PROSPECTING FOR MICROBES

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Abstract

In the alumina industry, the removal of contaminants such as organic acids and metals from Bayer streams will facilitate re-cycling which in turn decreases both the amount of water used and the amount of wastewater released. It is known that microbes can be used for bioremediation of organics and metals from industrial sites.

The objective of the study was to survey microbial populations from an alumina refinery site and to identify those that may be useful for bioremediation in the alumina industry using both a molecular and conventional microbiology approach.

We used conventional microbiology combined with molecular tools to identify microbes, including those that may resist conventional culturing techniques. Cyanobacteria, sulfate reducing and sulfide oxidising microbes that play an important role in sulfur cycling in undisturbed environments were identified at various sites at the refinery. The molecular approach was used to identify two genera that may degrade hydrocarbons and we also identified another species associated with metal detoxification. Two aerobic, alkalophilic, oxalate degrading bacteria strains were isolated through selective media cultivation. A range of alkalophilic *Bacillus* sp. were identified through cultivation and molecular approaches. Sulfate reducing bacteria were observed in media containing acetate or formate as a carbon source.

This prospecting using a range of samples from Rio Tinto Alcan Gove revealed a diverse array of potentially useful microbes. In this study we identified microbes with potential for bioremediation of potentially re-usable liquors currently containing unacceptable levels of organic acids and metals.

1. Introduction

Organic acids are a by-product of alumina processing from bauxite. These organic contaminants limit the recycling of water through the plant because ions can accumulate in the refinery leading to impurities in alumina. Five chemical based methods have been patented for the removal of organics from the Bayer process (http://www.freepatentsonline.com). Two of these approaches (US Pat No 4, 046, 855 and US Pat No 4, 101, 629) result in a loss of alumina due to the formation of precipitates and require additional processing. Removal of organics through the addition of lime (US Pat No 4, 335, 082) or a copper catalyst (US Pat No 4, 215, 094) is expensive and burning of liquor to remove contaminants (US Pat No 4, 215, 094) requires a license due to air pollution.

Microbes are considered to be a safer and less expensive form of remediating waste and contaminated sites. Microbes capable of degrading organic acids exist in a wide range of environments and some are currently being used to bioremediate sodium oxalate waste at mine sites (Morton *et al.* 1994). Rio Tinto Alcan International Ltd patented a method that exploits the oxalate degrading capabilities of microbes that were isolated from the rhizosphere of oxalate producing plants. Worsley patented the use of Bacillus AGAL N91/005579 in a bioreactor for the removal of oxalate from bayer liquor (Morton *et al.* 1994). These two treatment systems only address the removal of oxalate from waste streams while the patent WO/1994/006719 from Rio Tinto Alcan International Ltd uses a *Pseudomonas* species to remove the organic acids; oxalate, formate, acetate and benzoate from the bayer liquor. These organic acid degrading bacteria were identified through bioprospecting of microbes at Alumina refineries.

Bioprospecting of microbes at mines or refineries other than those associated with Alumina processing revealed a range of bacteria that can be exploited for the treatment of waste or remediation of contaminated sites (Jong & Parry 2003, Ma *et al.* 2006). Surveys at acid mine drainage sites revealed sulfate reducing bacteria (SRB) suitable for the development of a bioreactor treatment system (Jong & Parry 2003). SRBs use organic compounds such as lactate, acetate, propionate, pyruvate, malate, ethanol and formate as an energy source when sulfate is reduced to sulfide (George *et al.* 2007). The sulfide produced by SRBs reacts with metals to form insoluble metal sulfides that precipitate out of solution (Kim *et al.* 1999). In addition, the bicarbonate ions produced by SRBs leads to a reduction in acidity of acid mine drainage (Jong & Parry 2003, Kim *et al.* 1999). The metabolic capabilities of SRBs mean they are candidates for the removal of heavy metals and organic contaminants.

