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Separation Science and Technology
Publication details, including instructions for authors and subscription information:
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The Separative Bioreactor: A Continuous Separation Process for the Simultaneous Production and Direct Capture of Organic Acids

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To cite this Article Arora, M. B. , Hestekin, J. A. , Snyder, S. W. , St. Martin, E. J. , Lin, Y. J. , Donnelly, M. I. and Millard, C. Sanville(2007) 'The Separative Bioreactor: A Continuous Separation Process for the Simultaneous Production and Direct Capture of Organic Acids', Separation Science and Technology, 42: 11, 2519 — 2538
To link to this Article: DOI: 10.1080/01496390701477238
URL: http://dx.doi.org/10.1080/01496390701477238

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Separation Science and Technology, 42: 2519–2538, 2007
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ISSN 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390701477238

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The Separative Bioreactor: A Continuous Separation Process for the Simultaneous Production and Direct Capture of Organic Acids

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Abstract: The replacement of petrochemicals with biobased chemicals requires efficient bioprocesses, biocatalysis, and product recovery. Biocatalysis (e.g., enzyme conversion and fermentation) offers an attractive alternative to chemical processing because biocatalysts utilize renewable feedstocks under benign reaction conditions. One class of chemical products that could be produced in large volumes by biocatalysis is organic acids. However, biocatalytic reactions to produce organic acids typically result in only dilute concentrations of the product because of product inhibition and

Received 27 October 2006, Accepted 5 April 2007
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acidification that drives the reaction pH outside of the optimal range for the biocatalyst. Buffering or neutralization results in formation of the acid salt rather than the acid, which requires further processing to recover the free acid product.

To address these barriers to biocatalytic organic acid production, we developed the "separative bioreactor" based on resin wafer electrodeionization, which is an electrodeionization platform that uses resin wafers fabricated from ion exchange resins. The separative bioreactor simultaneously separates the organic acid from the biocatalyst as it is produced, thus it avoids product inhibition enhancing reaction rates. In addition, the separative bioreactor recovers the product in its acid form to avoid neutralization. The instantaneous separation of acid upon formation in the separative bioreactor is one of the first truly one-step systems for producing organic acids.

The separative bioreactor was demonstrated with two systems. In the first demonstration, the enzyme glucose fructose oxidoreductase (GFOR) was immobilized in the reactor and later regenerated *in situ*. GFOR produced gluconic acid (in its acid form) continuously for 7 days with production rates up to 1000 mg/L/hr at >99% product recovery and GFOR reactivity >30 mg gluconic acid/mg GFOR/hour. In the second demonstration, the E. coli strain CSM1 produced lactic acid for up to 24 hours with a productivity of >200 mg/L/hr and almost 100% product recovery.

Keywords: Bioprocessing, separative bioreactor, electrodeionization, organic acids, fermentation, resin wafer

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INTRODUCTION

Integrated biorefineries promises to reduce dependence on fossil feedstocks and to shift society to renewable biobased feedstocks for the production of fuels, chemicals, materials, and heat/power. Biocatalysis, including enzyme reactions and fermentations, are the most common processes to produce biobased chemicals and materials. Biocatalysts can convert biobased feedstocks under benign conditions to produce desired products with limited byproducts. Biocatalysis does not require high temperatures or pressures, and typically do not require environmentally toxic organic solvents or heavy metal catalysts (1).

Organic acids are among the most promising biobased chemicals that could be produced in integrated biorefineries. In a recent report identifying the top chemicals from biomass to replace petrochemicals, 2/3 of the candidates were organic acids (2). That report, as well as others, has identified product inhibition of biocatalysts and product recovery as two significant technical barriers that could limit the success of integrated biorefineries. We develop a new integrated process for continuous conversion and recovery of organic acids. The process, coined the "separative bioreactor," enables continuous product formation and recovery of organic acids limiting product inhibition. In addition, the separative bioreactor is capable of producing organic acids in the acid form, avoiding the need to neutralize the product to a salt and subsequently regenerate that acid.

Evolution solved the dilemma of product inhibition in organisms by actively pumping the accumulating product to the outside of the cell. The separative bioreactor was engineered to mimic this process. The separative bioreactor uses resin-wafer electrodeionization (RW-EDI is described below) to electrically pump out charged chemical products, with the biocatalysts localized within the separation system, providing simultaneous product removal upon formation (3–5). Applying biological principles to engineering issues has led to a novel and effective solution to the dilemma of product inhibition in continuous bioreactors.

