INL/EXT-16-39515-Revision-0

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August 2016

The INL is a U.S. Department of Energy National Laboratory
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Idaho National Laboratory
Idaho Falls, Idaho 83415

http://www.inl.gov

Prepared for the
U.S. Department of Energy
Office of Energy Efficiency and Renewable Energy
Under DOE Idaho Operations Office
Contract DE-AC07-05ID14517

Developing a Scalable System to Harvest Critical Materials from Industrial Waste with *Gluconobacter oxydans*

Michael Jindra

Office of Science, Science Undergraduate Laboratory Internship Program

Ohio State University, Columbus, OH

Idaho National Laboratory, Idaho Falls, ID

August 2016

Prepared under the direction of David W. Reed in the Biological and Chemical Processing Department at the Idaho National Laboratory.

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ABSTRACT

Organic acid production by *Gluconobacter oxydans* has been proposed as part of a potential economical and environmentally friendly heterotrophic bioleaching process for recovery of rare earth elements from industrial waste. To provide organic acids in a quantity and at a rate useful for commercial bioleaching, continuous culture of the acid-producing organism will be necessary. The objective of this research project was to determine optimal conditions for continuous cultivation of *G. oxydans* for production of organic acids. Cultures were subjected to dilution rates ranging from .05 hr^{-1} to .38 hr^{-1} to determine the optimum conditions for production of lixiviant agents. Previous studies have identified methods for maximizing production of gluconic acid and 2,5-diketogluconic acid from *G. oxydans*, yet the utilization of these and other unidentified *G. oxydans* metabolites for leaching of rare earth metals is largely unexplored. Continuous cultures exhibited sustained organic acid production for at least 110 hours, while batch fermentations showed slowed growth rates after 16 hours. Several parameters, namely optical density, dissolved oxygen, organic acid concentration and pH, were monitored to characterize the steady state conditions of the fermentation process. The observed trend was increasing acidity with decreasing dilution rates, as the lowest dilution rate of 0.05 hr^{-1} yielded the highest gluconic acid concentration of 176 mM and lowest pH of 2.20. Trace amounts of 2,5-diketogluconic acid were detected only at the 0.05 hr^{-1} dilution rate, likely because the pH of the bioreactors was not maintained within the window for gluconate dehydrogenase activity [Silberbach, M., et al., Appl Microbiol Biotechnol, 2003. **62**(1): p. 92-8].

I. INTRODUCTION

In response to anticipated rare earth element (REE) supply constraints, the Critical Materials Institute was initiated by the U.S. Department of Energy (DOE) to support research to develop alternative procurement methods, sources, and substitutes for REE and other identified strategic metals. The capital inputs and environmental externalities associated with current domestic REE mining practices have made the United States unable to compete with other global REE producers, namely China [1]. The DOE has identified three key strategies to satisfy the REE demand associated with clean energy technology advancement: diversifying REE global supply chains, developing functional substitutes, and recycling waste streams rich in REE [2]. Leaching technologies are ideal for extraction of trace elements from solid materials and are therefore the primary strategies to recover REE from industrial waste streams and virgin ores alike. Conventional REE extraction from virgin bastnaesite ore entails roasting with concentrated sulfuric acid at temperatures above 300°C, leaching with water, and acid neutralization with magnesia. As noted above, the environmental externalities from acidic roasting off-gasses and radioactive leaching residue compounded with mining process costs have discouraged new domestic REE mining operations from attempting to compete with Chinese operations [3]. Heap bioleaching approaches, however, could involve less expensive chemical and energy inputs and provide an economical inorganic extraction approach for low-grade ores [4]. Bioleaching is widely practiced in copper production. In copper bioleaching environments, sulfuric acid is produced by autotrophic microbes to facilitate solubilization of copper from the chalcocite ore at temperatures between 12°C and 27°C [5]. Increasing world copper demand and decreasing supply of primary ores have expanded the role of microbe-assisted leaching in copper hydrometallurgy [6]. This success has inspired examination of bioleaching to develop environmentally-friendly and economical processes for recovering other metals from low-grade ores or low-grade ore analogs, such as industrial wastes.