Microbial mats composed of SRBs and cyanobacteria are being used to treat acidic mine waste (Bender & Phillips 2004). The cyanobacteria in these mats have multiple roles, they provide an anoxic environment for SRBs (Bender *et al.* 1995), excrete organic ions that can be used by SRBs (Stal & Moezelaar 1997) and remove metals from waste (Bender *et al.* 1995). Cyanobacteria also have potential application in the removal of pesticides and chlorinated pollutants due to their ability to degrade aromatic hydrocarbons (Kuritz & Wolk 1995). Microbial treatment systems can be applied to address environmental problems such as oil spills and toxic contaminants (Bender *et al.* 1995, Kuritz & Wolk 1995).

2. Objectives

The objective of this study was to survey microbial populations from an alumina refinery site using both a molecular and conventional microbiology approach.

3. Materials and methods

Sampling

Five water and 9 sediment/soil samples were collected in replicate from sites on and adjacent to the Rio Tinto Alcan Gove refinery (Figure 1). The sampling sites for the rehabilitated area and an undisturbed location near the Gove mine site are not visible on Figure 1. For soil sampling, the top 2-3 cms were removed from a 20cm^2 area and for water sampling 200ml was collected.

Figure 1. Sediment/soil and water sampling sites at RioTinto Rio Tinto Alcan Gove (circles- soil/sediment, squares – water)

Isolation of bacteria 16S ribosomal RNA genes

Bacteria DNA was isolated from these samples using the MoBio UltraClean Soil DNA isolation kit and water samples were extracted using MoBio ultraclean water DNA isolation kit (Geneworks) according to the manufacturer's protocol. DNA quality and quantity were determined by separating samples on a 1% agarose gel, stained with ethidium bromide and visualised by UV transillumination.

When taking a molecular approach, bacteria are generally identified using their 16S ribosomal RNA (16S rRNA) gene, so this gene was amplified from environmental DNA samples by the polymerase chain reaction (PCR) using the primers of Buchholz-Cleven *et al.* (1997). The amplified gene was visualised by ethidium staining and UV transillumination. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and cloned into the pGEM-T easy vector kit (Promega) according to the manufacturers' protocols.

Typing and characterisation of bacteria

The bacteria 16S rRNA genes were amplified from pGEM-T easy vectors by PCR using the M13 forward and reverse primers (Promega) and 25-40 clones were selected for fingerprinting. The bacteria 16S rRNA genes amplified from pGEM-T easy clones were digested with the restriction enzyme *Taq*I (Promega) and fragments separated by polyacrylamide gel electrophoresis (PAGE). Digestion of bacteria 16S rRNA genes generates a DNA banding pattern (DNA fingerprint) for each cloned bacteria and this fingerprint is used to identify different bacteria types. The banding patterns were visualised by UV transillumination and recorded using Gel logic 100 imaging system (Kodak). The banding patterns for each bacteria 16S rRNA gene were typed and compared using Adobe Photoshop software (Adobe). The bacteria phylotype diversity was calculated through the website http://www.aslo.org/lomethods/free/2004/0114a.html (Kemp & Aller 2004). Clones containing the 16S rRNA genes from impacted Inner Gove Harbour sediment and relatively un-impacted mangrove sediment site were selected for sequence analysis based on banding patterns. Plasmid DNA was sequenced using the Big Dye Terminator Reaction kit (Australian Genome Research Facility). M13 primers were used for sequencing reactions. All steps were performed according to the manufacturer's protocol. Sequence reactions were separated at Bioscience North Australia's sequencing facility. The data were initially analysed using MacVector V9 (Eastman Kodak Co.). Nucleotide sequences of clonal inserts were analysed using the BLASTN search engine that was accessed through National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Putative species names were assigned to bacteria 16S rRNA genes that shared greater than 97% with reference sequences in NCBI non-redundant (nr) database.

