Biodegradation of thiocyanate by a mixed microbial population

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Abstract The activated sludge tailings effluent remediation (ASTER™) process was developed to biologically treat tailings water, laden with cyanide compounds and especially high concentrations of thiocyanate (SCN^-) and has been demonstrated for low (± 100 mg/L) concentrations at demonstration scale. The ability of the mixed culture to degrade higher concentrations (up to 1000 mg/L) was investigated in continuous reactors, with an 8 h residence time, at temperatures from 5 °C to 40 °C. The culture was not significantly inhibited at feed concentrations of 1000 mg/L, with maximum degradation rates of 70—80 mg/L/h achieved at 22 °C and 40 °C. Operation at 5 °C reduced efficiency by over 50%. Component species of the mixed population were identified, based on ribosomal RNA gene sequence, and included several species known to degrade cyanide species. The results show that the ASTER™ process has the potential to treat effluents from most BIOX® operations without further process optimisation.

Key Words Biodegradation of thiocyanate; ASTER™; Cyanide biodegradation; BIOX®

Introduction

Cyanide is extensively used in the mining and minerals processing industries. Of the 600,000 tons of sodium cyanide produced in 2001 utilisation for gold extractions accounted for over 90% (Dzombak *et al.*, 2006). The reaction of cyanide with reduced sulphur species (eg pyrite) during extraction leads to thiocyanate (SCN^-) formation. Waste water containing high concentrations (up to 3500 mg/L) of thiocyanate are formed during gold extraction dependent on the efficiency of the upfront sulphide oxidation step (roasting, pressure leaching or biooxidation). The thiocyanate and residual free cyanide are deported with the tailings as components of the effluent fraction after carbon in pulp (CIP) processing. A major concern is the catastrophic consequences associated with failure of tailings dams (Dzombak *et al.*, 2006). A second concern is the susceptibility of bioleaching micro-organisms to SCN^- (for example in the BIOX® process) which consequently prevents recycling of water upfront of the leaching circuit. This has major operating and economic implications, especially in arid regions. Furthermore, the concentration of thiocyanate typically exceeds legislated discharge specifications, necessitating on-site treatment if the effluent is not retained in impoundment facilities.

The biological degradation of thiocyanate and cyanide in effluents presents an alternative to the more traditional treatment processes, which include chemical and physical treatment technologies (Mosher and Figueroa, 1996; White *et al.*, 2000; Akcil., 2003; Akcil *et al.*, 2003). Thiocyanate and cyanide are utilised by a number of aerobic micro-organisms, primarily as a nitrogen source and in the case of SCN^- a sulphur source. Additional removal of metal-cyanide complexes is achieved by adsorption of metal ions to the cell surface or extracellular polymeric substances (EPS) secreted by the cells. These phenomena formed the basis for the development of a biological treatment system by researchers at Billiton Process Research and Gold Fields Ltd. The activated sludge tailings effluent remediation (ASTER™) process consists of aerated reactors, in which the cyanide species are oxidised, and a clarifier to facilitate water recovery and recycling of the thickened sludge (Figure 1). The ASTER™ process has been successfully operated at a pilot and demonstration scale at the Consort operation in South Africa, consistently achieving effluent thiocyanate concentrations below 1 mg/L (regulatory specification). The influent thiocyanate concentrations have been relatively low (< 100 mg/L).

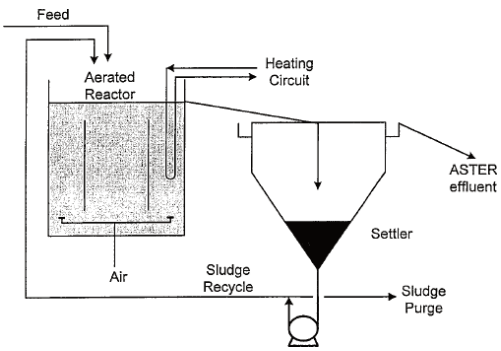

Figure 1 *Schematic representation of the ASTER™ (Marais and van Niekerk, 2009).*

There is a desire to commercialise the ASTER™ technology and export the technology to BIOX® operations outside South Africa, where effluent thiocyanate concentrations may reach 3500 mg/L. To achieve this, additional test work to characterise the performance and tolerance of the microbial population and determine the optimum design and operating parameters is required. The project centres on developing a robust and well characterised microbial consortium to drive an industrial scale ASTER™ process, as well as defining the optimal operating conditions and functional operating window for the process.