Leaching efficiencies of 90% to 100% of yttrium from retorted phosphor powder waste streams have been achieved, albeit concentrated (4N) mineral acid solutions and elevated temperatures were necessary [7]. Aung et al. reported improved extraction of metals from spent fluidized catalytic cracking (FCC) catalyst when using biotically produced leaching agents instead of synthetic leaching solutions of the same nominal acid concentration; recoveries were 2.7% to 20% higher with organic acids produced by *Aspergillus niger* than with the abiotically prepared organic acid solutions. The primary organic acids produced by *A. niger* were identified as citric, gluconic (GA), and oxalic acids. These data suggest micro-organisms positively contribute to the extraction of rare earths from spent catalyst pulp [8]. Most relevant to this study, *Gluconobacter oxydans* culture supernatants demonstrated higher levels of REE leaching from spent FCC catalysts than abiotically prepared solutions of GA at higher concentrations than measured in the *G. oxydans* supernatants; GA is the primary product of *G. oxydans* cultures grown on glucose [9]. Spent FCC catalyst is of interest because it is a substantial waste product of petroleum refineries, with 400,000,000 kg generated each year and is a known source of lanthanum and cerium [4], [10]. The DOE classified both cerium and lanthanum as "near-critical" materials with an endangered short-term supply [2]. Leaching with *G. oxydans* offers an appealing alternative to conventional REE recovery. *G. oxydans* is a non-pathogenic,

industrially important bacterium that incompletely oxidizes sugars, alcohols, and acids in the presence of oxygen [11]. When fed glucose, the primary oxidation product is GA, a non-toxic, non-caustic, and non-corrosive biodegradable acid [12]. The terminal product of glucose oxidation from *G. oxydans* is 2,5-diketogluconic acid (2,5-DKG), which has a lower pKa than GA and has potential for improving leaching of REE. With an annotated genome sequence and a developed network model, *G. oxydans* may also be engineered to increase production of certain metabolites, including 2,5-DKG [13], [14], [15].

The objective of this study was to develop an economic and environmentally friendly process for leaching of rare earth elements from industrial waste. *G. oxydans* was evaluated for continuous production of biodegradable lixiviant agents ideal for leaching of inorganics. Optimal conditions for cultivation of *G. oxydans* and production of organic acid solutions were investigated by continuously feeding glucose-rich media at various dilution rates. Optical density, dissolved oxygen, organic acid concentration, and pH were monitored to determine the effect of dilution rates on cell count and acid production.

II. MATERIALS AND METHODS

A. Preparation of chemostat

An Infors AG Sixfors Fermenter (Infors HT, Laurel, MD) was used for continuous culture of *G. oxydans*. This system consisted of six identical 500 mL working volume chemostats with online pH and dissolved oxygen (DO) monitoring. Pikovskaya medium (Pkm) without glucose was added to the reactor vessels and autoclaved with the pH and DO probes in place [16]. The medium was modified by substituting $CaCl_2 \cdot 7H_2O$ (0.3 mM) and KH_2PO_4 (2.7 mM) for the insoluble calcium phosphate. Filter-sterilized glucose solution was added to the reactor vessel until a starting concentration of 40 g / L was reached. The vessels were then placed in the Sixfors system and the DO probes were allowed to polarize for at least twelve hours. After polarization, temperature control to 30°C, stir speed of 600 RPM, and constant oxygen flow of 2 vessel volumes per minute (vvm) were set. Iris software allowed for online monitoring of pH and dissolved oxygen. Measurements were averaged over ten minute intervals.

B. Culture growth

G. oxydans strain NRRL B58 was obtained from the Agriculture Research Service, USDA (Peoria, IL). Stock cultures were grown in 100 mL of modified Pkm with 10 grams of glucose per liter in shake flasks at 28°C and 180 RPM for up to 48 hours.

C. Inoculation

Stock cultures were harvested by centrifugation at 6000 x *g* for 20 minutes and the cells were resuspended in glucose-free modified Pkm. The suspension was used to inoculate each SixFors reactor vessel to a starting optical density (OD) at 600 nm of 0.08-0.10.