In comparison to a conventional bioreactor, the main advantages of the separative bioreactor are:

1. Simultaneous product formation and removal upon product formation within the same localized area providing efficient product capture, separation, and recovery.
2. Simultaneous product removal upon formation reduced product inhibition enhancing biocatalyst reactivity.
3. The protons produced by the bioreaction are efficiently removed by the electrical current, stabilizing the pH without buffering or neutralization.
4. By avoiding the need for neutralization, the separative bioreactor produced organic acids in their acid form.
5. Because the product stream can be separated from the biocatalyst during continuous operation, very high concentrations of the product could be achieved, significantly beyond the titer of most biocatalysts.

In this paper we describe the potential uses and limitations of the separative bioreactor for continuous bioconversions with both enzymatic and fermentative reactions.

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BACKGROUND

Separation of Organic Acids

One of the largest barriers to biobased organic acid production is the separation of organic acids from biocatalytic reactions. Biocatalysis produces dilute concentrations of the acids of interest (6, 7), which relegates many conventional separation approaches economically impractical. For example, Grieves et al. (8) and Danner et al. (9) separated organic acids by electrodialysis, a commonly studied technique (8–14). At high pH (i.e., $>95\%$ ionic dissociation) they observed very good organic acid transport (8). The transport of these dilute organic acids is electrically inefficient requiring high power consumption (1.47–1.76 kWh/kg) to separate lactate from the fermentation broth. Several technologies have been utilized to perform organic acid separations including reverse osmosis (6, 7, 15), nanofiltration

(6, 7, 16), ion exchange (7, 17), ion chromatography (18), Donnan dialysis (19), and supercritical fluid extraction (7).

Bipolar electrodialysis has received a lot of interest for the separation of organic acids (15, 20–26). Bipolar electrodialysis uses a bipolar membrane containing a positively and a negatively charged surface on opposite sides of the membrane. Unlike the cation or anion ion-exchange membrane that allows selective single-charged ion transport across the membrane, the bipolar membrane blocks ion transport. Instead, in an electric field, the bipolar membrane splits the internal water molecules to deliver protons and hydroxyl ions to opposite sides of the membrane. Wodzki and Nowaczyk (20) showed that in a bipolar electrodialysis system with a Neosepta BP-1 membrane (Tokuyama), diffusional mass transfer coefficients increased in the order, citric acid < oxalic acid < lactic acid < tartaric acid < propionic acid < acetic acid, suggesting some possible problems in the selective separation of organic acids due to diffusional leakage. Bipolar electrodialysis also suffers from fouling that may occur on the bipolar membrane due to the generation of hydroxyl ions on the positively charged membrane surface. This limits its functionality for organic acid recovery from fermentations.

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Combined Reactors

Several researchers have also looked at combined reactors for sequential product formation and separation of organic acids (27–31). Ferraz et al. (27) immobilized Zymomonas mobilis cells on hollow fibers and then coupled this unit directly to an electrodialysis cell unit. During 60 hours of continuous operation, gluconic acid production was increased 80% in comparison to NaOH neutralization. Immobilizing cells on the shell side reduced enzymatic activity, possibly because of acidification, and therefore, tube side immobilization was preferred (28). Godjevargova et al. (29) reported that a polyacrylonitrile co-polymer membrane in close proximity to an anion exchange membrane could attach ~30 fold more immobilized enzyme than on the anion exchange membrane alone. In general, immobilization increased acid production in comparison to salt production due to the higher transport rate of H^+ as opposed to Na^+. However, we do not know of any process that could achieve high organic acid production rates without buffering.

MATERIALS AND METHODS

Chemicals and Cells

The sugars (glucose and fructose) and organic acids (gluconic and lactic) were acquired from Fisher and Aldrich Chemical Companies. Glucose oxidase (GOx) and catalase from the organism Aspergillus niger were obtained

from Sigma-Aldrich. Sugar and organic acid concentrations were analyzed using a Waters HPLC with a modified method of one previously described in literature (32).