Isolation and characterisation of bacteria

Sulfate reducing bacteria (SRB) were enriched using Postgate medium B (Jong & Parry, 2003, 2006) inoculated with sediment/soil samples (Table 1). The Postgate media was supplemented with lactate, acetate, formate or oxalate as a carbon source. Oxalate degrading bacteria were enriched using OM-2 media (Zaitsev *et al.* 1998), which is a selective media for this type of bacteria, using sediment/soil or water (Table 1). To enrich for target bacteria, liquid media was inoculated with sediment or water samples. After 2-4 weeks, aliquots were taken from these starter cultures and used to inoculate fresh media. After 2-4 weeks, aliquots from the second enrichment culture were streaked onto solid media. Individual colonies were isolated from solid agar and liquid media inoculated.

For isolation of *Bacillus* species, supernatant liquor (SNL) was inoculated with silage (Table 1). To determine if the bacteria in cultures originated from silage, sterile water was inoculated with silage (Table 1). Silage was made by mixing grass clipping with molasses and wrapping the mixture in plastic wrap. The grass mixture was left outdoors for two months. The SNL silage and control cultures were incubated for a month. Aliquots were removed from the cultures and streaked onto solid nutrient agar. Individual colonies were isolated and used to inoculate liquid nutrient agar.

DNA was extracted from liquid cultures, inoculated with individual colonies, using the MoBio Microbial DNA isolation kit (Geneworks). To characterise the oxalate degrading bacteria and possible *Bacillus* species their 16S rRNA genes were amplified by PCR using the 8F/1492r primer pair (Buchholz-Cleven *et al.* 1997). While for SRBs, the *dsr* primers (1F & 4R) were used to amplify their dissimilatory sulfite reductase genes (Karr *et al.* 2005). The amplified products were purified using the QIAquick PCR purification kit (Qiagen). The purified products were sequenced and analysed as outlined previously. The 16S rRNA gene sequences from cultures were further analysed by GAP analysis (MacVector) to calculate percentage similarities. The morphology of the oxalate degrading bacteria was examined by transmission electron (TEM) and scanning electron microscopy (SEM). Gram stain was used for differential staining and slides examined using an Olympus BH2 light microscope. Samples from SEM were prepared according to Gates and Muscatine (1992) with the slight modification that water was used instead of buffer. SEM slides were examined using Jeol SEM5610LV. TEM positive contrast samples were prepared according to Marin *et al.* (1993) and examined on a Jeol TEM 1200Ex.

Although sequence analysis revealed oxalate degrading bacteria were isolated, the following experiment was performed to confirm the bacteria used this substrate. OM-2 media adjusted to the pH values 7, 8, or 9 was inoculated with individual bacterial strains. Bacterial growth was monitored at absorbance 540nm. The oxalate in cultures was measured by capillary electrophoresis at Rio Tinto Alcan Queensland Research and Development Centre.

4. Results and Discussion

The estimated species richness (data not shown) could not be compared between the different sites because the species richness curves did not reach an asymptote (Hughes *et al.* 2001, Kemp & Aller 2004). When the curves reach an asymptote it indicates that the sampled phylotypes give a strong representation of the bacteria populations at the site. This result simply means that more clones would have to be sequenced to reach the limit of diversity.

Table 1. Cultures used for enrichment of oxalate degrading bacteria, sulfate reducing bacteria and *Bacillus* species.

