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Oxalate degradation by alkaliphilic biofilms acclimatised to nitrogen-supplemented and nitrogen-deficient conditions

Short title: Oxalate degradation under N supplemented and N deficient conditions

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Abstract

BACKGROUND: Sodium oxalate is a key organic contaminant in many industrial wastewater such as alumina industry, which diminish the process yields and product quality. Given that Bayer process liquor is typically deficient in nitrogen (N), there is external supplementation of N in current full-scale biological treatment processes. This study, for the first time examines
oxalate degradation under N deficient conditions in a comparative study using two parallel biofilm-reactors, one N-supplemented and the other under N-deficient conditions. Oxalate degradation rates and oxygen uptake rates (OUR) were determined at different bulk water dissolve oxygen (DO) set-points.

RESULTS: The results revealed that oxalate removal rates (33 – 111 mg/h.g biomass) linearly correlate with OUR (0 – 70 mg O2/h.g biomass) in the N-supplemented reactor. However, in the N-deficient reactor, a linear increase of oxalate removal was recorded only with DO upto ≤ 3 mg/L. Surprisingly, anaerobic oxalate removal was evident even in the presence of DO (up to 8 mg/L) in both reactors. Further elucidation revealed formate, acetate and methane by-products during anaerobic oxalate removal in both reactors.

CONCLUSION: This study revealed the feasibility of aerobic oxalate oxidation and fermentation under alkaline and N-deficient conditions. Further, this study confirms the critical role of DO in aerobic oxalate biodegradation.

Keywords: bioreactor, dissolved oxygen, nitrogen fixation, oxalate degradation, oxygen uptake rate, anaerobic

NOMENCLATURE

N Nitrogen
OUR Oxygen uptake rate
DO Dissolve oxygen
TOC Total organic carbon

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<table>
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<th>Abbreviation</th>
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<tr>
<td>HRT</td>
<td>Hydraulic retention time</td>
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<td>IC</td>
<td>Ion chromatography</td>
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<td>SS</td>
<td>Suspended solids</td>
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<td>VSS</td>
<td>Volatile suspended solids</td>
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<td>RFIC</td>
<td>Reagent free ion chromatography</td>
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<td>FID</td>
<td>Flame ionisation detector</td>
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INTRODUCTION

Bauxite ore is typically refined in a process known as the Bayer process to produce alumina (aluminium oxide). In brief, the Bayer process involves the digestion of crushed bauxite in a hot concentrated solution of sodium hydroxide (NaOH). During digestion most aluminium containing minerals such as gibbsite (Al(OH)₃), boehmite (γ-AlO(OH)) and diaspore (α-AlO(OH)), are solubilised to form sodium aluminate (NaAlO₂) 1,2. Any insoluble impurities are subsequently separated via settling and filtration of process liquor. To recover the solubilised Aluminium (Al), the process liquor is cooled and an Al(OH)₃ crystallisation seed is introduced to trigger crystallisation of Al. Finally, the spent NaOH is recycled back to the beginning of the process 3.

Recycling of spent NaOH-rich liquor is important for the Bayer process to be economical. Continuous recycling of NaOH-rich liquor, however, results in the build-up of metals (e.g. gallium (Ga) and vanadium (V)) and organics (naturally abundant in bauxite) in the process liquor. The total organic carbon (TOC) content of bauxite is typically ranging from 0.02 to 0.2 % (w/w) in the other regions of the world 4. However, in Australia, the bauxite ores can have a much higher TOC content in the range of 0.15 to 0.5 % (w/w) 2,4,5. The organic compounds in bauxite (e.g. polybasic acids, polyhydroxy acids, alcohols and phenols, humic and fulvic acids, and other carbohydrates) form organic sodium salts during the Bayer process, and of these organic salts, sodium oxalate (Na₂C₂O₄) has been identified as the most detrimental to the process 4,6. The recycling of spent Bayer liquor results in a build-up of Na₂C₂O₄ in the process liquor. Once supersaturation concentrations are reached, Na₂C₂O₄ tends to crystallise together with Al(OH)₃, impacting the quality, quantity and production cost of Al(OH)₃ 7-10. Therefore, effective removal of Na₂C₂O₄ from the Bayer process liquor is important for alumina refineries.
Different strategies have been adopted to facilitate Na$_2$C$_2$O$_4$ removal from Bayer process liquors$^9$. The most common strategies involve crystallisation of Na$_2$C$_2$O$_4$ and liquor burning in a side stream process$^{11-13}$. However, these conventional approaches suffer from a range of problems such as high energy consumption and emission of air pollutants. Recently, the alumina industry has embraced biological processes as a more environmentally benign alternative to oxidise Na$_2$C$_2$O$_4$$^{14,15}$. This process involves the use of microorganisms to drive aerobic oxidation of Na$_2$C$_2$O$_4$ into carbonate (reaction 1).

$$2C_2O_4^{2-} + 4OH^- + O_2 \rightarrow 4CO_3^{2-} + 2H_2O$$ (1)

Biological oxidation of Na$_2$C$_2$O$_4$ was first reported by Alcan International Limited in 1989$^{16}$. The company patented an aerobic bioreactor process, which consisted of a three compartment rotating biological contractor (with 73 rotating disks and a total biofilm surface area of 3.7 m$^2$) inoculated with Pseudomonas-like isolates from rhizospheres of oxalate producing plants. Due to the intolerance of the isolates to high pH, the pH of the bioreactor influent had to be adjusted to 7.5$^{16,17}$. The bioreactor successfully decreased an initial oxalate concentration of 2,600 mg/L to 50 mg/L within a short residence time of 5 hours. Following this study, other reactor systems and microbial strains have been explored under various conditions for biological oxalate removal$^{13,17,18}$. Today, some alumina refineries have successfully adopted full-scale biological treatment plants to degrade oxalate and other organic impurities from Bayer process liquor.

