% 16S rRNA gene similarity. Members of the genus *Halomonas* can grow using simple organic acids such as acetate, formate, malonate and succinate as sole sources of carbon and energy (Caton et al. 2004; Joshi et al. 2008; Mormile et al. 1999; Romano et al. 1996). However, species of *Halomonas* have not been reported to use oxalate as a sole source of carbon and energy. The detection of *Halomonas* spp. in all three bioreactor process suggests that they may have a key role in the biological degradation of oxalate and other organic acids in these biological processes.

Bacteria related to *Azoarcus (Az.) denitrificans* and *Az. tolulyticus* belonging to the *β-Proteobacterium* subgroup were detected in all three bioreactor configurations. These microorganisms are described as facultatively anaerobic denitrifiers capable of growth on organic environmental pollutants such as toluene and other hydrocarbons (Zhou et al. 1995; Anders et al. 1995). The percentage 16S rRNA gene similarity of uncultured bioreactor strains with the described strains of *Azoarcus* was low (<95%) suggesting that a new genus of *β-Proteobacterium* populates these bioreactors. Nitrogen is supplied to the bioreactor in excess in the form of a crude urea: ammonium: nitrate fertilizer and would be readily available within the bioreactor medium. The genus *Azoarcus* is typically known for containing many nitrogen-fixing and denitrifying microorganisms (Anders et al. 1995; Zhou et al. 1995), so it is possible that the microorganisms most closely related to the *β-Proteobacteria* subgroup may play a key role in the nitrogen cycle of the bioreactor process. However, analysis into the roles of these microorganisms in biological oxalate degradation is required and is currently underway.

Matches to microorganisms belonging to the *α-Proteobacterium* group were detected in the biofilm of MBBR1 and MBBR2 but were absent from effluent samples obtained from ASGB. The closest matches by percentage 16S rRNA gene similarity (99%) were to *Roseinatronobacter monicus*, *Rhodobaca (Rca.) barguzinensis* and *Rca. bogoriensis.* Microorganisms belonging to the genus *Rosinatronobacter* are typically described as obligately aerobic, heterotrophic, haloalkaliphilic and natronophilic and are able to produce pigments such as carotenoid and bacteriochlorophyll α under reduced light conditions (Boldareva et al. 2007). The species belonging

to the *Rhodobaca* genus are haloalkaliphilic, purple nonsulfur bacteria capable of anoxygenic photosynthesis under anaerobic conditions and also produce photosynthetic pigments. These microorganisms can also grow heterotrophically under aerobic conditions (Milford et al. 2000; Boldareva et al. 2008). The noticeable absence of this subgroup of microorganisms in the ASGB configuration and in the suspended biomass of both MBBR configurations suggests that these microorganisms may require a support for growth under the bioreactor operating conditions.

Strains of *Halomonas* spp. were isolated in a haloalkaline medium containing oxalate and citrate as sources of carbon and energy. Like all members of the *Halomonas* genus, the strains were capable of growing organotrophically in pure culture with oxalate when an additional source of carbon and energy was supplied (Arahal and Ventosa 2006; Mormile et al. 1999; Joshi et al. 2008; Romano et al. 1996). Both strains HE0.1.1 and HB0.1.1 showed the ability to simultaneously oxidise both oxalate and citrate when citrate was supplied as an additional carbon source. No species of the genus *Halomonas* has been described as using oxalate as a sole source of carbon and energy, without an additional carbon substrate. Feed into all bioreactor configurations had relatively high concentrations (1 g/l) of a combination of sodium salts of acetate, formate, malonate and succinate. This additional carbon source is most likely preferentially utilised over oxalate and may act as a kick start for oxalate oxidation. The microbial species responsible for the destruction of oxalate in these bioreactor processes were not isolated in this study, continuing work to isolate and characterise these bacteria using medium containing only oxalate as a source of carbon and energy are currently underway.

