Development of Reactor Configurations for an Electrofuels Platform Utilizing Genetically Modified Iron Oxidizing Bacteria for the Reduction of CO₂ to Biochemicals

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Abstract

Electrofuels processes are potentially promising platforms for biochemical production from CO₂ using renewable energy. When coupled to solar panels, this approach could avoid the inefficiencies of photosynthesis and there is no competition with food agriculture. In addition, these systems could potentially be used to store intermittent or stranded electricity generated from other renewable sources. Here we develop reactor configurations for continuous electrofuels processes to convert electricity and CO₂ to isobutyric acid (IBA) using genetically modified (GM) chemolithoautotrophic *Acidithiobacillus ferrooxidans*. These cells oxidize ferrous iron which can be electrochemically reduced. During two weeks of cultivation on ferrous iron, stable cell growth and continuous IBA production from CO₂ were achieved in a process where media was circulated between electrochemical and biochemical reactors. An alternative process with an additional electrochemical cell for accelerated ferrous production was developed, and this system achieved an almost three-fold increase in steady state cell densities, and an almost 4-fold increase in the ferrous iron oxidation rate. Combined, this led to an almost 8-fold increase in the steady state volumetric productivity of IBA up to 0.063±0.012 mg/L/hr, without a decline in energy efficiency from previous work. Continued development of reactor configurations which can increase the delivery of energy to the genetically modified cells will be required to increase product titers and volumetric productivities.

1. Introduction

Harvesting renewable energy for the creation of fossil fuel replacements is a grand engineering challenge. Agricultural approaches to biofuel production are limited by the inherent inefficiencies in photosynthesis and by competition with food production. Solar cells can have efficiencies of 10-40%, which is an order of magnitude higher than the efficiency of biological photosynthesis [2,16]. However, the storage of electricity obtained from intermittent solar power, or other renewable sources, in batteries is problematic. The widely employed lithium-ion batteries have energy densities ranging from 0.40 MJ/kg to 0.65 MJ/kg [13], whereas gasoline has an energy density around 46 MJ/kg [6]. Liquid fuels will likely continue to dominate the market, particularly in the transportation sector, considering their high power densities and the existing delivery infrastructure. Electrofuels processes aim to deliver renewable electricity to microorganisms, which then are genetically modified to produce chemicals and fuels from CO₂ [3,12]. This approach potentially combines the best efficiencies of renewables with advances in synthetic biology to efficiently create valuable reduced carbon compounds.

Several research groups have begun to demonstrate the feasibility of the electrofuels concept [12]. Li et al. developed an integrated electromicrobial system to convert CO₂ to higher alcohols using *Ralstonia eutropha* as the production host and achieved a production of 140 mg/L of isobutanol and 3-methyl-1-butanol [7]. Nevin et al. grew biofilms of *Sporomusa ovata* on graphite electrodes to reduce CO₂ to acetate and 85% of the electrons were retained in the product [11]. Torella et al. integrated a water splitting device with genetically modified *Ralstonia eutropha* to achieve solar-to-biomass yields up to 3.2% and isopropanol production yield up to

216 mg/L [14]. In general, these have been proof-of-concept demonstrations operated in batch modes, and continuous, steady state operations are not often reported.

We have been developing an alternative electrofuels process which utilizes iron as an electron mediator and the bacterium *Acidithiobacillus ferrooxidans* as the production host [5,9,8,10]. *A. ferrooxidans* is a gram-negative, aerobic, acidophilic chemolithoautotroph which is able to oxidize Fe^{2+} or sulfur for energy while fixing atmospheric CO_2 [15]. Fe^{2+} as an electron mediator has several advantages such as low cost, facile electrochemical reduction, and stability under the *A. ferrooxidans* growth environment [8]. In addition, *A. ferrooxidans* cells grow planktonically, and its genome has been sequenced [15]. All these factors make *A. ferrooxidans* an attractive candidate for potential electrofuel production.

Recently, we reported the metabolic engineering of *A. ferrooxidans* for biochemical production for the first time, and the cell lines were genetically modified to produce isobutyric acid (IBA) or heptadecane [5]. The IBA-producing cells were created by the conjugal transfer of the AF-KDC plasmid into *A. ferrooxidans* cells from *E. coli* donor cells. The plasmid was constructed by cloning a codon optimized version of the 2-keto decarboxylase (KDC) gene from *L. lactis* into the pJRD215 broad host range plasmid and selections were performed with kanamycin. The low pH growth medium was also engineered to enable increases in overall process performance. Citrate and gluconate (which cannot be used as carbon sources by the cells) have been included as chelators to increase the solubility of the Fe^{3+} ions and to reduce Fe^{3+} inhibition [5,9]. Mg^{2+} ions have also been included to increase medium conductivity which improves the energy efficiency during electrochemical iron reduction in continuous cultures [8].