The gene for the enzyme glucose fructose oxidoreductase (GFOR) was obtained from Zymomonas mobilis strain (ATCC 29191). The gene was cloned into Escherichia coli with a six-histidine tag at the amino terminus of GFOR to permit a one step purification by the IMAC method with a Ni-NTA resin, (Qiagen Superflow). After elution, the His-tag was not cleaved from GFOR to enable specific attachment to the Ni-NTA resin in a modified RW.

A prototrophic strain of Escherichia coli was engineered to produce primarily lactic acid from glucose by introduction of a deletion mutation of *pflB* (33). This strain (CSM1) grew vigorously in strictly minimal medium under anaerobic conditions and converted 1 mol of glucose to 1.65 mol lactic acid, 0.14 mol succinic acid and 0.04 mol each of acetic acid and ethanol. For application in the separative bioreactor, CSM1 was aerobically grown to log phase and then diluted 1/75 to provide approximately 1 g dry weight of cells to the separative bioreactor.

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Electrodialysis Unit

The electrodialysis unit used for all experiments was built using a Tokuyama TS-2 electrodialysis (ED) stack with 1–4 cell pairs. Neosepta® membranes, AMH (anion-exchange membrane) and C6610F (cation-exchange membrane) were used to form each cell pair. The only exception to this was that a bipolar membrane was used in the product stream adjacent to the anodic electrode rinse compartment to prevent ion (e.g., Na^+) transport from the electrolyte rinse solution to the product stream. In this configuration, each cell pair had approximately 0.2 ft^2 (200 cm^2) of membrane area. Each stream was connected to a tank containing between 1–4 gallons (4–15 L), and flow rates were approximately 250–300 mL/min for all streams. The system had pressure gauges on all streams and the pressures were kept between 5 psig and 15 psig for all streams below the 20 psig system limit. The temperature of the reaction was either controlled at 37°C or uncontrolled (at room temperature). pH meters and conductivity meters were used in all feed and product streams.

Wafer Fabrication

Ion-exchange resin beads were immobilized in a porous matrix to form a resin wafer (RW). The RW was inserted into the feed compartment of the electrodeionization (EDI) stack. Equal ion-exchange capacities of strong acid cation and strong base anion (PFC100E and PFA444 from Purolite) were blended

into a 70% Latex emulsion with a water-based polymerization agent, CA30 from FLUOROLAST Inc. The mix was set inside a mold and cured for 24–48 hrs to form a 3 mm thickness RW with ion-exchange resin beads bonded by the elastomer network. The resin wafer is supported in a gasket of approximately equal thickness and compressibility. The porous matrix of the resin wafer has a free-flow-void-space of approximately 30–40%. Pressure drop across the resin wafer in the EDI stack was approximately 5 pisg when a 100 g/l sugar solution was fed at a rate of 50 ml/minute/cell pair (or RW). Details of fabrication and characterization are described elsewhere (3).

Enzyme Immobilization in the Resin Wafer

GFOR was genetically engineered to enable specific localization within the RW by tagging the amino-terminus with six histidines (His-GFOR). The histidine tag coordinates to nickel-resin (Ni-resin) beads. The modified RW wafer contained up to 10 wt% Ni-resin beads incorporated into the wafer. The Ni-resin beads provided in situ regeneration, by allowing His-GFOR to be attached and detached in the RW using procedures similar to capturing and releasing His-tagged proteins from Ni-esin during purification. GFOR stripping was accomplished by flowing 1 M imidazole solution through the feed compartment to remove the immobilized His-GFOR.

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RESULTS AND DISCUSSION

The Resin Wafer EDI

The ion exchange resin beads used in a conventional EDI are packed as a loose resin bed enclosed between two membranes (33–35). We designed a new resin EDI system with the resins immobilized and molded into a wafer to form a "resin wafer" (Fig. 1). The wafer is very uniform and is flush with the surface of the gasket that contains it. The wafer behaves similarly to a piece of plastic in that it is pliable yet not very compressible. Thus, when the wafer is placed in the feed flow solution it acts as a porous media and allows flow through all of the open channels. It is important when fabricating the wafer that the right amount of binding polymer is used. If too much binding polymer is used, the system has little or no increase in ionic transport due to the blinding of the ion exchange resins. If too little binding polymer is used, the wafer does not have sufficient structural integrity and the system exhibits leaks similar to conventional EDI.