D. Chemostat operation

After chemostat inoculation the cultures were grown under batch conditions for about 15 hours to ensure the cultures had reached mid-log growth phase. Continuous culture was initiated by feeding modified Pkm amended with glucose (40 g/L) at various dilution rates: 0.05 hr^{-1}, 0.16 hr^{-1}, 0.25 hr^{-1}, 0.30 hr^{-1}, and 0.38 hr^{-1}. A scheme of the bioreactor is shown in Figure 1.

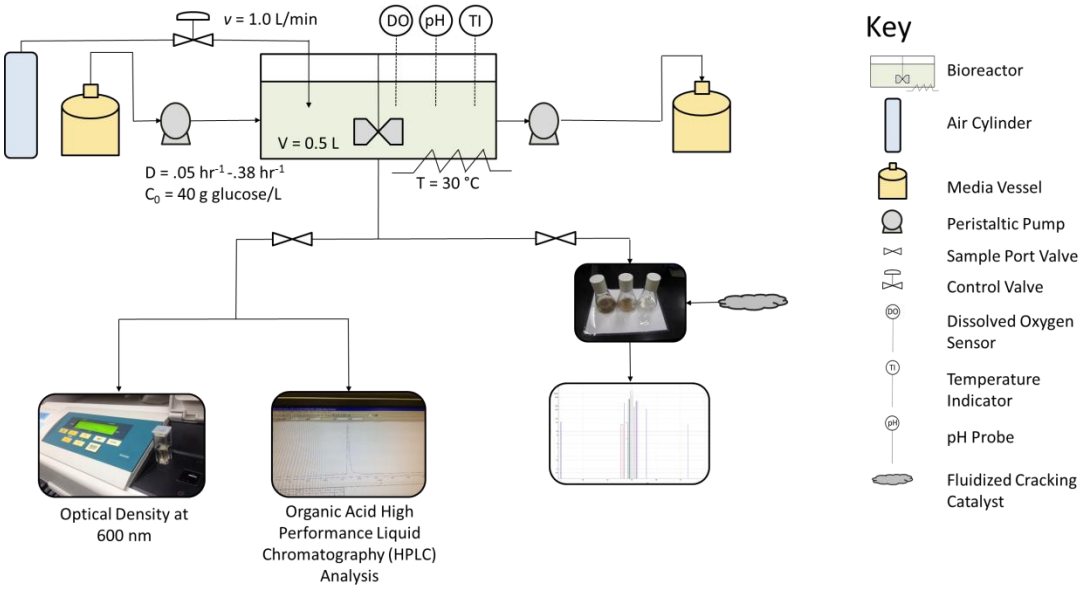

FIG. 1: Piping and instrumentation diagram for Sixfors bioreactor used to continuously culture *G.oxydans*

Samples of 1.5 mL were drawn from each bioreactor vessel at the end of the startup phase and periodically during the continuous feeding for measurement of optical density (OD) of the bacterial culture (an approximation of cell density) and organic acid production. The samples for organic acid analysis were centrifuged at 20,000 x *g* for 5 minutes and filtered with Millex-LH 0.22 µm PTFE filters immediately after sampling and frozen at 4°C prior to analysis, to halt further metabolic activity.

E. Analytical Methods

OD was measured using a spectrophotometer at 600 nm. Organic acids were measured by high-performance liquid chromatography (HPLC) with a BioRad Aminex HPX-87H column (300 mm x 7.8 mm, 5 µm particle size) using 0.004 M sulfuric acid as the mobile phase at 0.6 mL /min and a column temperature of 35°C; organic acids were monitored by a photodiode array detector and quantified at 210 nm using external standards. Standard curves for oxalate, citrate, malate, succinate, formate, and acetate were generated using a BioRad organic acid

standard mixture. Separate standards for gluconic, 2-ketogluconic acid, 5-ketogluconic acid, and 2,5-DKG were utilized. The GA and 2-ketogluconate were obtained from Sigma-Aldrich (Saint Louis, Missouri). The 5-ketogluconate was obtained from Carbosynth (Berkshire, United Kingdom). The 2,5-DKG hemicalcium salt was generously provided by Masaaki Tazoe and Tatsuo Hoshino (NRL Pharma, Inc., Kawasaki, Japan).