Culture number	Enrichment media	Inoculum	Growth condition
1C	OM-2	Labyrinth water	Light aerobic
2C	OM-2	Bore 44 water	Light aerobic
3C	OM-2	Seawater discharge channel water	Light aerobic
4C	OM-2	Rehabilitated stream water	Light aerobic
5C	OM-2	Unknown bore water	Light aerobic
6C	OM-2	Bore 44 soil	Light aerobic
7C	OM-2	Red mud ponds soil	Light aerobic
8C	OM-2	Sand near seawater discharge channel	Light aerobic
9C	OM-2	Dry Hydratalcite soil	Light aerobic
10C	OM-2	Duck creek soil	Light aerobic
11C	OM-2	Labyrinth soil	Light aerobic
12C	OM-2	Undisturbed site soil	Light aerobic
13C	OM-2	Rehabilitated site seepage soil	Light aerobic
14C	OM-2	Bore soil	Light aerobic
15C	OM-2	Mangrove soil	Light aerobic
16C	OM-2	Inner Gove Harbour sediment	Light aerobic
20CD	OM-2	Bore 44 soil	Dark aerobic
21CD	OM-2	Sand near seawater discharge channel soil	Dark aerobic
22CD	OM-2	Dry Hydratalcite	Dark aerobic
23CD	OM-2	Labyrinth soil	Dark aerobic
24CD	OM-2	Mangrove soil	Dark aerobic
25	Silage	Supernatant liquor (SNL)	Light aerobic
26	Silage	Supernatant liquor (SNL)	Light aerobic
27	Silage	Sterile water	Light aerobic
28	Postgate	Labyrinth soil	Dark anaerobic
29	Postgate	Undisturbed site soil	Dark anaerobic
30	Postgate	Sand near seawater discharge channel soil	Dark anaerobic
31	Postgate	Inner Gove Harbour soil	Dark anaerobic
32	Postgate	No inoculum	Dark anaerobic

Cyanobacteria

Comparison of banding patterns showed that 35 clones from 15 sites contained inserts that were the same as reference cyanobacteria species, *Microcoleus chthonoplastes*, *Nodularia spumigena*, and *Symploca* PCC8002. Cyanobacteria synthesise biologically active compounds such as antibiotics, toxins, siderophores and immunosuppressants (Ehrenreich *et al.* 2005). The siderophores (Ito & Bultler 2005), polysaccharide bioflocculents (Bender *et al.* 1994), stress protein GroEL and metal binding metallothionein (Ybarra & Webb 1999) allow cyanobacteria to regulate metal ion accumulation so they can survive in metal contaminated sites. This cyanobacteria trait is being exploited to develop treatment systems such as microbial mats for the removal of heavy metals (Bender & Phillips 2004). In addition, a test for determining heavy metal levels in environmental samples has been developed using cyanobacteria (http://www.freepatentsonline.com/5776681). Further studies of cyanobacteria at Rio Tinto Alcan Gove would provide insight into whether these bacteria could be used for the removal of metal contaminants from Bayer liquor.

Bacteria from Marine Sediment

Sequence analysis of clonal sequences showed that bacteria at the Inner Gove Harbour site were from only 4 genera while at the mangrove site 11 genera were identified (Table 2). At the Inner Gove Harbour site, 18 bacteria phylotypes were from unknown species and 17 of these phylotypes were related to bacteria isolated from metal contaminated sediments (Table 2). Four bacteria from the impacted site were closely related to *Idiomarina loihiensis*, which can detoxify metals such as arsenate, cadmium and mercury (Hou *et al.* 2004). Analysis of the *L. loihiensis* genome revealed 32 genes involved in the synthesis of extra cellular polysaccharides important for the formation of biofilms (Huo *et al.* 2004). This lead Lopez *et al.* (2006) to propose that *L. loihiensis* may be involved in the formation of biofilms and the subsequent attachment of microorganisms and the accumulation of metals. Another two bacteria from this site were members of the *Idiomarina* genus but with no assigned species name – these bacteria may also be resistant to heavy metals. The ability of *Idiomarina* species to detoxify metals means they may have potential application in the removal of metal contaminants.

Most bacteria (14) found at the Inner Gove Harbour site were members of the *Planococcus* or *Planomicrobium* genera (Table 2). *Planococcus* are abundant bacteria in marine environments but have been identified in various environments such as hot sulfur springs (Romano *et al.* 2003) and cyanobacterial mats from Antarctica (Reddy *et al.* 2002). *Planococcus* and *Planomicrobium* species can reduce the metalloids pertechnetate to technetium (Lovely 1993) and selenite to selenium (Siddique *et al.* 2005). *Planococcus* species are also able to degrade diesel and kerosene indicating they are candidates for remediating accidental spills of these compounds (Engelhardt *et al.* 2001, Jacobucci *et al.* 2001). More recently, a *Planococcus* species was reported to have potential application in the starch industry due to its ability to degrade starch (Choi *et al.* 2007). Further studies will provide insight into the metabolic capabilities of the Northern Australia *Planococcus* bacteria and their potential for remediation.