Although rich in biodegradable carbons, the process liquor of the alumina industry is deficient of nitrogen (N). Nitrogen is a major essential element required for the growth of all living organisms including bacteria. At present nitrogen requirements of biological treatment plants in alumina refineries are met with an external dose of N, and a commonly used nitrogen source
is urea\textsuperscript{14}. Compared to ammonium, urea is relatively stable under alkaline conditions. However, for bacteria to gain access to the nitrogen in urea, urea has to be first hydrolysed to form ammonia. At alkaline conditions, much of this ammonia can be easily stripped-off (by aeration) from the bioprocess via volatilisation. Therefore, urea is typically supplied to the bioreactor in excess to avoid N limitations \textsuperscript{14}. However, excessive dosage of urea is not only costly, the elevated ammonia volatilisation may also cause undesirable emission of odours, leading to health and safety hazards in the refinery.

One alternative would be to explore the use of alkaliphilic bacteria capable of fixing atmospheric dinitrogen (N\textsubscript{2}) to facilitate oxidation of oxalate and other organic impurities in process liquor. To our knowledge, no previous attempt has been made to oxidise organic impurities present in Bayer process liquor under nitrogen deficient conditions. Hence, a comparative assessment of the performance of N-deficient and N-supplemented microbial communities is imperative to ascertain whether the alumina industry could capitalise from the use of N-fixing inocula for bio-treatment of Bayer process liquor. Therefore, the aim of this study was to compare oxalate degradation under N-supplemented and N-deficient conditions in two packed bed biofilm reactors. The two bioreactors were operated aerobically for a period of 275 days using a synthetic medium that simulated the Bayer process liquor in terms of salinity and alkalinity. The effect of dissolved oxygen (DO) concentration on oxalate degradation was evaluated under N-supplemented and N-deficient conditions. This study will offer insights on the pros and cons of adopting N-fixing microorganisms to remove organics in Bayer liquor.
MATERIALS AND METHODS

Aerobic bioreactor systems

Two identical laboratory-scale aerobic bioreactor systems, consisting of packed bed columns and recirculation bottles were setup and operated as detailed below (Figure 1). The columns (total volume 650 mL) had an internal diameter and height of 55 mm and 400 mm, respectively, and were packed with graphite granules (3-5 mm diameter, KAIYU Industrial (HK) Ltd) to a bed volume of 600 mL. The weight of the air-dried graphite granules in each column was 480 g and once packed, the void volume of the granular column was 210 mL. The graphite granular media in the column reactors was exposed to a synthetic Bayer liquor that was continuously aerated in a 2 L glass recirculation bottle. The two systems were operated in sequencing-batch mode with a cycle length of 4 h. In the first 2 mins of the cycle, concentrated solutions (carbon and nutrients) were pumped in together with deionised water into the 2 L bottle to form 1.3 L of fresh influent. Thereafter, to maintain a near saturation level of DO, compressed air was sparged through the liquid in the 2 L bottle throughout the entire cycle. The liquor (a total working volume of approximately 1.5 L) was continuously recirculated through the packed bed column in an up-flow direction at a flow rate of 9.6 L/h. At the end of each cycle, the liquid (~1.3 L) in the 2 L bottle was decanted, while the packed column remained submerged (~200 mL).

The reactors were monitored and controlled using data acquisition and control hardware (CompactRio National Instruments, USA) and software (Labview, National Instrument, USA). Online monitoring of DO and pH was carried out using a luminescent DO probe (PDO2, Barben Analyzer Technology, USA) and an intermediate junction pH probe (Ionode IJ44, Ionode Pty Ltd, Australia), respectively. Two DO probes were used to measure the DO concentrations before and after the pack column, with one immersed in the liquid of the 2 L bottle and the
other placed at the outlet of the column reactor (Figure 1). All experiments were carried out at room temperature (~ 23°C).

**Synthetic Bayer Process Liquor**

*Influent for N-supplemented reactor*

The feed solution contained 2.0 g/L of sodium oxalate (Na₂C₂O₄) as the carbon source and 25 g/L of sodium chloride (NaCl, salinity 2.5 %) in order to simulate the salinity of Bayer liquor. The pH of the solution was adjusted to 9.0 - 9.5 with 2 M NaOH. Additionally, the solution contained (per L): 25 mg NH₄Cl, 125 mg NaHCO₃, 51 mg MgSO₄·7H₂O, 15 mg CaCl₂·2H₂O, 20.5 mg K₂HPO₄·3H₂O, and 1.25 mL of trace element solution. The trace element solution contained (per L): 0.43 g ZnSO₄·7H₂O, 5 g FeSO₄·7H₂O, 0.24 g CoCl₂·6H₂O, 0.99 g MnCl₂·4H₂O, 0.25 g CuSO₄·5H₂O, 0.22 g NaMoO₄·2H₂O, 0.19 g NiCl₂·6H₂O, 0.21 g NaSeO₄·10H₂O, 15 g ethylenediaminetetraacetic acid (EDTA), 0.014 g H₃BO₃, and 0.05 g NaWO₄·2H₂O.