Key challenges in the maturation of electrofuels technologies will be the development of processes with high energy efficiencies, high product titers, and high volumetric productivities. In this manuscript, we explore two integrated electrofuels platforms with different reactor configurations for the continuous production of IBA from ambient CO₂ by the genetically modified (GM) *A. ferrooxidans* cells (Figure 1). We hypothesized that continuous reactor configurations where the iron reduction rate could be decoupled from the hydraulic residence time could be developed to improve process performance. A single loop system (shown by the solid lines in the Fig 1 flow diagram), where media is recycled between electrochemical and biochemical reactors, was used to characterize both wild type and GM *A. ferrooxidans* bacteria. A two-loop system (shown by the dotted lines in the Fig 1 flow diagram) was designed and explored to increase energy delivery to the bioreactor by increasing the iron regeneration rate. The impact of the two designs on IBA production by the GM cells were investigated, and the two-loop system was found to significantly increase the cell density, iron oxidation rate, and volumetric IBA productivity.

2. Materials and Methods

2.1 Microorganisms

Wild type *A. ferrooxidans* (ATCC 23270) cells were obtained from American Type Culture Collection (ATCC). Genetically modified (GM) *A. ferrooxidans* cells were obtained by inserting a synthetic codon-optimized 2-ketoacid decarboxylase (KDC) gene into wild type cells as described previously [5]. Actively growing cells were maintained by weekly subculturing in 1 L

batch cultures at 30 °C. An ATCC 2039 *A. ferrooxidans* medium was used for batch cultures which contained 0.8 g/L (NH₄)₂SO₄, 2 g/L MgSO₄•7H₂O, 0.4 g/L K₂HPO₄, 5 mL/L Wolfe's Mineral Solution and 20 g/L (72 mM) of FeSO₄•7H₂O at an initial pH of 1.8 adjusted by concentrated sulfuric acid. Logarithmic-growth phase cells were harvested by centrifugation at 12,000 RCF for 10 minutes. All chemicals were from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

2.2 Single loop reactor designs

The single-loop bioreactor was made from a 5 L spinner flask (Chemglass, Vineland, NJ) with four side arms. Two side arms were fitted with stainless steel 3-way port assemblies where both air and liquid could be exchanged aseptically. A 0.2 µm vent filter (Whatman, GE Healthcare, Piscataway, NJ) was fitted with the air outlet of the reactor to create a sterile environment. Flow of medium into and out of the bioreactor was driven by a Masterflex L/S Precision Modular Drive pump (Cole-Parmer, Vernon Hills, IL). The bioreactor was placed on a Super-Nuova Single-Position Digital Stirring Hotplate (Thermo Scientific, Waltham, MA) which agitated the bioreactor at 225 RPM with a stirrer bar and temperature was maintained at 30 °C.

The electrochemical reactor was created in a stacked cell design, consisting of an anode, cathode, and proton-exchange membrane. The anode and cathode chambers were fed separately. The cathode was made of a porous carbon felt (Alfa Aesar, Ward Hill, MA). The felt was activated in a Plasma Cleaner/Sterilizer PDC – 32G (Harrick Plasma, Ithaca, NY) for ten minutes. Carbon Toray paper (Alfa Aesar, Ward Hill, MA) was attached to one side of the felt with a conductive carbon cement. A stainless steel 316 foil was used as the current collector. The

anode was made of a platinized-titanium DSA (De Nora). Nafion was used for the membrane separating the two chambers. A peristaltic pump (Masterflex, Cole Palmer) controlled the flow of anolyte and catholyte into the reactor.

2.3 Cell culture medium for fermentation and electrochemical cell electrolyte

The culture medium used for continuous fermentation was optimized for both electrochemical and biological performance. The medium was made with 0.8 g/L $(\text{NH}_4)_2\text{SO}_4$, 23.3 g/L $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.4 g/L K_2HPO_4 , 21.5 g/L $\text{Mg}_3(\text{C}_6\text{H}_5\text{O}_7)_2 \times 9\text{H}_2\text{O}$, 5 mL/L Wolfe's Mineral Solution and 40 g/L $\text{FeSO}_4 \times 7\text{H}_2\text{O}$. Thus it contained 200 mM Mg^{2+} , 70 mM citrate and 144 mM Fe^{2+} . The pH of the medium was adjusted to 2.0 using concentrated sulfuric acid.