Table 2. Putative bacteria species identified by sequence analysis (*Both – identified at Inner Gove Harbour and un-impacted mangrove).

Source of inserts	Clone Number	Closest match	Accession #	% similarity
Mangrove	18.01	*Pseudoaltermonas* sp.	AY187028	96%
Mangrove	18.05	Uncultured bacteria PC1446B-88	DQ270631	97
Mangrove	18.18	Uncultured bacteria FAC79	DQ451518	92
Mangrove	18.21	Uncultured delta proteobacteria	AY771945	95
Mangrove	18.22	Uncultured delta proteobacteria	AY771960	96
Mangrove	18.23	Proteobacteria SCB11	Z31658	92
Mangrove	18.24	Uncultured gamma proteobacterium	DQ811844	95
Mangrove	18.25	Unidentified delta proteobacterium	AF026993	94
Mangrove	18.26	Uncultured bacteria SB1093	AF269002	97
Mangrove	18.29	uncultured bacteria clone B6	DQ228366	92
Mangrove	18.30	Uncultured bacteria clone B93	AY375063	94
Mangrove	18.33	Uncultured bacteria	AM176867	99
*Both	18.34	Desulfacinum subterraneum	AF385080	95
Mangrove	18.35	Uncultured bacteria	AM176867	99
Mangrove	18.36	Uncultured delta bacteria	DQ811817	95
Mangrove	18.37	Uncultured deltaproteobacteria	AJ704687	97
Mangrove	18.38	Uncultured deltaproteobacteria	AM039962	96
Mangrove	18.42	Uncultured deltaproteobacteria	AY771936	95
Mangrove	18.43	Lucina nassula gill symbiont	X95229	96
Mangrove	18.46	uncultured alpha proteobacteria	DQ289899	96
Inner Harbour	19.01	Uncultured acidobacteriales	DQ351783	97
Inner Harbour	19.02	Uncultured bacteria	AJ532701	98
Inner Harbour	19.03	*Planomicrobium* spEP22	AM403524	97
Inner Harbour	19.07	uncultured low G+C gram +ve bacteria	AY642552	96
Inner Harbour	19.09	Uncultured bacteria	AJ532701	98
Inner Harbour	19.14	*Planococcus rifiiensis*	AJ493659	99
Inner Harbour	19.15	*Planomicrobium* spEP19	AM400938	98
Inner Harbour	19.17	*Idiomarina* so. NT	AB167017	99
Inner Harbour	19.19	Uncultured bacteria	AJ532701	98
Inner Harbour	19.20	uncultured bacteria	DQ513057	99
Inner Harbour	19.22	*Idiomarina* so. NT	AB167017	99
Inner Harbour	19.24	*Idiomarina loihiensis*	AE017340	98
Inner Harbour	19.25	uncultured acidobacteriaceae	AY225646	91
Inner Harbour	19.30	*Halomonas* sp. Claire	AJ969933	99
Inner Harbour	19.36	*Planomicrobium* sp.	AM403522	99
Inner Harbour	19.37	*Idiomarina loihiensis*	AE017340	99
Inner Harbour	19.41	Uncultured bacteria	AJ532701	98
Inner Harbour	19.44	*Idiomarina loihiensis*	AE017340	99
Inner Harbour	19.46	*Idiomarina loihiensis*	AE017340	99