III. RESULTS AND DISCUSSION

The synthesis pathway for the oxidation of glucose by *G. oxydans* as described by Qazi et. al. is shown in Figure 2.

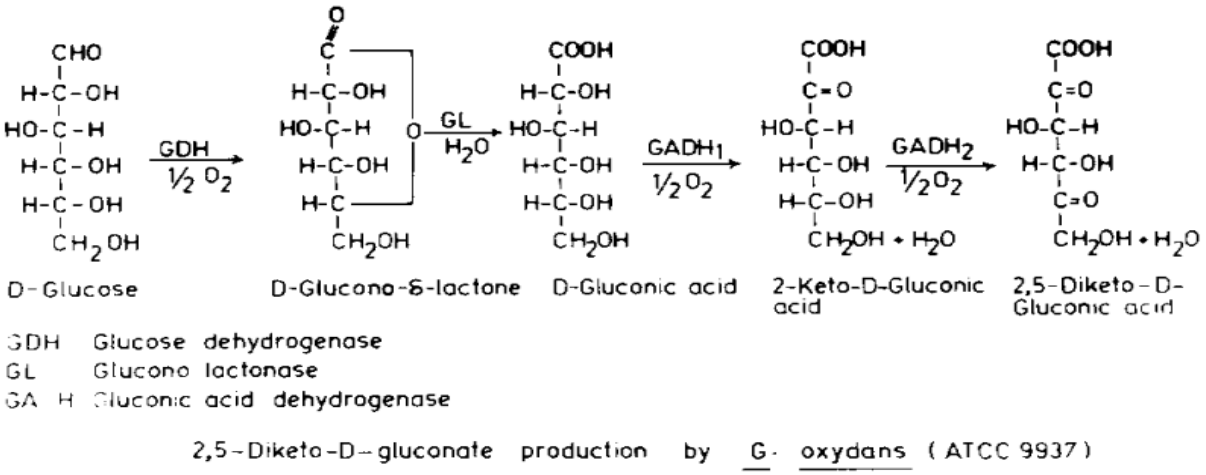

FIG. 2. Glucose oxidation pathway in *G. oxydans* [17]

Previous bioleaching experiments have suggested that pH could be the dominant factor in the leaching efficiency of *G. oxydans* exudates applied to FCC catalyst [9]. GA, 2-keto gluconic acid and 2,5-DKG have pK_a values of 3.6, 2.66, and 2.52, respectively; therefore, the goal of this study was to maximize 2,5-DKG production, the most acidic product. While chelation by organic acids may also have an effect on rare earth element extraction, the chelating ability of the ligands in this study was not directly considered. The maximum 2,5-DKG concentrations measured under the various dilution rates are shown in Table 1 in the Appendix.

2,5-DKG production was not observed during batch growth with the strain used in this study. This is in contrast to other literature reports of 2,5-DKG reaching 200 mM in batch growth [18], [19] although those studies did show that 2,5-DKG production was highly strain dependent. *G. oxydans* DSM 3503 produced only minor amounts of 2,5-DKG while *G. oxydans* NCIMB 8084 produced high levels [18] . Additionally, the studies with the 8084 strain maintained a steady pH at 3.1 which may also have contributed to high 2,5-DKG yields as the enzymes that catalyze the conversion of GA to 2,5-DKG are optimally active at a pH less than 3.5 [17].

In continuous studies using *G. oxydans* subsp. *melanogenum* (ATCC 9937) 2,5-DKG concentration was highest at the lowest dilution rate tested of 0.07 hr^{-1} [20] and the levels

decreased as dilution rate increased. This may be explained by the multiple dehydrogenation enzymes that must contact the substrate in order to yield the 2,5-DKG. Higher dilution rates will shorten substrate residence time, hindering the complete oxidation of the substrate. As shown in Figure 1, *G. oxydans* dehydrogenase enzymes progressively convert the glucose substrate into GA, 2-ketogluconic acid, and 2,5-DKG. Production of 2,5-DKG was observed in this study only at the lowest dilution rate tested in this study, 0.05 hr^{-1}.