Each RW is placed into the EDI stack similar to how a gasket and spacer would be placed in conventional EDI as shown in Fig. 2. Because the flow

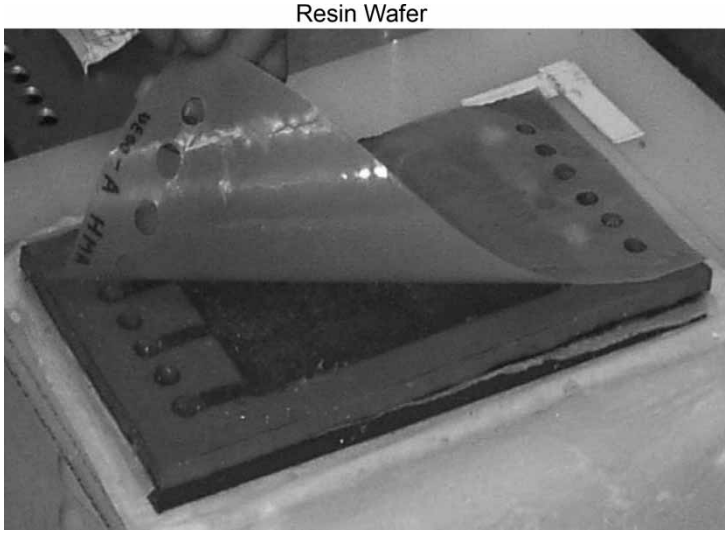

Figure 1. Engineered resin wafer. The electrodialysis wafer was molded in a gasket and sandwiched between two electrodialysis membranes.

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occurs near the ion exchange resin of the wafer, the wafer acts very similar to a spacer in promoting good flow throughout the feed channel. However, a traditional ED spacer is uncharged and does not promote ion diffusion through the feed channels. Thus, RW-EDI has much lower diffusional resistance than ED at low ion concentrations, nearly eliminating the typical limiting current density exhibited by ED (3). It should be also pointed out that there have been some reports using charged spacer materials (36, 37); however, the spacer does not provide nearly as much functionality or surface area as the loosely linked ion exchange beads.

Another feature of the separative bioreactor, takes advantage of the water splitting on the membrane surface in bipolar ED. We paired the bipolar membrane with anion-exchange membranes inside the RW-EDI stack (i.e., instead of a paired cation-exchange membrane and anion-exchange membrane), allowing the bipolar electrodialysis to selectively separate the organic anion from the biocatalyst but leave behind the counter cations. Thus, this process is able to produce pure organic acid in the acid form, even in the presence of buffering cations. (i.e., replacing the cation-exchange membranes with bipolar membranes in Fig. 2)

Bioreactor Process Simulations

Using a simple mass-balance model, we evaluated the product removal rate required to operate the bioreactor as a true separative bioreactor, i.e.,