The dominant groups of microorganisms detected in the pilot-scale oxalate-degrading bioreactors in this study are significantly different to the microorganisms in other described oxalate-degrading bioreactors. Morton et al. (1991), Chinloy et al. (1993) and Thè et al. (1990) have described processes for the biological destruction of oxalate using a pure culture of *Psuedomonas* sp. strain B-1 (ATCC 53883) for microbially-mediated oxalate destruction. This current study showed that there was mixed communities of microorganisms present in all three bioreactor configurations and that these groups probably work in unison to achieve complete oxalate degradation. No *Pseudomonas* spp. was detected by 16S rRNA gene analysis. The bioreactors described in this study operate in continuous flow with oxalate slurry influent feed mixed with recirculated process waters, indicating that the microorganisms contained within the bioreactors are indigenous to the refinery environment surrounding the processes. Strains of *Halomonas* detected and isolated from the influent of both MBBR bioreactors also shows that these microorganisms are indigenous to the refinery environment, and that activated sludge may not have been a necessary addition to achieve successful operation of the oxalate-degrading bioreactors. This is also reinforced by the PCR-DGGE molecular fingerprint which did not detect any microorganisms which have been previously detected in activated sludge.

The successful establishment and efficiency of these pilot-scale oxalate-degrading bioreactors has substantial advantages to the bauxite refining, alumina and aluminium industries by decreasing the overall economic and environmental costs associated with the production and storage of sodium oxalate organic waste. The use of both culture-dependent and -independent techniques simultaneously ensured that a more complete picture of the microbial ecology and community dynamics of biological oxalate degradation was attained. Further characterisation of pilot-scale bioreactor isolates is currently under investigation to determine the role of *Halomonas* spp. and the other microbial groups detected by PCR-DGGE in the biological destruction of oxalate. Development of methods to monitor the microbial communities involved in the biological degradation of oxalate and to monitor establishment, maintenance and activity of these microorganisms during bioreactor operation is required to optimise the biological oxalate destruction process in further pilot-scale and full-scale operations. Batch studies to determine which reactor configuration optimally removes oxalate from influent streams are currently underway to confirm conclusions made from this study.

Acknowledgments This study was funded by Alcoa World Alumina. Sequence purification and analysis was performed at LotteryWest State Biomedical Facility Genomics, c/o Department of Clinical Immunology and Immunogenetics, Level 2 North Block, Royal Perth Hospital, Box X2213 GPO Perth Western Australia 6847, Australia. Gas chromatography and total organic carbon analysis was performed by Alcoa

World Alumina, Technology Delivery Group, Kwinana Refinery, PO Box 161, Kwinana, WA, 6966, Australia.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3): 403–410

Anders HJ, Kaetzke A, Kampfer P, Ludwig W, Fuchs G (1995) Taxonomic position of aromatic-degrading, denitrifying pseudomonad strains k-172 and kb-740 and their description as new members of the genera thauera, as thauera aromatica sp Nov., and azoarcus, as azoarcus evansii sp. Nov., respectively, members of the beta subclass of the proteobacteria. Int J Syst Bacteriol 45(2):327–333

Arahal DR, Ventosa A (2006) Proteobacteria: gamma subclass. In: Dworkin M (ed) Prokaryotes. A handbook on biology of bacteria, vol 6, 3rd edn. Springer, Berlin

Boldareva EN, Bryantseva IA, Tsapin A, Nelson K, Sorokin DY, Tourova TP, Boichenko VA, Stadnichuk IN, Gorlenko VM (2007) The new alkaliphilic bacteriochlorophyll a-containing bacterium *roseinatronobacter monicus* sp. Nov., from the hypersaline soda mono lake (California, United States). Microbiology 76(1):82–92

Boldareva EN, Akimov VN, Boychenko VA, Stadnichuk IN, Moskalenko AA, Makhneva ZK, Gorlenko VM (2008) *Rhodobaca barguzinensis* sp. Nov., a new alkaliphilic purple nonsulfur bacterium isolated from a soda lake of the Barguzin valley (Buryat Republic, Eastern Siberia). Microbiology 77:206–218

Brassinga RD, Fulford GD, McCready RCG, Gould WD, Beaudette LA (1990) Biodegradation of oxalate ions in aqueous solutions. Australian patent no. AU-B-39465/89

Brown N, Cole T (1980) The behaviour of sodium oxalate in a bayer alumina plant. Light Met 15:105–117

Caton TM, Witte LR, Ngyuen HD, Buchheim JA, Buchheim MA, Schneegurt MA (2004) Halotolerant aerobic heterotrophic bacteria from the great salt plains of oklahoma. Microb Ecol 48:449–462