GA was also at the highest concentration at the 0.05 hr^{-1} dilution rate and GA concentration decreased as the dilution rate increased. In contrast, the ATCC 9937 strain had low concentrations of GA at low dilution rates with increasing concentrations of GA as dilution rate increased until it peaked at a dilution rate of 0.45 hr^{-1} and then decreased [20].

As mentioned above, lower pH was associated with increased leaching of REE from FCC catalysts in previous studies. Although the batch experiments generated the highest yields of organic acids and the lowest pH, continuous production of organic acids would not be possible in batch mode (Figure 3). GA concentration also exhibited minimal increases under batch conditions after mid-log phase was reached. The lowest dilution rate tested in this study, 0.05 hr^{-1}, was associated with lower GA concentrations (176 mM versus 220 mM for the batch) but had a pH similar to the batch system (2.17 versus 2.20). Thus the lowest dilution rate appears to produce the best organic acid solution for leaching of REE from FCC.

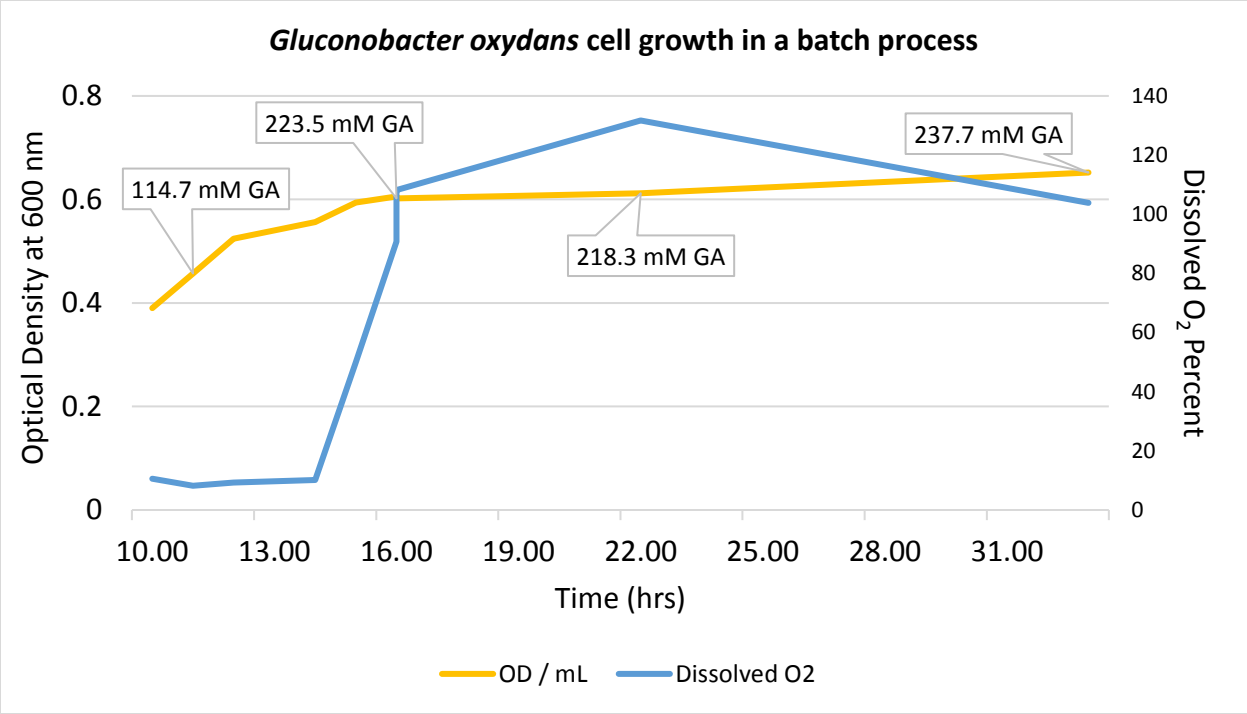

FIG. 3. The optical density suggests *G. oxydans* growth is arrested when substrate is not replenished. In addition, the sharp increase in dissolved oxygen suggests that metabolic activity significantly slowed after around 14 hours. The steady decrease in dissolved oxygen 22 hours after inoculation may be attributed to the activity of the gluconate dehydrogenase enzymes, which are dependent on oxygen.