The anolyte of electrochemical cell contained 0.5 M sulfuric acid with 140mM Fe^{2+} to minimize iron crossover between anolyte and catholyte.

2.4 Single loop reactor system operation

The single loop reactor configuration is shown in Figure 1 (flows indicated with solid lines). 2 L of growth medium was aseptically transferred into the bioreactor. The bioreactor was inoculated with an initial cell density of approximately 10^8 *A. ferrooxidans* cells/ml. Sterile aeration with air was provided using an air pump with a 0.22 μm HEPA filter at 7.5 L/min. The reactor was operated in batch mode with just air sparging for the first 3 days until more than 80% of the Fe^{2+} was oxidized by the cells. Flow then commenced from a 5 L of feedstock with the same medium formulation but with a pH of 2.7 at a dilution rate of 0.8 d^{-1} . During operation with wild type cells, the Fe^{3+} rich effluent from the bioreactor was collected in a 5 L media bottle

fitted with 0.2 μm vent filter (Whatman, GE Healthcare, Piscataway, NJ). Every other day, the collected effluent was filtered with a 0.2 μm Polycap AS filter (Whatman, GE Healthcare, Piscataway, NJ) to remove the cells. The medium was pumped through the electrochemical reactor, which reduced Fe^{3+} effluent back to Fe^{2+} with >95% conversion, and this was added manually to the feedstock. This process was run for 12 residence times (15 days).

A continuous process was implemented for the GM cell experiments where the Fe^{3+} rich effluent from the bioreactor was filtered through the Polycap AS filter and sent to the electrochemical reactor without storage. The reduced medium was directly fed back to the bioreactor. A total cell potential of -1.9V was applied, and the current was monitored for the duration of the reduction using an Ivium-n-Sat multi-channel electrochemical analyzer (Ivium Technologies). The entire process was run for 10 residence times (12 days).

Cell densities, Fe^{2+} concentration in both inlet and outlet stream, and the pH in both inlet and outlet streams were monitored daily in both reactor configurations. Ammonia concentrations, citrate concentrations and phosphate concentrations in the bioreactors were measured (see below) on the first and last day of the process.

2.5 Two-loop reactor system and operation conditions

The two-loop reactor configuration was operated with a smaller bioreactor (1L) otherwise outfitted and operated the same as the single loop system and using the same medium formulation and aeration. The configuration of the two-loop system is shown in Figure 1 (flows indicated with dotted lines). To initiate experiments, 0.5 L of growth medium was aseptically

transferred into the bioreactor. The bioreactor was inoculated with an initial cell density of approximately 10^8 cells/ml. The reactor was run as a batch for the first 3 days until more than 80% of the Fe^{2+} was oxidized. Flow then commenced and the effluent from the bioreactor was split into two streams at different flow rates, one stream flowed through the electrochemical reactor at 420 mL/hr (without bacteria cell removal). The other stream was passed through the biomass removal filter at 17 mL/hr, producing a bioreactor dilution rate of 0.8 d^{-1} . The electrochemical reactor operation conditions were the same as in the single loop system. This two-loop configuration allowed for the decoupling of the rates of iron reduction from biomass removal. This system was run for 7 residence times (9 days).

2.6 Product toxicity experiments

Batch cultures of *A. ferrooxidans* medium (pH 1.8) were inoculated with GM cells and four different initial IBA concentrations (0, 20ppm, 50ppm, 100ppm). The initial cell concentrations were OD 0.1 in all cultures. All experiments were performed in triplicate with 5 mL volumes of media in 15 mL BD falcon tubes. The tubes were incubated in a rotary shaker at 30 °C and iron concentrations were measured over time.

2.7 Iron Concentration Measurements

The Fe^{2+} concentrations in all culture media samples were measured by titrating mixtures of medium (1 mL) and ferroin indicator (0.1 mL) with 0.1 M cerium (IV) sulfate and observing the color change [1].