No bacteria belonging to *Planococcus*, *Planomicrobium* and *Idiomarina* genera were identified at the un-impacted mangrove site. The majority of bacteria from the mangrove site were closely related to sulfate reducing or sulfide oxidising microbes. These bacteria play an important role in energy production through sulfur cycling in intertidal systems (Elshahed *et al.* 2003). The SRB, *Desulfacinum subterraneum* was identified at both the Inner Gove Harbour and mangrove site. Three members of the sulfate reducing *Desulfotomaculum* genera were isolated from the Inner Gove Harbour site using conventional microbiology. Growth of these SRBs was not supported on media containing oxalate, which suggests they are unable to use this carbon source. The isolated *Desulfotomaculum* genera did grow on media supplemented with the carbon sources, lactate, acetate and formate. These findings suggest these SRBs species may be exploited to remove these organic contaminants from wastewater if other environmental parameters are suitable for growth. Furthermore, these SRBs can potentially be used for removal of metal contaminants from water or effluent. Previous studies have proven this concept through the successful treatment of metal rich, acid mine drainage (Jong & Parry 2003, 2006).

Oxalate degrading bacteria

Enrichment experiments for oxalate degrading bacteria showed that after 20 days, 17 of the OM-2 cultures inoculated with field samples (Table 1) had pH values >10 which signalled that oxalate was being oxidised. There was no change in pH for three cultures. Microscopy examination of cultures showed a mixture of bacteria-like structures in all cultures (data not shown). After 26 days, eight cultures had an increase in pH to 9.5-10 and the

absorbance values increased to >0.500. These findings suggest the bacteria were growing in the OM-2 media and oxalate was being degraded. Light microscopy of these cultures revealed bacteria-like structures in the cultures (data not shown). The remaining cultures either had no change in pH or a slight increase to 8. Some of the cultures that had a slight change in pH had absorbance values >0.500 which indicated bacterial growth but only low rates of oxalate degradation. Cultures that had no change or a slight change in pH were not analysed further.

After 28 days, none of the cultures reached a pH value above 9 but there was an absorbance increase for most cultures. The bacteria-like structures observed in the cultures on days 20 and 26 were more abundant in day 28 cultures. Bacteria from these cultures were streaked onto solid OM-2 media, and after 3 to 5 days colonies were observed. The colonies were generally convex and circular, and were white, cream or brown. Salt crystals were present on most plates. A 500bp fragment of the 16S ribosomal RNA gene was characterised for the bacteria cultures; 6C colony 1 (6C.1), 6C colony 2 (6C.2), 8C colony 1 (8C.1), 8C colony 2 (8C.2), 10C colony 1 (10C.1), 10C colony 2 (10C.2), 12C colony 1 (12C.1), 14C colony 1 (14C.1), 14C colony 2 (14C.2), 16C colony 1 (16C.1), 16C colony 2 (16C.2), 17C colony 1 (17C.1), 17C colony 2 (17C.2), 23C colony 1 dark (23C.1 dark) and 23C colony 2 (23C.2 dark). Comparative analysis of these nucleotide sequences showed that the bacteria from cultures 14C.1, 14C.2, 16C.1, and 16.2C were identical to each other and shared 100% identity with the 16S rRNA gene of *Ammoniphilus oxalaticus* RAOx-FF (AOY14579). The bacteria from cultures 6C.1, 6C.2, 8C.1, 8C.2, 10C.1, 10C.2, 12C.1, 17C.1, 17C.2, 23C.1 dark and 23C.2 dark shared 100% identity with each other and 99.6% identity with the corresponding gene from *A. oxalaticus* RAOx-FF. These findings suggest two different strains of *A. oxalaticus* may have been isolated from the Rio Tinto Alcan Gove site.