*Influent for N-deficient reactor*

The feed solution for the N-deficient bioreactor system was otherwise similar to that of the N-supplemented bioreactor, except that the Na₂C₂O₄ concentration was only 0.8 g/L depending on maximum oxalate removal capacity, NH₄Cl was omitted, and 10 mg/L of yeast extract was added to supplement specific nutrient requirements such as amino acids.

**Reactor start up**

The inoculum for the bioreactors was sourced from two locations. One sediment sample was collected from a local beach (Floreat Beach) and another two were collected from Perry Lake Reserve of Western Australia. An equal weight (75 g) of sediment from each location was
mixed together and suspended in two separate 2 L glass vessels, which contained a 1.5 L of N-supplemented and N-deficient feed solutions, respectively. The Na$_2$C$_2$O$_4$ concentrations in the working solutions were initially maintained low (100 mg/L) to prevent substrate inhibition. The two vessels were aerated at room temperature for 3 weeks to enrich aerobic oxalate degrading microorganisms. During the enrichment, aeration was regularly (weekly) terminated for a period of 1 h to allow settling of the reactor contents. Subsequently, the supernatants (1 L) were discarded and replenished with respective fresh feed solutions.

After the enrichment, the sediments in both bottles were vigorously disturbed by shaking to dislodge microbial cells from the sediment material. Subsequently, the two column reactors were connected to the two vessels as illustrated in Figure 1. The feed solutions with the dislodged biomass were re-circulated through the column reactors to facilitate biofilm formation on the graphite granules. With intermittent disturbance of the sediment in each of the bottles, the reactors were operated with sediment in the vessels for another 2-3 d. Thereafter, all sediment material in each of the bottles was completely removed and microbial enrichment in column reactors were allowed to continue with weekly replenishment of feed solutions (1.3 L) in the 2 L bottles.

During the enrichment period, oxalate removal in the reactors was monitored every two weeks and the cyclic length of reactors were decreased as oxalate removal performance improved. Once a 4 h cycle length was achieved, the oxalate loading rate was increased (by increasing oxalate concentration in the feed) until no further increase of oxalate removal rate was detected. The final feed Na$_2$C$_2$O$_4$ concentrations in the N-deficient and N-supplemented reactors during stable performance were 0.8 g/L and 2.0 g/L, respectively.

**Cyclic studies**
Cyclic studies were performed both during enrichment and steady state operation of reactors. Cyclic studies were performed every two weeks and the frequency of sampling during a cycle depended on cycle length. Hourly sampling was carried out when cyclic studies were carried out on 4 h cycles. During sampling, 3 mL samples were withdrawn from the 2 L bottles and were immediately filtered through 0.22 μm pore size syringe filters (Cat. No. SLGN033NK, Merck Pty Ltd, Australia) into 2 mL Eppendorf tubes. The samples were then stored at 4 °C prior to analyses.

**Investigating the oxygen uptake rate of established biofilm**

Once a stable reactor performance was reached, the oxygen uptake rate of the biofilm was examined by exposing the biofilm to different influent DO concentrations (between 0 and 8 mg/L) in the column reactors. All of these experiments were carried out at an influent pH of 9.5 and with oxalate concentrations used during normal operation of reactors. DO set points were feedback-maintained by sparging compressed air or nitrogen into the 2 L vessel. The DO probe in the vessel (DO\textsubscript{in}) was used as the input variable for the feedback control algorithm and together with the second DO probe (DO\textsubscript{out}) located at the outlet of the column reactor, specific oxygen uptake rates (OUR) of biofilm were determined in accordance to equation 2.

\[
OUR = \frac{DO_{in} - DO_{out}}{HRT \times Weight\ of\ dry\ biomass \times Reactor\ liquid\ volume}
\] (2)

Where DO\textsubscript{in} and DO\textsubscript{out} are influent and effluent dissolved oxygen concentrations (mg O\textsubscript{2}/L), HRT (h) is the hydraulic retention time of the column reactor, the reactor liquid volume (L) is the total liquid volume of the reactor and weight of dry biomass (g) is the dry weight of biofilm attached onto graphite media. HRT was calculated as follows (equation 3):
The biofilm was exposed to each influent DO set point over a single 4 h cycle. In the subsequent 5 cycles, the biofilm was allowed to recover using normal operational conditions (i.e. at a DO of 8 mg/L) before the impact of the next DO concentration was examined. During these studies hourly liquid samples were collected and immediately filtered through 0.22 µm syringe filters (Cat No SLGNO33NK, Merck Millipore, USA) for analyses for oxalate and chemical oxygen demand.

The effect of initial oxalate concentration on OUR was also investigated by determining the OUR of the biofilm exposed to various initial concentrations of oxalate (80 – 550 mg). At first the biofilm was exposed to an influent DO concentration of 8 mg/L in complete absence of oxalate. Once a steady background OUR was observed, varying initial concentrations of Na$_2$C$_2$O$_4$ were introduced into the reactor at different time intervals and the response of the biofilm was quantified by measuring OUR, residual oxalate and COD concentrations. The total oxygen consumed (mmol) for a given quantity of Na$_2$C$_2$O$_4$ removed (mmol) was calculated by integrating the area under the OUR profile.