2.8 Cell Density Measurements

An absorbance method was used to measure cell densities. Medium samples (1 mL) were centrifuged at 12,000 RCF for 5 min and resuspended in pH 1.8 basal salt solution (0.8 g/L $(\text{NH}_4)_2\text{SO}_4$, 2 g/L $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.4 g/L K_2HPO_4 and 5 mL/L Wolfe's Mineral Solution, pH of 1.8). The absorbance at 600 nm was measured using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) after background subtraction. The total cell number was calculated from the OD_{600} reading as previously described [9]. An OD_{600} of 1.0 was found to equal a total cell density of approximately 8.3×10^9 cells/mL.

2.9 Citrate, Phosphate and Ammonia Concentration Measurements

Citrate assay kits (Abcam, Cambridge, MA), phosphate assay kits (Abcam, Cambridge, MA) and ammonia assay kits (Sigma-Aldrich, St. Louis, MO) were used to measure the citrate, phosphate and ammonia concentrations in the medium samples. On the first day and last day of the experiments, 25 mL samples were taken from the bioreactor and deproteinized using Amicon 10,000 NMWL centrifugal filter units (Millipore, Billerica, MA). The samples were then analyzed in triplicate for citrate, phosphate and ammonia concentrations following the instructions of the manufacturer.

2.10 IBA Concentration Measurements

IBA concentrations were measured daily in the bioreactor and the feed tank for the single loop system, and in the bioreactor and the anolyte in the two-loop system. IBA crossover to the anolyte in the single loop system was assumed to not affect total IBA concentration measurements because of the much larger working volume of the system. A Shimadzu GCMS-QP2010 Ultra was used to measure IBA concentrations in the medium samples. 8 μL of each

sample was injected into a Zebtron ZB-WAX plus column on split mode at a ratio of 1:10. The oven temperature was held at 40°C initially for 2 min, then ramped up to 200°C at a rate of 20°C/min and finally held at 200°C for 2 min. IBA peaks were identified at 6.5 min by comparing retention times and mass to charge ratios of fragments with a standard reference solution. A calibration curve (1 ppm, 5 ppm, 10 ppm, 30 ppm) was made to quantitatively measure IBA concentrations.

2.11 Statistics

All calculations were conducted after the system reached steady state, defined as the last five days of each trial for the single-loop system, and last three days for the two-loop system. Unpaired *t*-tests were used to assess the significance of different cell strains and reactor configurations on various steady state process parameters. Paired *t*-tests were used to assess the significance of citrate, phosphate and ammonia concentrations before and after experiments using the single loop system and wild type cells. A p-value less than 0.05 indicated statistical significance.

3. Results

To demonstrate the long-term stability of the continuously-operated single loop system, wild type and GM cells were cultivated in the system for a period of 12 days (15 for wild type). Cell densities, Fe²⁺ concentrations and pH values inside the bioreactor were selected as indicators for cell performance and monitored on a daily basis as shown in Figure 2. Cell densities of GM cells decreased initially after inoculation and began to increase after day 3 and stabilized after 8 days at $0.46 \pm 0.01 \times 10^9$ cells/mL. This roughly mirrored the trends observed with the Fe²⁺

concentrations where the values for the GM cell cultures increased initially and began to decrease after 3 days and reached a steady state concentration of 12 ± 1 mM after 8 days. The pH values of the GM cell cultures were stable throughout the experiments. Compared to the GM cells, the wild type cells exhibited more stable behavior in terms of all three parameters. Despite initial fluctuations, the steady state values of all cell density and pH were similar between wild type and GM cells as shown in the first two columns in Table 1. The effluent Fe^{2+} concentration was lower in the GM cells, and this was the only value found to be statistically significantly different between the wild type and GM cell culture systems.

The addition of iron chelators presents the opportunity for cells to potentially metabolize these molecules and this could confound the analysis of the electrofuels platform. This has been tested before in short batch cultures [9] but not in longer term continuous experiments. In addition, cells need trace nutrients such as ammonia and phosphate for efficient growth. Citrate, phosphate and ammonia concentrations were measured before and after the continuous wild type cell cultures (Table S1). Changes in the values after 12 days were found to be statistically insignificant indicating citrate is not used as a carbon source and adequate trace nutrients were available.