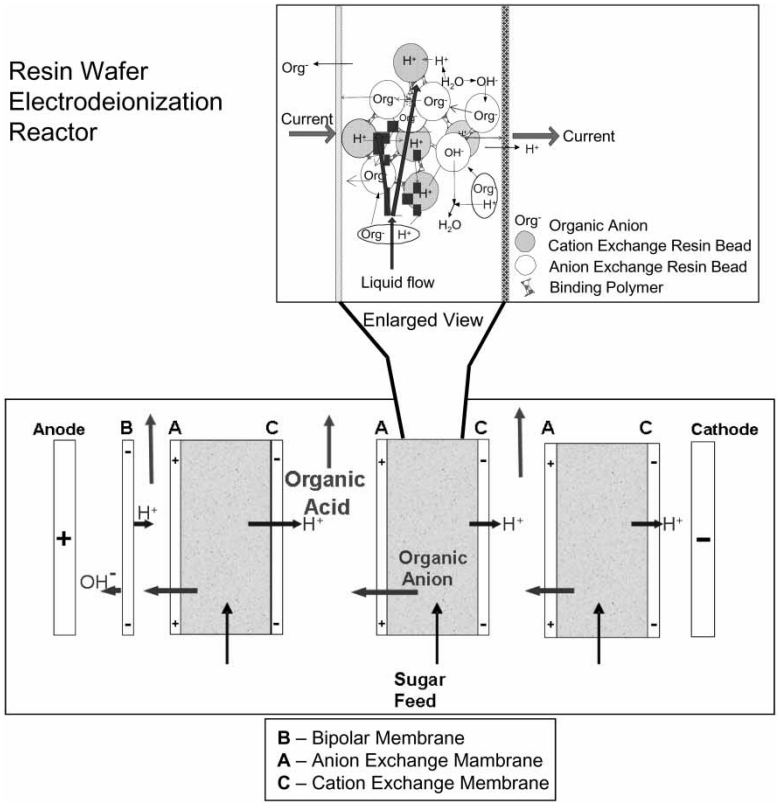

Figure 2. General scheme of the RW-EDI separative bioreactor. The separative bioreactor stack below and enlarged view of resin wafer system above. The resin wafer is sandwiched between anion and cation exchange membranes. The RW sandwiches are stacked between the anode, bipolar membrane, and cathode. The organic anion is pulled through the RW to the anion exchange membrane between RWs and recombines with the protons that are pulled through the RW to the cation exchange membrane to form the pure organic acid between RWs. The resin wafer is enlarged above as a schematic of cations and anions flowing between resins in an electrical current.

continuous product formation and separation of an organic acid without buffering or neutralization. Product removal rate is determined by several factors:

1. the substrate feeding flow rate,
2. bioconversion rate, and
3. the product separation efficiency. Two separation schemes (Fig. 3) were considered.

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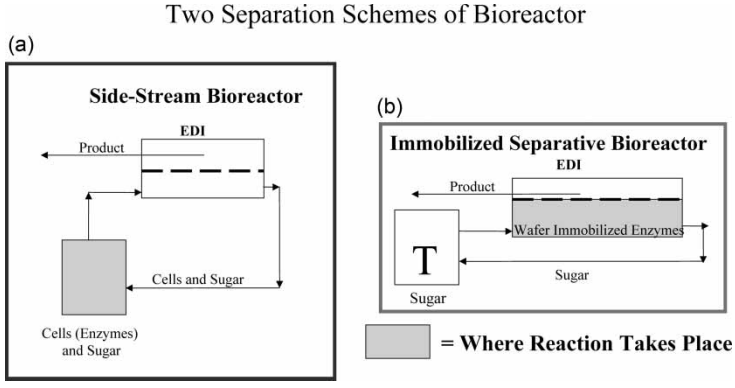

Figure 3. Schematic representation of the side-stream bioreactor and immobilized separative bioreactor. (a) Side-stream bioreactor scheme has the reaction taking place before separation for sequential product formation and separation. (b) Immobilized separative bioreactor scheme where product formation takes place within the separation process for a one-step simultaneous product formation and separation bioreactor process.

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The first scheme, a side-stream bioreactor, is a traditional bioreactor containing biocatalysts (enzymes/cells), sugars, and the organic acid products all in the solution feeding to the EDI separation unit (Fig. 3a) demonstrating a traditional sequential reaction and separation. The only component typically in a traditional bioreactor that is absent from the side-stream bioreactor is buffer. In the second separation scheme (Fig. 3b), the biocatalyst is immobilized directly in the RW-EDI to enable simultaneous reaction and separation. Immobilizing the enzyme within the RW integrates product formation and separation into a simultaneous continuous process, mimicking organisms in nature, which prevent product inhibition by pumping out products as they are made. It was assumed that the maximum flow rate that could pass through the system was one complete reactor volume per minute in a side stream bioreactor, and that the maximum transport of gluconic acid through the system is dictated by a current of 25 A/ft^2 (38). Figure 4 shows that for the various schemes, an efficient and high rate of separation of a pure acid, with no buffering, was only possible using an immobilized biocatalyst within the separation unit. For example in a sequential side-stream bioreactor, at pH 6, the concentration of unbuffered gluconic acid is <0.2 mg/L. At this low concentration, production rates are limited by the large volumes that the RW-EDI would have to manage for separation. However, in the immobilized case of the separative bioreactor, the product formation is constant regardless, independent of the feed solution pH. Therefore, it was calculated by Faraday's Law that a rate of 2.6 g/L/hr was a good product formation rate for the separative bioreactor.