**Anaerobic oxalate degradation**

Anaerobic removal of oxalate was examined by withdrawing two subsets of biofilm coated granules from each reactor (16mL and 20 mL of media from N-supplemented and N-deficient reactor respectively) at the end of a reactor cycle. The granules from the two reactors were then transferred into two 150 mL serum bottles that contained their respective growth media but without the presence of oxalate. The serum bottles were subsequently capped (using butyl rubber stoppers) and crimped with aluminium seals and flushed for 3 min using N$_2$. To begin the time-course experiment, specified volumes of an oxalate stock solution (22 g/L) were added
to the N-supplemented and N-deficient serum bottles to obtain an initial oxalate concentrations of 1200 and 930 mg/L, respectively. The initial pH was 9 and liquid volume was 50 mL. Liquid and headspace gas sampling were carried out from both anaerobic bottles at time intervals of 32, 48, 72, 96 and 120 h. The liquid samples were immediately filtered through 0.22 µm syringe filters (Cat No SLGNO33NK, Merck Millipore, USA) and the filtrates were analysed for residual oxalate. The head space gas samples were analysed for methane gas. The experiment was carried out in duplicate to examine reproducibility of the results.

To explore possible routes of methanogensis, another duplicate set of biomass samples of N-supplemented and N-deficient reactors were exposed to H2/CO2 in complete absence of oxalate. Similar to previous experiment, the serum bottles with respective biomass (16 - 20 mL of media) and growth media were capped (using butyl rubber stoppers) crimped (with aluminium seals) and flushed for 3 min using N2. Subsequently, the head space of each bottle was flushed again using a H2/CO2 mixture for another 3 min. Headspace gas sampling for analysis of methane was then carried out at time intervals of 0, 16, 24, 40 and 48 h.

Assessment of nitrogen-deficient conditions

Two methods were used to monitor prevalence of N-deficient and N-supplemented conditions in the reactors, namely concentrations of soluble nitrogen species in the reactors, and acetylene reduction assays.

Concentrations of soluble nitrogen species

Ammonia-N, nitrite-N and nitrate-N concentrations in both N-deficient and N-supplemented reactors were frequently analysed using ion chromatography (IC). Approximately 2 mL solution samples were withdrawn and immediately filtered through 0.22 µm syringe filters (Cat No SLGNO33NK, Merck Millipore, USA) and the filtrates were stored at 4 °C until analysed.
Acetylene reduction assays

A modified assay of Sprent\textsuperscript{19} was used for the acetylene reduction assay. Six and two biofilm coated granules samples (16 - 20 mL medium) were withdrawn from the N-deficient and N-supplemented reactors respectively and the samples were placed in eight 150 mL bottles. Subsequently, 50 mL of N-deficient and N-supplemented growth media (pH 9) were introduced into the corresponding bottles. Two of the N-deficient and two of the N-supplemented bottles received 2 g/L oxalate. Of the remaining four N-deficient bottles, two received 1.2 g/L of acetate and the other two received 1.2 g/L of formate. All bottles were subsequently capped (using butyl rubber stoppers), crimped (with an aluminium seal) and flushed with helium for 3 min to remove any N\textsubscript{2} from the samples. Thereafter, 2 % of the headspace helium was removed and replaced with pure oxygen in one N-supplemented bottle and one oxalate, one acetate and one formate containing N-deficient bottle. In the remaining four bottles, the headspace was completely flushed with pure oxygen. Finally, 2 % headspace volumes of all eight bottles were replaced with acetylene (produced by reacting tap water with calcium carbide in a 1 L conical flask and acetylene captured with displacement of water in a measuring cylinder). The bottles were then incubated in an environmental shaker at 28 °C. Liquid and headspace gas sampling were carried out on all eight bottles at time intervals of 3, 16, 24, 40 and 48 h. The liquid samples were immediately filtered through 0.22 μm syringe filters (Cat No SLGNO33NK, Merck Millipore, USA) and the filtrates were stored at 4 °C until analysed.

Sample analysis

Estimation of dry biomass weight in reactors

The amount of biomass coating the graphite media in the reactors was determined by removing a known volume of media from each of the reactors. The graphite media was then immersed in
a known volume of deionized water in a 50 mL Falcon tube and sonicated in an ultrasonic water bath (Sanophon ultrasonic cleaner - 90 watts and 50 Hz) for 3 min to dislodge the attached biofilm. The suspension was then collected in a new Falcon tube (50 mL) and the graphite media was sonicated once more for 3 min in fresh deionized water. The suspensions from the two sonications were combined and suspended solids (SS) and volatile suspended solids (VSS) of the suspension were measured using standard methods\textsuperscript{20}.

Analysis of nitrogen species and organic carbon

A Dionex ICS-3000 reagent free ion chromatography (RFIC) system equipped with an IonPac\textsuperscript{®} AS18 4 x 250 mm column was used to measure oxalate, acetate, formate, nitrite and nitrate in liquid samples. Potassium hydroxide was used as an eluent at a flow rate of 1 mL/min. The eluent concentration was 12-45 mM from 0-5 min, 45 mM from 5-8 min, 45-60 mM from 8-10 min and 60-12 mM from 10-13 min. Ammonium (NH\textsubscript{4}\textsuperscript{+}-N) was measured with the same RFIC but with a IonPac\textsuperscript{®} CG16, CS16, 5 mm column. Methansulfonic acid was used as an eluent with a flow rate of 1 mL/min. The eluent concentration was 30 mM for 29 min. The temperature of the two columns were maintained at 30°C. Suppressed conductivity was used as the detection signal (ASRS ULTRA II 4 mm, 150 mA, AutoSuppresioin\textsuperscript{®} recycle mode).