GM cells were cultivated in the two-loop configuration, and these results were compared to the single-loop operation. The two loop system was operated so that the iron could be reduced at a higher flow rate, and this led to an increase in the iron oxidation rates by the cells in the systems. The cells in the single loop system oxidize iron at a rate of 3.8 ± 0.3 mM/hr and this nearly tripled in the two loop system to 15 ± 1 which was a statistically significant increase. The

cumulative IBA concentrations and cell densities in both single loop and two-loop configurations are shown in Figure 3. Cell densities doubled in the two-loop system during the first two days and then stabilized around $(1.3 \pm 0.1) \times 10^9$ cells/ml after 7 days. The cell densities in the single loop system were less dynamic and reached a steady state value of $(0.46 \pm 0.01) \times 10^9$ cells/ml as mentioned above. This difference was also statistically significant. Since IBA was not removed from the reactors, the cumulative IBA concentrations in both systems increased steadily over time. The two-loop systems reached an IBA concentration of 7.7 ± 0.7 ppm after 9 days, while the single loop systems reached 4.2 ± 0.4 ppm during a similar period of time.

Steady state performance metrics from both single loop and two-loop systems were calculated (see Supplemental Information) and are summarized in Table 1. Higher cell densities and higher iron oxidation rates in the two loop system led to a statistically significant, almost 8-fold increase in the volumetric productivities (0.008 ± 0.001 to 0.063 ± 0.012). As one might expect, the inner-loop of the two loop system effectively provides additional energy flow into the bioreactor for a given overall dilution rate. Higher IBA yields (~2-fold increase) and Gibbs free energy efficiencies (also ~2-fold) were observed in the two loop system as compared to the single loop system. These results are consistent with previous batch results showing the impact of $\text{Fe}^{2+}/\text{Fe}^{3+}$ on efficiency and yield [10]. The current versus time graphs for GM cells in both the single loop and two-loop systems are shown in Figures S1 and S2. Through Faraday's law, the reduction rate of Fe^{3+} to Fe^{2+} provides the measure of the extra energy available to the cells.

Since the IBA concentration accumulated in both of the reactors with GM cells, the effect of IBA on cell growth was explored in batch cultures. Figure 4 shows the effect of varying initial

IBA concentrations on Fe^{2+} oxidation rates by GM cells. The Fe^{2+} concentrations decreased steadily in all cultures at different rates. The initial consumption rate of Fe^{2+} was 2.3 ± 0.1 , 2.1 ± 0.2 , 1.3 ± 0.4 , and 1.1 ± 0.2 mM Fe^{2+} /min when exposed to IBA concentrations of 0, 20, 50, and 100 ppm, respectively. These results suggest that external IBA concentrations beyond 50 ppm have a significant inhibition effect on cell growth. In the present study, the reactor concentrations are significantly below this threshold, and we thus assume that the influence of IBA on cell behavior is not important.

4. Discussion

As renewable electricity resources are developed, it will become increasingly important to develop technologies to create fuels and chemicals using this energy while using CO_2 as a feedstock. We have previously reported on media formulations for the growth of wild type *A. ferrooxidans* cells in chemostat cultures; and furthermore, we have reported on the genetic modification of *A. ferrooxidans* enabling the production of IBA using CO_2 as the carbon source under batch and continuous flow conditions [5,9,8,10]. Here, we have coupled the bioproduction to electricity by integration with an electrochemical reactor. Two laboratory-scale reactor configurations (Figure 1) were explored as electrofuels platforms for IBA production. By increasing the rate of iron reduction compared to the rate of cell removal a significant increase in volumetric productivity was achieved.

There are several advantages to the use of *A. ferrooxidans* in the electrofuels platform. In the integrated system, proton production in the electrochemical reactor is stoichiometrically matched to biological proton consumption. Thus, there is no stoichiometric requirement for acid, and this

is important for development of large-scale, sustainable fuel production platform. The pH in the single loop system stabilized around 1.7 without addition of extra acid or base. Protons were consumed in the biological oxidation of Fe^{2+} to Fe^{3+} but regenerated in the anodic compartment of the electrochemical cell via water oxidation and diffused to the cathode side, balancing the proton consumption (Figure 1). The anolyte solution, with an initial sulfuric acid concentration of 0.5 M, was chosen to ensure that the pH in the bioreactor stabilized at a reasonable value for cell growth.