Chinloy D, Doucet J, McKenzie M, The K (1993) Processes for the alkaline biodegradation of organic impurities. US patent no. 5 271 844

Driscoll C, Schecher W (1990) The chemistry of aluminium in the environment. Environ Health 12:28–49

Hawkes RB, Franzmann PD, O'Hara G, Plumb JJ (2006) *Ferroplasma cupricumulans* sp. Nov., a novel moderately thermophilic, acidophilic archaeon isolated from an industrial-scale chalcocite bioleach heap. Extremophiles 10:525–530

Hind AR, Bhargava SK, Grocott SC (1999) The surface chemistry of Bayer process solids: a review. Colloid Surf A Physicochem Eng Asp 146(1–3):359–374

Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Vani AA, Borgave SB, Sarnaik SS (2008) Cultivable bacterial diversity of alkaline Lonar lake, India. Microb Ecol 55:163–172

Katayama Y, Hiraishi A, Kuraishi H (1995) *Paracoccus thiocyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. with emendation of the genus. Microbiology-Uk 141:1469–1477

Lane DJ (1991) 16s/23s rrna sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wylie, Chichester, pp 115–175

Lee SD (2007) *Devosia subaequoris* sp. nov., isolated from beach sediment. Int J Syst Evol Microbiol 57:2212–2215

Milford AD, Achenbach LA, Jung DO, Madigan MT (2000) *Rhodobaca bogoriensis* gen. Nov., and sp. Nov., an alkaliphilic purple nonsulfur bacterium from African rift valley soda lakes. Arch Microbiol 174(1-2):18–27

Mormile MR, Romine MF, Garcia T, Ventosa A, Bailey TJ, Peyton BM (1999) *Halomonas campisalis* sp. Nov., a denitrifying, moderately haloalkaliphilic bacterium. Syst Appl Microbiol 22(4):551–558

Morton R, Dilworth M, Winecke B (1991) Biological disposal of oxalates. International patent no. WO 91/12207

Muyzer G, Hottenträger S, Teske A, Wawer C (1996) Denaturing gradient gel electrophoresis of pcr-amplified 16s rRNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities. Mol Microbial Ecol Man 3:1–23

Plumb JJ, Gibbs B, Stott MB, Robertson WJ, Gibson JAE, Nichols PD, Watling HR, Franzmann PD (2002) Enrichment and characterisation of thermophilic acidophiles for the bioleaching of mineral sulphides. Miner Eng 15(11): 787–794

Ratkowsky DA, Lowry RK, McMeekin TA, Stokes AN, Chandler RE (1983) Model for bacterial culture growth rate throughout the entire biokinetic temperature range. J Bacteriol 154:1222–1226

Romano I, Nicolaus B, Lama L, Manca MC, Gambacorta A (1996) Characterization of a haloalkaliphilic, strictly aerobic bacterium, isolated from Pantelleria Island. Syst Appl Microbiol 19:326–333

Smeulders DE, Wilson MA, Armstrong L (2001) Insoluble organic compounds in the Bayer process. Ind Eng Chem Res 40(10):2243–2251

Song BK, Palleroni NJ, Haggblom MM (2000) Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soils and sediments. Appl Environ Microbiol 66(8):3446–3453

Tamura K, Dudley J, Nei M, Kumar S (2007) Mega4: molecular evolutionary genetics analysis (mega) software version 4.0. Mol Biol Evol 24(8):1596–1599. doi:10.1093/molbev/msm092

Thè K, Brassinga RD, Beaudette LA, Gould WD, McCready RCG (1990) The destruction of sodium oxalate. In: Technical session of the light metals committee at 119th meeting of the minerals, metals and material society (LIGHT METALS 1990), Feb 18–22, 1990. Anaheim, CA, pp 53–56

Thè K, Beaudette LA, Gould WD, McCready RCG (1992) Oxalate degrading bacterium. International patent no. WO 92/00370

Zhou JZ, Fries MR, Cheesanford JC, Tiedje JM (1995) Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *azoarcus tolulyticus* sp. Nov. Int J Syst Bacteriol 45(3): 500–506