A closed reflux dichromate method (HACH Method 8000, HACH Ltd) was used in accordance to manufacturer’s instructions to measure chemical oxygen demand in the liquid samples.

Gas analysis

Headspace gas analysis was carried out using a Trace 1300 gas chromatograph (ThermoFisher Scientific, USA) fitted with a flame ionisation detector (FID). Methane, acetylene and ethylene gases were measured using a Rt\textsuperscript{®}-U-BOND (30 m, 0.32 mm ID, 10 µm film, Cat.# 19752, Restek, USA) capillary column. A gas volume of 100 µL was manually transferred using a gas
tight syringe into a split injector (split flow 40 mL/min) maintained at 200 °C. Helium was used as the carrier gas and the flow rate through the capillary column was maintained at a constant pressure of 53.1 kPa. The initial oven temperature was set at 100 °C for 1 min. Subsequently the temperature was raised to 150 °C at a rate of 25 °C/min and was finally held for 4 min. FID temperature was maintained at 230 °C and analysis was carried out using Chromeleon software (Version 7.1.2.1478).

RESULTS AND DISCUSSION

Start-up of the N-supplemented and N-deficient reactors

Although started with the same inoculum and operating conditions, the oxalate removal rates recorded for the two reactors were notably different (Figure 2). The N-supplemented reactor demonstrated immediate ability to oxidise oxalate (~ 34 mg/L.h), whereas the N-deficient reactor only demonstrated a measurable oxalate-degrading activity after approximately 75 d. Since the difference between the two reactors was the availability of ammonium, the inability of the N-deficient reactor to readily acclimatise an active oxalate-degrading culture was likely due to the lack of ammonium.

The oxalate removal rate of the N-supplemented reactor rapidly increased (with a linear increase from 34 to 364 mg/L.h \((R^2 = 0.98)\)) during the first 48 d of reactor operation. During this period, the cycle length was gradually shortened down to 4 h and the oxalate concentrations in the feed was systematically increased. At a cycle length of 4 h, the highest oxalate degradation rate was 430 mg/L.h and the system completely removed an initial oxalate Na2C2O4 concentration of 2 g/L. In contrast, a slow increase of oxalate removal (from 7 to 187 mg/L.h) was noted after a prolonged operation (after 84 d) of N-deficient reactor, and a relatively stable
performance was only observed after 224 d of operation. The acclimatisation period of the N-deficient reactor was five times longer than that of the N-supplemented reactor. Moreover, the N-deficient reactor only could remove approximately half (0.8 g/L) the quantity of oxalate that was fed into the N-supplemented reactor (2 g/L).

The lower oxalate degradation rate in the N-deficient reactor was likely attributed to the lower concentration of biomass and lower specific oxalate removal rate as compared to the N-supplemented reactor. The VSS and specific oxalate removal rate of N-supplemented reactor (9.6 mg/mL of graphite media and 111 mg-oxalate /h. g-biomass respectively) was found to be approximately 1.25 times higher than that of N-deficient reactor (7.6 mg/mL of graphite media and 87 mg-oxalate /h. g-biomass respectively). The slow increase of oxalate removal rate in the N-deficient reactor during the period of enrichment, could be a result of low initial abundance and/or slow growth rates of microorganisms capable of oxidising oxalate under N-deficient conditions. In general, the growth rates of nitrogen fixing bacteria are magnitude lower than the growth rates of bacteria that can assimilate ammonia \(^{21}\). While slower growth rates of N-fixing microorganisms may have contributed towards the long enrichment period, potentially sub-optimal environmental conditions (e.g. pH, temperature etc.) for growth may also have contributed to the observed outcome.

**Detailed studies revealed that both N-supplemented and N-deficient biofilms had a similar capacity to degrade oxalate**

Only a comparative assessment of oxalate degradation between an N-supplemented and an N-deficient process can reveal whether the N-deficient process could perform as well as the N-supplemented process. This study, for the very first time compared the oxalate degradation of
an N-supplemented and an N-deficient reactor. The oxalate degradation studies were executed once both reactors achieved a stable performance at a cycle length of 4 h.

Understanding the dependence of oxalate biodegradation on oxygen transfer or availability is useful for optimising aerobic biofilm treatment processes \(^{22-24}\). Hence, the effect of DO concentration on oxalate degradation was examined by exposing the N-supplemented and N-deficient biofilms to different bulk water DO concentrations (Figure 3). The responses of the N-supplemented and N-deficient biofilms to various DO concentrations were notably different. The N-supplemented reactor showed a higher oxalate removal rate with an increase in bulk water DO concentration (Figures 3A and 3B), and the highest removal rate was observed with a bulk water DO concentration of 8 mg/L (Figure 3B). Linear relationships were recorded between DO concentration and (1) oxalate removal rate \((R^2 = 0.94)\) and (2) OUR \((R^2 = 0.99)\) (Figure 3B). These results indicated that oxalate degradation in the N-supplemented reactor was limited by DO availability.

In contrast, the N-deficient reactor did not show an increase of oxalate removal with an increase of bulk water DO concentration. Oxalate degradation improved until DO concentrations reached 3 mg/L, but no further increase occurred at higher DO concentrations (Figures 3D and 3E). A linear correlation \((R^2 = 0.99)\) between increase of bulk water DO concentration and oxalate removal rate was only observed up to a bulk water DO concentration of approximately 3 mg/L.