In addition to being pH balanced, the reactor configurations were found to be robust by other metrics as well. *A. ferrooxidans* cultivation on electrochemically reduced Fe^{2+} as a sole energy source was demonstrated and no degradation in cell growth kinetics was observed for over 10 residence times (Figure 2). These results confirm that the electrochemically reduced Fe^{2+} was able to support *A. ferrooxidans* growth without any sign of accumulation of toxic byproducts. Han *et al.* reported anodic generation of toxic compounds such as superoxide free radicals (O_2^-) and nitric oxide (NO) that inhibited GM *Ralstonia eutropha* growth when an electrochemical cell for formate production was incorporated into the bioreactor [7]. The Nafion membrane in the electrochemical reactor in this work effectively prevented reactive compounds from diffusing to the cathodic chamber. Ammonium, phosphate and citrate concentrations were measured at the beginning and end of each run conducted in single loop system and their concentration changes were measured (Table S1). In particular, changes in the citrate concentration over time were not statistically significantly different, which confirmed that carbon in the IBA product was derived from autotrophic transformation of CO_2 . Phosphate and ammonia were, on average, consumed at

a rate of 0.0011 g/L/d and 0.0036 g/L/d which was also insignificant during the time of the experiments.

The GM cells performed similarly to the wild type cultures in the single loop reactor configuration. GM *A. ferrooxidans* cells cultured in the single loop system demonstrated similar steady state values for all parameters including cell density and pH, and only the Fe^{2+} concentrations were significantly different (reduced by 40%) from the wild-type cells as shown in Figure 2 and Table 1. Cell yields and iron oxidation rates per cell were also similar between wild type and GM cells, leading to no significant changes in steady state performance metrics. This indicates that the cell physiology was not significantly perturbed by the genetic modification. The GM cells are transiently transformed with a recombinant KDC gene and this enzyme decarboxylates 2-ketoisovalerate, an intermediate in the valine synthesis pathway, into isobutyraldehyde. Isobutyraldehyde is then biologically or abiotically oxidized to IBA [5]. These results indicate that although valine is an essential amino acid for bacterial growth, conversion of carbon from this pathway to a secreted biofuel does not affect cell performance at these low product fluxes.

To further increase IBA production in *A. ferrooxidans*, Li *et al.* suggested that a lower oxidation reduction potential (ORP) value of the culture medium could increase IBA yield [10]. One of the biggest factors determining the ORP value is the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio and the Fe^{2+} concentration in the single loop system was only 12 mM at steady state for the GM cells. The Fe^{2+} concentration could be increased by either increasing total initial iron concentration or by increasing the Fe^{2+} delivery rate. The total iron concentration in the medium was fixed due to

solubility and product inhibition [4], and therefore, we explored increasing the Fe^{2+} consumption rate by increasing flow rate through the electrochemical cell. However this could no longer be accomplished in the single loop system as an increased rate of cell removal would eventually lead to bioreactor wash out.

Therefore, a two-loop system was used to decouple the electrochemical loop from the cell filtration loop (Figure 1) enabling independent control of the hydraulic residence time and the iron reduction rate. Significantly higher Fe^{2+} consumption rates and steady state cell densities were achieved compared to those in the single loop system as shown in Table 1. There was significant, approximately eight-fold increase in IBA volumetric productivity in the two-loop system after 9 days. The increased productivity is the result of two important factors, the higher cell concentrations and the higher cellular yield. The significantly higher cell concentrations are a result of the higher Fe^{2+} feed rates to the bioreactor, while this higher cellular yield, as discussed above, is a consequence of the low ORP maintained by the process.

To understand the performance of the system, the Gibbs free energy efficiency for IBA production was calculated. The yield of IBA per Fe^{2+} is compared to 68 mol Fe^{2+} /mol IBA (calculated from Gibb's free energies of formation [5]), assuming an electrochemical cell operating with 100 % Faradaic efficiency at the thermodynamic cell potential. For the single loop system, the efficiency was $(0.17 \pm 0.03)\%$ and this was found to double in the two loop system to $(0.33\% \pm 0.07)\%$.

Previously we reported the short term operation (6 days) of a chemostat with GM cells. The bioreactor was operated without an electrochemical reactor, and fresh medium was constantly fed into the system without recycle. The Fe^{2+} concentration in the feed was the same as used here (40 g/L), but the citrate concentration was lower (50mM) than what was used in this work (70mM). In the single loop system, we obtained a volumetric productivity of 0.011 ± 0.007 mg IBA/L/hr and we estimated a Gibbs free energy efficiency of 0.34% [5]. In the present work, the two-loop system with electrochemically reduced and recycled iron operating for twice the time period resulted in a volumetric productivity of 0.063 ± 0.012 mg IBA/L/hr and with an almost identical Gibbs free energy efficiency of $(0.33 \pm 0.07)\%$. These results demonstrate that reactor designs can be developed to dramatically increase reactor productivities without negatively compromising energy efficiency. We have also recently a Gibbs free energy efficiency of 1.3 % under batch operation and 0.88 % in continuous, single pass chemostat operation through additional engineering of the growth medium [10]. This suggests further improvements in efficiencies in the integrated continuous reactors are readily attainable.