Dilution rate also had a significant impact on dissolved oxygen content. It was observed that increasing the dilution rate not only lowered GA yields but also lowered dissolved oxygen content. After sixty-five hours in continuous operation, bioreactors with a dilution rate of 0.05 hr^{-1} and 0.38 hr^{-1} had dissolved oxygen contents of 74% to 79% and 2% to 17%, respectively. Direct oxidation, an oxygen-dependent metabolic pathway, is the dominant mechanism for converting glucose to GA at glucose feed concentrations above 15 mM [21]. Since substrate concentrations in this experiment were well above that, the lower DO levels at higher dilution rates was likely inhibitory to GA production. Additionally, dissolved oxygen content above 30% of air saturation at 1 bar has been shown to maximize 2,5-DKG production .

Previous leaching experiments by Reed *et al.* utilized organic acids from *G. oxydans* generated in a batch reactor. Achieving high efficiency and reliability of organic acid production with a continuous culture would support the development of a heap bioleaching approach similar to those already utilized in the copper metallurgical industry [9]. Steady state conditions were difficult to identify in some experiments as cell number, acid production, and DO exhibited cyclic behavior up to 110 hours after inoculation. Figure 4 displays oscillatory DO patterns exhibited at a dilution rate of .30 hr^{-1}.

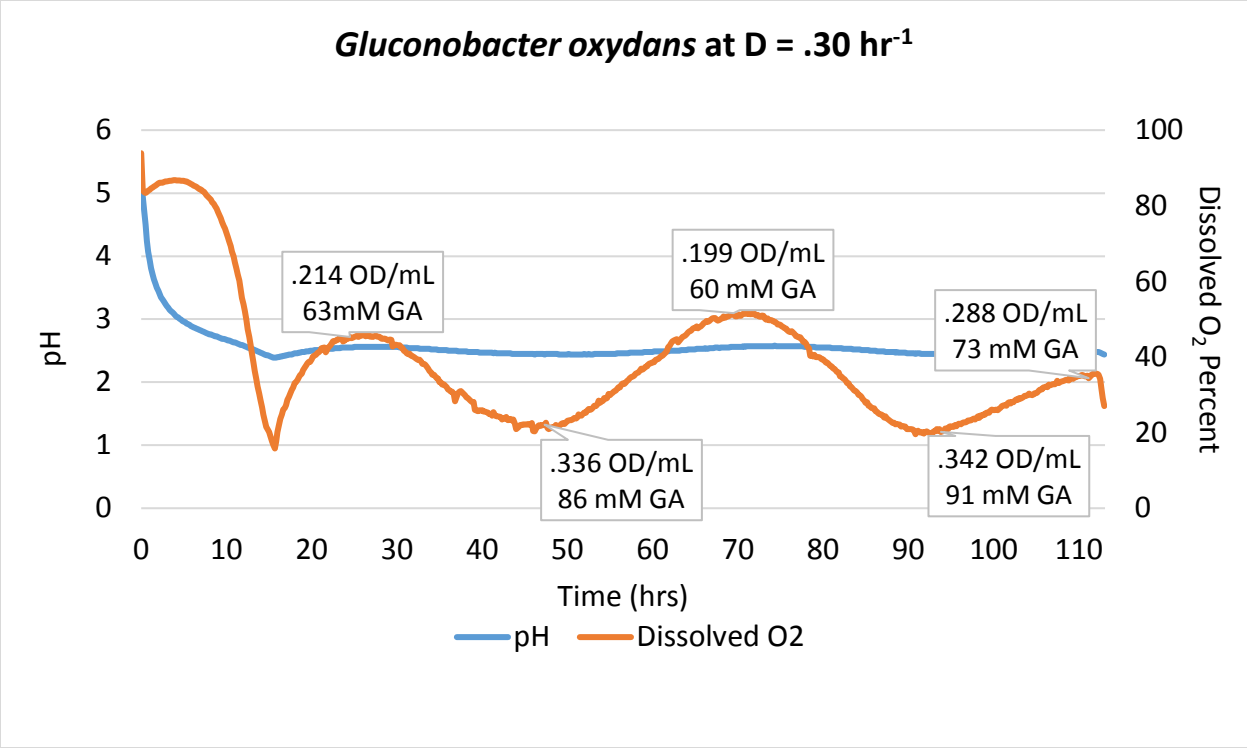

FIG. 4 Displays cell count and GA concentration superimposed onto online pH and DO measurements for a dilution rate of .30 hr^{-1}

IV. CONCLUSION

G. oxydans was grown in continuous culture at various dilution rates which were found to have significant effects on cell number, culture pH, organic acid concentrations, and dissolved oxygen. Dilution rates greater than 0.25 hr^{-1} resulted in markedly lower GA concentrations and higher pH compared to lower dilution rates. For REE leaching from waste materials, generally lixiviants with higher GA concentrations and lower pH are favored. The highest GA concentration and lowest pH were attained with a dilution rate of 0.05 hr^{-1}. When averaged over the duration of the continuous phase of the experiment, the GA concentration and pH for the 0.05 hr^{-1} level were 176 mM and 2.20, respectively. 