In addition to aerobic degradation of oxalate, oxalate also can be degraded anaerobically to form methane \(^{25}\). Even at saturated concentrations of DO, anaerobic pockets may still be present in biofilm reactors as reported in literature \(^{26}\). The experimental oxalate removal and the theoretically expected aerobic oxalate removal (calculated according to reaction 1) were plotted against the total experimental oxygen consumption at different DO concentrations (Figure 3G).
The amount of oxalate degraded was higher in both reactors compared to the expected amounts of oxalate removed based on the actual oxygen consumption. Moreover, both reactors were capable of removing oxalate in complete absence of DO (0 mg/L, Figure 3G). Compared to the N-supplemented reactor, the anaerobic removal of oxalate was marginally lower (3 mmol) in N-deficient reactor. Approximately 7.5 mmoles of oxalate was anaerobically removed alongside aerobic oxalate degradation in the N-supplemented reactor at all tested DO concentrations. This result suggested that even a high DO concentration of 8 mg/L failed to eliminate the occurrence of anaerobic pockets in the biofilm of N-supplemented reactor. A linear relationship \((R^2 = 0.91)\) between OUR and oxalate removal rate (Figure 3C) confirms that oxygen was limiting in this reactor allowing anaerobic removal of oxalate also to continue. With an increase of DO from 0 to 8 mg/L, the \% anaerobic removal \((\frac{(Total\ oxalate\ removed - Theoretical\ aerobic\ removal\ of\ oxalate)}{Total\ oxalate\ removed} \times 100)\) decreased from 100 to 45 \% (due to increase of aerobic oxalate removal) suggesting that anaerobic removal of oxalate was a significant component in N-supplemented reactor even at a higher concentration of DO.

In contrast, the N-deficient reactor only showed a linear relationship \((R^2 = 0.99)\) between OUR and oxalate removal rate (Figure 3F) with bulk water DO concentrations lower than 3 mg/L. When the bulk water DO concentration was above 3 mg/L, the increase in OUR did not corroborate with an increase in oxalate removal rate, which largely remained unchanged (80 mg/h.g biomass). At higher OUR (e.g. at 68 mg O\(_2\)/h.g biomass; ~ at DO 8 mg/L), the N-deficient reactor showed a low constant oxalate removal rate (83 mg-Oxalate/h.g biomass) compared to an increasing removal rate noted in N-supplemented reactor (110 mg-Oxalate/h.g biomass).

The graph showing oxalate degradation versus oxygen consumption for N-deficient reactor was also parallel to theoretical oxygen consumption graph when oxygen consumption was below
1.8 mmol (Figure 3G). Approximately 4.5 mmoles of oxalate were anaerobically removed alongside aerobic degradation of oxalate even in the N-deficient reactor when oxygen consumption was below 1.8 mmol. An increase of OUR with no increase of oxalate removal rate at oxygen consumption above 1.8 mmol suggested an increase of aerobic and a simultaneous decrease of anaerobic removal of oxalate maintaining a constant net removal of oxalate (~ 10 mmol) in the N-deficient reactor (Figure 3G). This indicated that oxygen limitation in the N-deficient reactor was not as severe as in the N-supplemented reactor at higher bulk water DO concentrations. This observation was perhaps due to the low biomass concentration (thinner biofilm) in the N-deficient reactor (1.25 times lower than that of N-supplemented reactor), enabling maintenance of possibly a much thinner biofilm in the reactor. Higher biomass densities on the other hand induce higher oxygen demands and also reduce void spaces in packed bed column reactors. A reduction of void spaces also results in short circuiting flow paths and anaerobic conditions in N-supplemented reactor was likely induced by all of the above factors.

In summary, both reactors showed a much higher level of oxalate removal (specifically at bulk water DO concentrations of less than 3 mg/L, or total oxygen consumption below 1.8 mmol) compared to the theoretical estimations of aerobic oxalate degradation (Figure 3G). This suggests that anaerobic degradation of oxalate also occurred in both reactors in the presence of oxygen. Anaerobic oxalate degradation, however, decreased with an increase of bulk water DO concentrations in the N-deficient reactor whereas this was not evident in N-supplemented reactor. Moreover, for all DO concentrations tested, the N-supplemented reactor was able to remove a marginally higher quantity of oxalate compared to the N-deficient reactor (Figure 3G). Overall the oxalate removal efficiencies of both reactor can be further enhanced by eliminating anaerobic pockets and enhancing mass transfer by replacing the packed bed columns with suspended biomass (e.g. activated sludge) or biofilm carriers.
To further verify whether the N-supplemented reactor was more efficient at removing oxalate, an alternative method was used to determine the relationship between oxygen consumption and oxalate removal in both N-supplemented and N-deficient reactors (Figure 4). This experiment enabled accurate quantification of oxygen demand (at a bulk water DO of 8 mg/L) for a spike of a known quantity of oxalate, and this was facilitated with an integration of area under the derived OUR curve. Both reactors, when starved of oxalate for an extended period of time, showed an endogenous, base line oxygen consumption rate of approximately 20 mg/h.g biomass (Figures 4A and 4B). Compared to the N-deficient reactor, the oxalate removal efficiency of the N-supplemented reactor was found to be approximately 1.3 times higher at a given level of oxygen consumption (Figure 4C). Nitrogen fixation was likely the main mechanism by which biological nitrogen requirements were met in the N-deficient reactor. The high energy demand of nitrogen fixation, may have negatively impacted oxalate removal efficiency of the N-deficient reactor. Future research should focus on developing strategies to improve the oxalate removal efficiency in N-deficient reactors.