The cell biomass (and IBA product from the GM cells) is produced from ambient CO_2 via fixation through the Calvin-Benson-Bassham cycle [5]. As chemolithoautotrophy-based electrofuels technologies mature, these systems should eventually become carbon limited. Since the cellular and volumetric productivities are still low in these present systems, the aeration used in the current reactor configurations delivers sufficient CO_2 . However as the productivities advance, reactor configurations will be required to achieve increased CO_2 delivery to the cells so that this does not become limiting.

4.1 Conclusions

As electrofuels technologies are developed to convert CO₂ to biochemicals using renewable energy, it will be increasingly important to transition from proof-of-principle batch reactions to characterizing continuous processes in order to better understand energy efficiencies and to predict large scale performance. In this work, we cultivated wild type and GM *A. ferrooxidans* cells in continuous bioreactors coupled with electrolysis reactors to regenerate the Fe²⁺ required for cell growth. All of the bioreactors obtained steady state performance values indicating no impact of the electrochemical reactions on the biological viability. A two-loop reactor configuration was explored to decouple the hydraulic residence time from the electrochemical iron reduction rate. This enabled an 8-fold increase in the volumetric productivity of the system, while maintaining a Gibbs free energy efficiency of $(0.33 \pm 0.07)\%$ similar to our previous studies.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

5. References

1. Acar S, Brierley JA, Wan RY (2005) Conditions for bioleaching a covellite-bearing ore. *Hydrometallurgy* 77:239-246

2. Agrawal R, Singh NR (2010) Solar Energy to Biofuels. *Annual Review of Chemical and Biomolecular Engineering* 1:343-364. doi:10.1146/annurev-chembioeng-073009-100955
3. Conrado R, Haynes C, Haendler B, Toone E (2013) Electrofuels: A New Paradigm for Renewable Fuels. In: Lee JW (ed) *Advanced Biofuels and Bioproducts*. Springer New York, pp 1037-1064. doi:10.1007/978-1-4614-3348-4_38
4. Kawabe Y, Inoue C, Suto K, Chida T (2003) Inhibitory effect of high concentrations of ferric ions on the activity of *Acidithiobacillus ferrooxidans*. *Journal of Bioscience and Bioengineering* 96:375-379
5. Kernan T, Majumdar S, Li X, Guan J, West AC, Banta S (2016) Engineering the iron-oxidizing chemolithoautotroph *Acidithiobacillus ferrooxidans* for biochemical production. *Biotechnology and Bioengineering* 113(1):189-197 doi:10.1002/bit.25703
6. Levine RB, Pinnarat T, Savage PE (2010) Biodiesel Production from Wet Algal Biomass through in Situ Lipid Hydrolysis and Supercritical Transesterification. *Energy & Fuels* 24:5235-5243
7. Li H, Ogenorth PH, Wernick DG, Rogers S, Wu T-Y, Higashide W, Malati P, Huo Y-X, Cho KM, Liao JC (2012) Integrated Electromicrobial Conversion of CO₂ to Higher Alcohols. *Science* 335:1596-1596. doi:10.1126/science.1217643
8. Li X, Mercado R, Berlinger S, Banta S, West AC (2014) Engineering *Acidithiobacillus ferrooxidans* growth media for enhanced electrochemical processing. *AIChE Journal* 60:4008-4013. doi:10.1002/aic.14628
9. Li X, Mercado R, Kernan T, West AC, Banta S (2014) Addition of Citrate to *Acidithiobacillus ferrooxidans* Cultures Enables Precipitate-Free Growth at Elevated pH and Reduces Ferric Inhibition. *Biotechnology and Bioengineering* 111:1940-1948. doi:10.1002/bit.25268
10. Li X, West AC, Banta S (2016) Enhancing isobutyric acid production from engineered *Acidithiobacillus ferrooxidans* cells via media optimization. *Biotechnology and Bioengineering* 113:790-796. doi:10.1002/bit.25837
11. Nevin KP, Woodard TL, Franks AE, Summers ZM, Lovley DR (2010) Microbial Electrosynthesis: Feeding Microbes Electricity To Convert Carbon Dioxide and Water to Multicarbon Extracellular Organic Compounds. *Mbio* 1. doi:10.1128/mBio.00103-10
12. Nybo SE, Khan NE, Woolston BM, Curtis WR (2015) Metabolic engineering in chemolithoautotrophic hosts for the production of fuels and chemicals. *Metab Eng* 30:105-120. doi:10.1016/j.ymben.2015.04.008
13. Tarascon J-M, Armand M (2001) Issues and challenges facing rechargeable lithium batteries. *Nature* 414:359-367
14. Torella JP, Gagliardi CJ, Chen JS, Bediako DK, Colon B, Way JC, Silver PA, Nocera DG (2015) Efficient solar-to-fuels production from a hybrid microbial-water-splitting catalyst system. *Proceedings of the National Academy of Sciences of the United States of America* 112:2337-2342. doi:10.1073/pnas.1424872112
15. Valdes J, Pedroso I, Quatrini R, Dodson RJ, Tettelin H, Blake R, 2nd, Eisen JA, Holmes DS (2008) *Acidithiobacillus ferrooxidans* metabolism: from genome sequence to industrial applications. *BMC Genomics* 9:597. doi:1471-2164-9-597
16. Zhu XG, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. *Annu Rev Plant Biol* 61:235-261. doi:10.1146/annurev-arplant-042809-112206