The morphological characteristics of the bacteria cultures, 8C.2, 14C.2 and 23C.1 dark were examined by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and gram staining. SEM revealed cells in all bacteria cultures were straight or slightly curved rods that occurred singly or in chains (Figure 2). The rods of bacteria 8C.2 and 23C.1 dark had a smooth appearance (Figure 2a & c) while 14C.1 had a rough appearance (Figure 2b). Zaitsev *et al.* (1998) reported *A. oxalaticus* strains as having electron dense granules on the external layer of the cell so the rough appearance for Rio Tinto Alcan Gove bacteria may be due to these granules. The authors suggested the name 'oxalosome' for these granules and postulated these granules were possibly organelles which were involved in attachment to plants and/or contained the enzymes for oxalate oxidation. Alternatively, the rough appearance of Rio Tinto Alcan Gove bacterial cells may be due to salt precipitation in the culture media. The cells from bacteria cultures need to be analysed by thin sectioning SEM to confirm the presence of 'oxalosomes'. The cells of 14C.1 were 2.9 - 3.2 µm long and 0.5 - 1.12 µm wide. The average bacterial cell sizes in 8C.1 cultures were 2.2 - 3.2µm long and 0.8 – 1.0µm wide, and for 23C.1 dark 1.2 – 2.8 µm long and 0.4 – 0.6 µm wide. However some cells in 8C.1 and 23C.1 dark cultures were twice to six times larger than the average cell (Figure 2a). These larger cells may be undivided cells. Peritrichous (distributed over the cell) flagella were revealed by TEM for bacteria 8C.2 and 23C.1 dark (Figure 2c). A number of cells in the bacteria cultures were connected by an unknown substrate (data not shown).

Aliquots were taken from bacteria cultures with absorbance values >1 (540nm) for gram staining. In all cultures the cells were variable. The gram - positive (blue colour) cells are spores that have been released or are forming in the cell. The gram - negative (pink – red) cells are younger cells that have not started to produce spores.

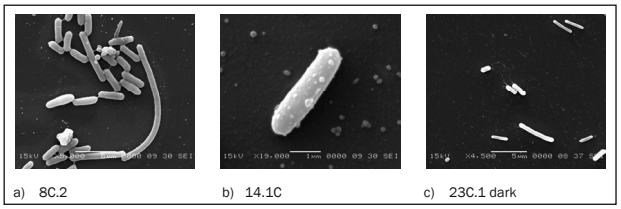


Figure 2. Scanning electron micrograph of cells of bacteria cultures 8C.2, 14C.1 and 23C.1 dark.

Based on microscopy the bacteria 8C.2, 14C.1 and 23C.1 dark share the following characteristics in common with *A. oxalaticus* strains:

a) Gram variable
b) Endospores present
c) Peritrichous flagella
d) Straight or slightly curved rods that occur singly or in chains
e) The cells sizes fell in the size range of *A. oxalaticus* strains RAOx-PF, RAOx-PM, RAOx-RF, RAOx-RM and DWOx-RM.

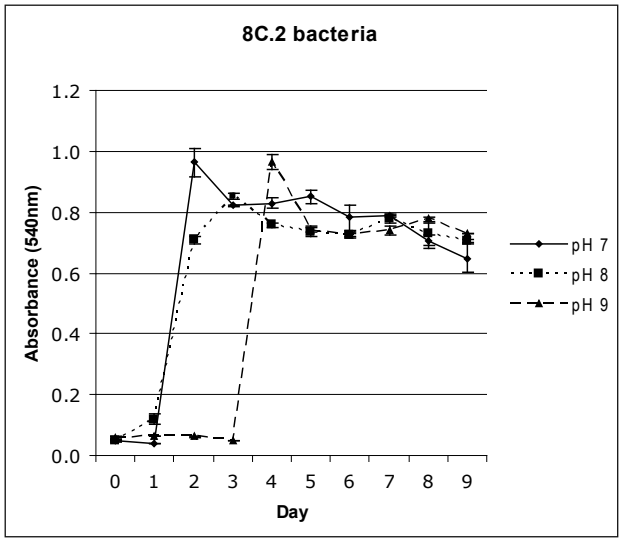
The culture experiments for 23C.1 dark and 8C.2 bacteria were performed in duplicate and the bacteria consistently grew at pH 7, 8, and 9 (Figure 3). The standard deviation ranged from 0 - 0.1 (Figure 3).