**Presence/absence of an easily utilisable nitrogen source influence pathway of methonogenesis during alkaline fermentation of oxalate**

Anaerobic fermentation of oxalate to methane is reported in literature. However, studies have not revealed the methane generation pathways, specifically when fermenting oxalate under alkaline and N-deficient conditions. Our study for the first time, compared fermentation of oxalate in an N-supplemented and an N-deficient reactor to determine the methane generation pathways of these two reactors. A separate serum bottle experiment was conducted to determine the anaerobic oxalate degradation pathway in each N-supplemented and N-deficient reactors. The N-supplemented and N-deficient bottles were exposed to an initial oxalate concentration of ~ 1 g/L under anaerobic conditions. Both samples showed a good removal of oxalate under
anaerobic conditions (Figure 5). The initial anaerobic oxalate fermentation rates in the N-supplemented and N-deficient reactors were 8.9 and 6.7 mg/g biomass.h, respectively. When oxalate concentrations decreased to ~100 mg/L, the oxalate fermentation rates in the N-supplemented and N-deficient reactors reduced to 0.5 and 0.6 mg/g biomass.h respectively. The decrease in anaerobic oxalate fermentation rate also coincided with a peak concentration of formate (510 and 202 mg/L in N-supplemented and N-deficient reactors, respectively), which is an intermediate by-product of both aerobic and anaerobic oxalate degradation. However, it remains unclear whether the decrease in oxalate fermentation was due to the build-up of formate (which might have inhibited oxalate fermentation) or the exhaustion of oxalate in both reactors.

Acetate accumulation was also detected in both reactors, but unlike formate, the concentration of acetate did not decrease over time (Figures 5A and 5B). Guyot and Brauman report the ability of bacteria to ferment formate to acetate. The detection of both formate and acetate in both N-supplemented and N-deficient reactors suggested two possible mechanisms of anaerobic oxalate mineralisation: (1) by a single bacterium having necessary metabolic pathways to ferment oxalate to acetate via the intermediate by-product formate and (2) by a group of bacteria (individually lacking a complete metabolic pathway) interacting symbiotically. If a single bacterium was responsible, the fermentation of formate to acetate appears to be the rate limiting step of the process resulting in a momentary accumulation of formate in the medium. The decrease in the formate concentration over time suggested the presence of hydrogenotrophic methanogens since some hydrogenotrophic methanogens are known to be capable of utilising formate as a substrate.

The formate and acetate that accumulated, far exceeded the amount of methane produced in both N-supplemented and N-deficient reactors during anaerobic fermentation of oxalate (Figure...
5). The N-supplemented reactor has a higher methane production rate (0.125 mg/g biomass.h) than the N-deficient reactor (0.05 mg/g biomass.h). While a low emission of methane could be an inherent feature of N-deficient systems, a difference in the thickness of biofilm in the two systems may also have contributed toward this outcome with a low and a high abundance of methanogens in N-deficient and N-supplemented reactors, respectively.

To further explore the possible route of methanogenesis, both N-supplemented and N-deficient reactors were exposed to H₂/CO₂ in the absence of oxalate. Over a 2 d incubation period, the N-supplemented reactor demonstrated continuous production of methane whereas no measurable quantity of methane was produced by the N-deficient reactor (Figure 6). The inability of the N-deficient reactor to produce methane with H₂/CO₂ suggests lack of hydrogenotrophic methanogens and methane production during fermentation of oxalate possibly occurred via acetoclastic methanogens in this reactor 32, 33. The methane production rates when fermenting oxalate (0.125 mg/g biomass.h) and when utilising H₂/CO₂ (0.131 mg/g biomass.h), on the other hand showed a great deal of similarity in the N-supplemented reactor. This indicates that the route of methanogenesis in N-supplemented reactor was perhaps predominantly via hydrogenotrophic methanogens. Although chemical data provides some insight towards route of methanogenesis of both N-supplemented and N-deficient reactors, more in depth microbiological analysis of methanogens in ecosystems similar to that of N-supplemented and N-deficient oxalate degrading reactors is required to better understand the influence of nitrogen on the selection of methanogens during oxalate fermentation.

**Are nitrogen requirements of N-deficient reactor fulfilled with atmospheric nitrogen fixation?**

The feed of the N-deficient reactor did not contain any inorganic nitrogen source. Frequent measurement of NH₄-N, nitrite-N and nitrate-N confirmed no measurable quantity of
ammonia, nitrite or nitrate in the N-deficient reactor. It is common practice to include a trace
amount of yeast extract to supplement essential nutrient requirements of nitrogen fixing bacteria
and, therefore 10 mg/L yeast extract was added into the feed of the N-deficient reactor.
This trace amount of yeast extract is unlikely to fulfil all nitrogen requirements of the N-
deficient reactor. Hence, nitrogen requirements of the N-deficient reactor were likely fulfilled
via biological nitrogen fixation.