Experimental Setup

Stock Reactor

The microbial inoculum was obtained, on two separate occasions, from the demonstration plant at the Consort mine, Barberton, Mpumalanga. The inoculum was maintained in a glass continuously stirred tank reactor (CSTR) connected to a glass clarifier unit. The working volumes of the reactor and clarifier were one and two litres respectively. This system mimics that of the ASTER™ unit design (Figure 1). The feed (pH 7.00 ±0.02) to the stock reactor contained molasses (0.15 g/L) as sole carbon source, potassium dihydrogen phosphate (0.027 g/L) as sole phosphorus source and potassium thiocyanate (100 mg/L SCN^-) as sole nitrogen source. The hydraulic retention time was maintained at 8h, with a sludge recycle rate of 900 mg/h. Aeration was set to 300 mL/min and the system was operated at room temperature (24—27 °C). Mixing was achieved using an overhead stirrer fitted with a pitched-blade impeller, set to 270 rpm. The sludge was purged from the clarifier underflow at a constant rate of 20 mL/h. Throughout the duration of the experiment the thiocyanate concentration in the stock CSTR was below 1 mg/L.

Microbial Species Identification

A sample from the stock reactor was removed and plated onto a nutrient agar medium designed to promote growth of aerobic, heterotrophic microorganisms. The plates were stored at 30 °C until visible colonies were observed. Distinctive colonies were picked off the plates and processed using the technique described below.

Typically, not all components of a mixed environmental sample can be cultured on solid media. For this reason a portion of the liquid culture was processed separately. A sample from the reactor was removed and spun down at 9000 rpm. The resulting pellets were resuspended and transferred into fresh collection tubes and the DNA extracted using the Roche High Pure Template Preparation kit according to the manufacturer's instruction. The DNA concentration was quantified using a Nanodrop ND-2000 (Thermo Scientific) and the samples stored for further analysis.

The extracted DNA from each sample (picked colonies and reactor liquid) was subjected to a polymerase chain reaction (PCR) amplification, using universal 16S primers for bacteria and archaea and 18S primers for fungi, resulting in a product containing several hundred million copies of the targeted gene from each of the species in the samples.

Half the amplified DNA was then separated on an agarose gel while the remainder was used as template for quantitative real-time PCR analysis, using universal 16S primer sets. Following the amplification cycles a melt curve analysis was performed. The number of peaks on the melt curve correspond to the number of distinct species in the sample. Bands of the correct size were excised from the agarose gels, ligated into pGEM-T Easy and transformed into *E. coli*. Successfully transformed colonies of *E. coli* were picked and the cloned insert purified for DNA sequencing and subsequent species identification.

Sampling and Analyses

A 3 ml sample was removed from the flask and filtered through a 0.22 um membrane filter (MilliPore). Filtered samples were analysed for thiocyanate and ammonium concentration using high pressure liquid chromatography (HPLC). The thiocyanate analysis was performed using the technique described by Tamosiunas *et al.* (2006), employing the Thermo Scientific HPLC spectra system with a UV detector. The technique was optimised by the group of Tamosiunas with regards to pH, UV-wavelength, tetrabutyl ammonium dihydrogen phosphate (TBA) concentration and acetonitrile concentration. The stationary phase used in this study was a reversed phase C_{18} Discovery HS column (5 µm, 250 mm × 4.6 mm). The mobile phase consisted of 40% (v/v) acetonitrile in double distilled H_2O with a final TBA concentration of 2 mM. A linear standard curve (0—100 mg/L thiocyanate) was generated for each analytical run using fresh reagents.

Analysis of the ammonium concentration was performed using the Waters 717plus HPLC system with a conductivity detector. The stationary phase was a Hamilton cation exchange column (10 µm, 150 mm × 4.1 mm). The mobile phase consisted of 30% methanol in double distilled water. A linear standard curve (0—100 mg/L NH_4^+) was generated for each analytical run using fresh reagents.