2,5-DKG was only detected at this dilution rate. A possible reason for low 2,5-DKG yields is operation outside of the pH window for optimal activity for the dehydrogenase enzyme responsible for synthesis of the ketogluconates. Forthcoming data on FCC leaching using the bioproducts are expected to support the supposition that dilution rates below 0.25 hr^{-1} for production of lixiviant by *G. oxydans* are preferable for the end use of REE extraction from recycled industrial waste. Validating the steady state responses of *G. oxydans* to low dilution rates will be critical for future use of this organism in a heap bioleaching process for REE recovery from industrial wastes.

V. ACKNOWLEDGMENTS

Many thanks are owed to David Reed, Vicki Thompson, Yoshiko Fujita, and Dayna Daubaras of the Idaho National Laboratory Biological and Chemical Processing Department.

This research was supported by the Critical Materials Institute, an Energy Innovation Hub funded by the U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Advanced Manufacturing Office and was conducted under Idaho National Laboratory Contract DE-AC07-05ID14517.

This work was supported in part by the U.S. Department of Energy, Office of Science, Office of Workforce Development for Teachers and Scientists (WDTS) under the Science Undergraduate Laboratory Internships Program (SULI).

HPLC analysis of products was greatly assisted by Masaaki Tazoe and Tatsuo Hoshino (NRL Pharma, Inc., Kawasaki, Japan), who provided a 25DKGA sample.

VI. APPENDIX

Table 1: Acid production, pH, and optical density measurements are shown for each dilution rate. Values for the continuous experiments were averaged from the time of initiation of continuous feed until the end of the experiment. Values for the batch experiment were averaged from the time of inoculation until the end of the experiment. Duplicate experiments were performed for dilution rates of 0.05 hr^{-1}, 0.25 hr^{-1}, and 0.38 hr^{-1}.

Dilution rate (hr^{-1})	**Average pH**	**Average Cell Number (cells / mL)**	**Average GA concentration (mM)**	**Maximum 2,5-DKG concentration (mM)**
N/A[a]	2.17 ± 0.01	6.03E8 ± 2.23E7	226 ± 8.0	n.d.[b]
0.05	2.20 ± 0.04	2.98E8 ± 1.44E8	173 ± 16	20.5
0.16	2.25 ± 0.04	2.77E8 ± 6.81E7	144 ± 33	n.d.
0.25	2.41 ± 0.10	2.78E8 ± 9.02E7	118 ± 35	n.d.
0.30	2.49 ± 0.04	2.21E8 ± 6.27E7	77 ± 10	n.d.
0.38	2.66 ± 0.10	1.92E8 ± 9.85E7	58 ± 16	n.d.

[a] denotes experiment was performed in batch reactor conditions

[b] not detected

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