Table 1. Steady state and efficiency values for the single loop systems and the two-loop system with a dilution rate of 0.8 d⁻¹. Experiments were conducted in triplicate and reported in average with errors representing standard errors.

	Single loop system (Wild type)	Single loop system (GM)	Two-loop system (GM)
Steady State pH	1.7±0.1	1.7±0.1	2.0±0.1
Effluent Fe ²⁺ concentration (mM)	20±1	12±1†	60±2
Fe ²⁺ oxidation rate (mM/hr)	3.7±0.1	3.8±0.3	15±1*
Cell density (10 ⁹ cells/mL)	0.51±0.01	0.46±0.01	1.3±0.1*
Cell yield per Fe ²⁺ (10 ⁹ cells/mol Fe ²⁺)	4500±200	4000±100	2800±100
Iron oxidized per cell (mmol Fe ³⁺ /10 ⁹ cells/hr)	0.0071±0.0001	0.0084±0.0001	0.013±0.001
IBA cellular productivity (ng IBA/10 ⁹ cells/hr.)	N/A	18±2	57±4
IBA volumetric productivity (mg IBA/L culture volume/hr.)	N/A	0.008±0.001	0.063±0.012 *
IBA yield per Fe ²⁺ (mg IBA/mol Fe ²⁺)	N/A	2.2±0.5	4.3±0.9
Gibbs free energy efficiency for IBA production	N/A	0.17%±0.03%	0.33%±0.07%

†Indicates the value for the GM cells is significantly different from the value for the wild type cells for the single loop system by an unpaired t-test (p<0.05). *Indicates the value for two-loop configuration with GM cells is significantly different from single-loop configuration with GM cells by an unpaired t-test (p<0.05).

Figure Legends

Figure 1. Schematics of two integrated processes with biological and chemical reactions involved. The flow diagram shown in solid lines (single loop system) was used to characterize both wild type and GM *A. ferrooxidans* cultures and demonstrates the long-term stability of both strains. The flow diagram in dotted lines (two-loop system) was designed to increase volumetric isobutyric acid (IBA) productivity by separating Fe^{2+} regeneration rate from biomass removal rate

Figure 2. Comparison of (A) Cell densities, (B) Fe^{2+} concentrations and (C) pH values over time in the single loop systems cultivated with wild type and GM *A. ferrooxidans* cells. Experiments were conducted in triplicate and reported in weighted average. Error bars represent standard errors.

Figure 3. Comparison of (A) cumulative isobutyric acid (IBA) concentrations and (B) cell densities over time in the single loop system and two-loop systems both cultivated with GM *A. ferrooxidans* cells. Experiments were conducted in triplicate and reported in average. Error bars represent standard errors.

Figure 4. Effect of various IBA concentrations on the Fe^{2+} consumption rate of GM *A. ferrooxidans* cells grown in batch cultures. Experiments were conducted in triplicate and reported in average. Error bars represent standard errors.

Figure 1

Figure 2A

A

Figure 2B

B

Figure 2C

C

Figure 3A

A

Figure 3B

B

Figure 4