To confirm the presence of nitrogen fixation activity, acetylene reduction assay was performed,
which facilitates an indirect measurement of nitrogen fixation by exploiting the ability of the
nitrogenase enzyme to reduce acetylene to ethylene. When the acetylene reduction assay was
carried out using the biomass of N-deficient reactor, no ethylene was detected over a 48 h
incubation period with both 2 % and 100 % of oxygen. Inactivation of nitrogenase enzyme with
exposure to high concentration of oxygen is well documented and there is a possibility that
the negative results obtained was a result of the use of 100% oxygen content. With Figures 3E
and 3F suggesting limited anaerobic activity in N-deficient reactor when the bulk water DO
was 8 mg/L, the biofilm in the reactor was assumed to be tolerant towards oxygen when fixing
nitrogen. Specifically, the 2 % oxygen used in the assay was unlikely inhibitory as this has been
widely reported in past literature where acetylene reduction assays have been carried out to
determine nitrogen fixation.

Although able to oxidise the carbon source, some nitrogen fixing bacteria have been shown to
have difficulties in reducing acetylene when exposed to particular carbon sources. For
example, a nitrogen fixing strain *Methylococcus capsulatus*, although able to oxidise both
methanol and glutamate, was only able to reduce acetylene when in the presence of methanol
and not glutamate. Accordingly, the negative result obtained with oxalate could also be a
result of a substrate inhibition (i.e. Oxalate) on the acetylene reduction. According to Figure 5,
acetate and formate are intermediate by-products of oxalate metabolism and hence, in addition to oxalate, the biofilm of N-deficient reactor is also capable of utilising both acetate and formate. Therefore, the acetylene reduction assay was subsequently carried out replacing oxalate with acetate or formate. When formate was used as the sole source of carbon, the outcome was similar to oxalate i.e. no ethylene was detected. However, when acetate was used as the sole source of carbon in the acetylene reduction assay, ethylene was detected (data not shown) confirming that the biofilm of N-deficient reactor was capable of fixing nitrogen.

Similar to what has been observed by Dolton et al. (1976), in this study the use of both oxalate and formate as substrate inhibited the reduction of acetylene to ethylene by the nitrogen fixing bacteria in the N-deficient reactor.

**Implications of findings**

Biological degradation of oxalate is a more environmentally benign approach to removing oxalate from Bayer process wastes when compared to traditional destruction methods such as liquor burning. With a comparative assessment, this study for the very first time experimentally demonstrated that an N-deficient biological process could be used to oxidise oxalate under alkaline conditions. An in-depth assessment of the oxalate degradation efficiency revealed that the oxalate removal relative to oxygen consumption in the N-supplemented reactor was only slightly higher than in N-deficient reactor, indicating that aeration costs would be similar if current N-supplemented systems were to be replaced with N-deficient systems.

The findings also suggest that anaerobic oxalate mineralisation in the biofilm systems tested was inevitable. However, conversion of oxalate to methane is of environmental concern because compared to carbon dioxide, methane is a much more potent (30 times) greenhouse gas. The methane yield per oxalate degraded was lower for N-deficient than N-supplemented reactor,
indicating that converting N-supplemented bioreactors to N-deficient ones could potentially reduce methane emissions.

CONCLUSIONS

This study evaluated and compared the effect of DO concentration on oxalate degradation of N-supplemented and N-deficient biofilm reactors under saline and alkaline conditions. Based on the results, N-supplemented reactor performs better in oxalate removal rate and also has a much shorter start-up period compared to the N-deficient reactor. In both reactors, oxalate degradation occurred in absence of DO, and the amount of oxalate removed per oxygen consumed exceeded theoretical aerobic oxalate removal estimates, suggesting that some of the oxalate was degraded anaerobically. Under anaerobic conditions, both reactors produced formate and acetate during fermentation of oxalate.

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REFERENCES


18. Author, Biological disposal of oxalate - by introducing oxalate into reactor contg. aq. soln. in which oxalate-degrading aerobic microorganism is present, etcWO9112207-A1; WO9112207-A; AU9173182-A; BR9105940-A; AU645065-B; US5314806-A; ES2056023-A6; CA2073758-C (1991).


Figure 1. Schematic diagram of the aerobic bioreactor system used in the study.
Figure 2. Change of influent oxalate concentration and cycle length of (a) N-supplemented and (b) N-deficient reactors at the start-up of the reactors, and (c) Oxalate removal efficiencies of the N-supplemented and the N-deficient reactor inoculated with the same starting inoculum.
Figure 3. Effect of (a and d) DO concentration on the removal of oxalate; (b and e) DO concentration on the removal rate of oxalate; (c and f) Oxygen uptake rate on the removal rate of oxalate; (g) Correlations of oxygen consumed and oxalate removed for N-supplemented and N-deficient reactors.
Figure 4. Influence of oxalate spikes on oxygen uptake rates in (a) N-supplemented and (b) N-deficient reactors. (c) Correlations of oxygen consumed and oxalate removed for N-supplemented and N-deficient reactors.
Figure 5. Conversion of carbon in N-supplemented and N-deficient reactors during anaerobic fermentation of oxalate. $C/C_0 = \text{molar ratio of carbon in degradation products versus initial oxalate carbon concentration.}$ $C = \text{Carbon moles in degradation products (such as oxalate, acetate, formate or methane)}$ and $C_0 = \text{Carbon moles in initial oxalate concentration.}$
Figure 6. Production of methane during anaerobic exposure to H₂/CO₂ (Mixture of H₂ 80\% and CO₂ 20\%) in the absence of oxalate.