Although there was only a slight change in pH values of these cultures, cultures 23C.1 dark and 8C.2 pH 7 contained no oxalate after 10 days (Figure 4). These findings indicate the bacteria 8C.2 and 23C.1 dark are candidates for oxalate remediation.

Nutrient agar media containing oxalate supported the growth of *Bacillus pseudofirmus*, *Bacillus* sp. and *Lysinibacillus fusiformis* isolated from silage. This suggests that these bacteria may also be suitable candidates for the remediation of oxalate. *Bacillus pseudofirmus*, *Bacillus* sp. and *Lysinibacillus fusiformis* were also isolated from silage and water cultures which indicates that these bacteria most likely originated from the silage and not the SNL because they were present in both cultures. *Lysinibacillus fusiformis* was previously named *Bacillus fusiformis* (Ahmed *et al.* 2007). *Bacillus* species have been used to remove organic pollutants and ammonia (Liu & Han 2004, Menezes Bento *et al.* 2005). Further investigation of the *Bacillus* and *Lysinibacillus* species isolated during this study will allow us to determine the remediation potential of these bacteria strains.

5. Conclusions

The molecular approach revealed a diverse range of potentially useful bacteria extracted from soil and water samples on or adjacent to an alumina refinery. Conventional microbiology revealed microbes with potential for bioremediation of potentially re-usable alumina refinery liquors.



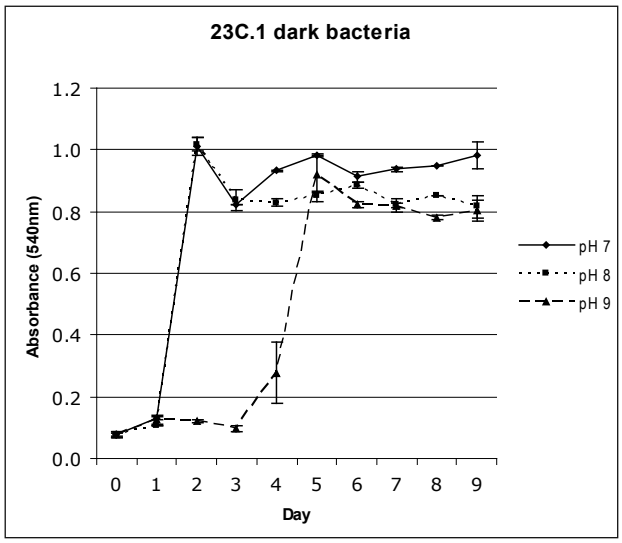


Figure 3. Growth for bacteria 8C.2 and 23C.1 dark during culturing experiments. (Error bars represent standard deviation values).

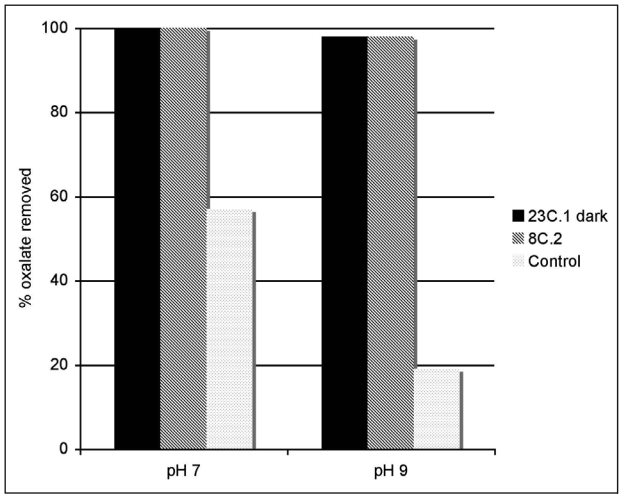


Figure 4. Oxalate removed (percentage) from OM-2 cultures inoculated with 8C.2 and 23C.1 dark bacteria cultures.

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