Experimental Design

Continuous stirred tank reactors were setup with a working volume of 1 L. The media contained molasses (0.15 g/L) and potassium phosphate (0.027 g/L). The sole nitrogen source was potassium thiocyanate and the concentration varied as dis-

cussed. The inoculum used was taken from the stock reactor and inoculated at 10% (v/v). The reactor stirrer speed was set to 270 rpm with a constant aeration rate of 800 mL/min and a hydraulic retention time of eight hours.

Results and Discussion

Microbial species identification

Three distinct colony morphologies were observed after incubation for 48 hours and these were picked off the plates and cultured in nutrient media for 48 hours, after which the cells were concentrated and DNA extracted as described previously. The DNA from the purified species were cloned and sequenced as previously described. No mould-type fungi were observed on the cultured plates.

DNA was successfully extracted from all samples. Amplification of the DNA with universal 16S and 18S primers yielded positive results in all cases. Successful amplification was not achieved with the archaeal primers, suggesting the archaea do not constitute a significant proportion of the population.

The 16S sequences were compared to previously deposited sequences using the Basic Local Alignment Sequence Tool (BLAST) available on the National Centre for Biotechnology Information (NCBI) website and the results summarised in the table below (Table 1). Species identified before to be active in the ASTER™ process were ordered from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and included Bosea thiooxidans and Ralstonia eutropha.

Several of the identified species (Pseudomonas and Ralstonia) have been explicitly demonstrated to have the ability to degrade cyanide and related compounds (Chapatwala *et al.*, 1998; Akcil *et al.*, 2003; Sewell *et al.*, 2003). Several of the other species identified in the samples have not specifically been recorded to contain nitrilase enzymes and this may be related to the choice of molasses as the carbon source. Scanning electron micrographs (SEM) were acquired as part of the ongoing process to characterise the individual microbial species from the mixed microbial sludge population (Figures 2: a-e). These SEMs show not only morphologies observed in the

Table 1 *Microbial species identity based on highest BLAST score.*

Sample	Species	% homology
Reactor 1 - 16S	*Pseudomonas stutzeri* isolate OC-10	98%
Reactor 1 - 16S	*Microbacterium schleiferi* strain 118	98%
Reactor 1 - 16S	*Pusillimonas noertemannii*	98%
Reactor 1 – 16S	*Pseudomonas alcaligenes*	98%
Reactor 1 – 16S	*Pseudomonas fluorescens* KDK8	98%
Reactor 1 – 18S	*Candida humulus*	99%

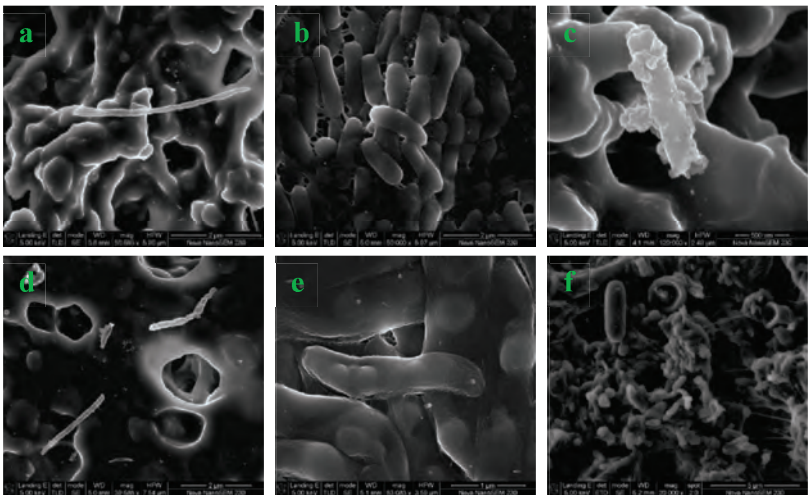

Figure 2 *Scanning electron micrographsof pure cultures isolated from the mixed microbial population including (a) Pseudomonas, (b) Pusillimonas, (c) Microbacterium,(d) Bosea, (e) Ralstonia as well as of the bio-sludge (f) Mixed Consort culture.*

mixed microbial population (Figure 2f) but also EPS production likely aiding in biofilm formation.

Effect of thiocyanate concentration on the thiocyanate biodegradation ability of the mixed microbial population

Continuous stirred tank reactors, CSTR1—3 operated at 5 °C, 22 °C and 40 °C respectively, were fed with a 100 mg/L SCN^- feed concentration during the start-up phase (Figure 3). CSTR2 and CSTR3 achieved 100% SCN^- biodegradation while CSTR1 achieved only 60% biodegradation by day 2. All three CSTRs were switched to continuous mode and operated for a further six residence times to achieve approximate steady state. The feed SCN^- concentration was subsequently increased to 250 mg/L, where CSTR2 maintained complete degradation, while CSTR3 achieved a 92% SCN^-biodegradation efficiency. The low temperature reactor (CSTR1) only achieved a reduction efficiency of 42%. This state was maintained for 20 days before increasing the feed SCN^- concentration to 500 mg/L. The rate on thiocyanate degradation only increased marginally, leading to a decrease in overall efficiency. CSTR1, CSTR2 and CSTR3 operated at 30%, 66% and 52% efficiency respectively. This was maintained for 25 days. During this period significant biofilm growth was observed on the impeller, baffles and walls in all three reactors. The biomass retention in the biofilm led to a higher effective cell concentration and greater thiocyanate degradation. The maximum SCN^- degradation rate was achieved at a feed concentration of 1000 mg/L, with CSTR2 and CSTR3 achieving rates of 80 and 70 mg/L/h respectively. The efficiency was reduced by approximately 50% at 5 °C. The increased rates coincided with the development of a substantial attached bioflim, suggesting this was a function of biomass retention, rather than the activation of an additional metabolic pathway or continued adaptation. The data suggest that the active population was not significantly inhibited at feed thiocyanate concentrations of up to 1000 mg/L. Based on the rate data, complete degradation could be achieved at 22 °C and 40 °C by increasing the residence time to 15—18 hours.

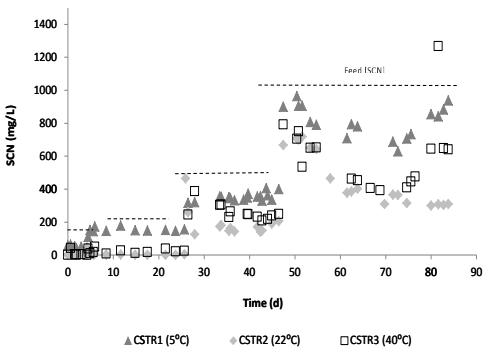

Figure 3 *Residual thiocyanate concentration as a function of time and feed concentration (———) for CSTRs1—3.*

The data show that temperature can have a significant effect on system performance. Work done by Gokulakrishnan and co-workers (Gokulakrishnan*et al.*, 2006) showed an increased specific microbial growth rate with increasing temperature, up to an optimum. Above 35 °C, the growth of Pseudomonas on caffeine decreased sharply, while significantly lower rates were observed at temperatures below 30 °C. In another study, the effect of temperature on ferrocyanide removal by biodegradation was clearly shown for temperatures between 20—40 °C (Dash *et al.*, 2009). Optimum removal of ferrocyanide was achieved at 30 °C. Increasing the temperature by 5 °C had a noticeable effect on the biodegradation rate and a 10% reduction in the degree of ferrocyanide removal was observed at 40 °C. In the current study the mixed population appeared to have a relatively broad effective temperature range. Preliminary batch experiments (data not shown), indicated an effective temperature range from 22 °C to 40 °C. Thiocyanate degradation efficiency decreased significantly below 20 °C and no activity was observed at 45 °C. It is not clear if changes in efficiency outside the optimum range are a function of metabolic inhibition, the loss of component species or both. The characterisation of mixed microbial populations is thus critical to determine the effect of temperature on the kinetics of the individual species making up the mixed population (Gurbuz *et al.*, 2009).

Conclusions

This study showed that the mixed microbial population consisted of a variety of heterotrophic organisms, several of which have been confirmed to have metabolic pathways capable of degrading cyanide species. The culture did not appear to be significantly inhibited by feed thiocyanate concentrations of up to 1000 mg/L, with degradation rates of between 70 and 80 mg/L/h achieved at this concentration at 22 °C and 40 °C. Lower temperatures significantly reduced degradation efficiency. The data show that the ASTER process has great potential to effectively treat effluents with thiocyanate concentrations significantly higher than at the current demonstration plant and suggest that complete degradation could be achieved by adjusting the residence time appropriately.

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