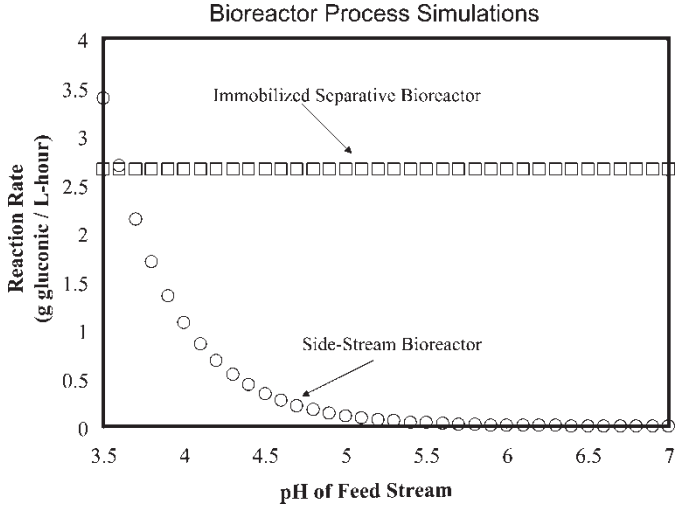

Figure 4. Comparison of processing rate simulations between the side-stream bioreactor and immobilized separative bioreactor. As pH changes in the feed stream, the reaction rate of a side-stream bioreactor decreases whereas the reaction rate of the separative bioreactor remains constant.

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Side-Stream Bioreactor

In the side-stream bioreactor, the introduced enzyme and sugars and produced organic acids were circulated to the RW-EDI, performing a sequential product formation and separation process. The goal was to evaluate how the RW-EDI would perform as a side-stream bioreactor and whether it could achieve the performance target of the immobilized separative bioreactor system (2.6 g/L/hr). Continuous product removal was first tested with the model enzyme glucose oxidase (GOx), which converts glucose to gluconolactone, which is subsequently hydrolyzed to gluconic acid. Glucose oxidase was not specifically immobilized within the resin wafer, but was free in the starting solution feed stream and is referred to as a side-stream bioreactor. The side-stream bioreactor was run for >80 hours through the RW-EDI system at a constant current of 0.01–0.05 A. The run was started with 2 L of 40 g/L glucose solution (without buffers or salts used to stabilize the enzyme) and the feed stream was run through the RW-EDI system at approximately 300 mL/min. Over the course of the bioreactor run, the concentration in the product stream increased from 0.31 g/L gluconic acid to 3.5 g/L gluconic acid (4 L volume). At the end of the bioreactor run, the concentration of gluconic acid in the feed stream was <10 mg/L, giving a separation factor of >350-fold. With only the trace product in the reaction feed tank, gluconic acid separation approached 100%. At the end of the run, the product stream was at pH 2.7. Calculating the amount of gluconic acid in the product

stream ($pK_a = 3.6$), we balanced the concentration of gluconic acid within 2% demonstrating that at least 98% of the gluconic acid existed as an acid. Any Na^+ impurities came from the electrode rinse (which is 0.3 N Na_2SO_4), which is separated from the product stream by the bipolar membrane.

Maintaining the pH of the feed stream, which is the enzyme milieu, is crucial for maintaining high enzymatic activity in the bioreactor and allows the RW-EDI bioreactor to function without buffering. pH control was also tested in the side-stream RW-EDI bioreactor. Experimental conditions (ion exchange resin composition, electrical current and flow rates) were selected to maintain a pH of ~4.5. As shown in Fig. 5, the pH of the feed side was maintained at ≈ pH 4.8 for over 80 hours. The results revealed that the RW-EDI maintained the targeted pH and provided an effective means by which an unbuffered biocatalytic reaction may be employed in a bioreactor.

Even with buffering, the GOx specific activity will decline as the product concentration exceeds the capacity of the buffering system. A comparison of the GOx enzymatic activity between the side-stream bioreactor and buffered solution will reveal the impact on product inhibition. Extrapolating from the cited rates of GOx (Sigma 49178 Batch G7016), the 30 mg load of GOx should have a calculated activity of 1370 mg/min (pH 5.3) and with 60% activity at pH 4.8 (39) calculated as 820 mg/min. However, product accumulation was observed to be only 2.4 mg/min (pH 4.8). Therefore GOx is limited to ~0.3% of the initial solution activity. The side-stream bioreactor captured about 72.5 mg/L-feed/hr. Although the RW-EDI side-stream bioreactor clearly demonstrated pH control and that most all of the gluconic acid was

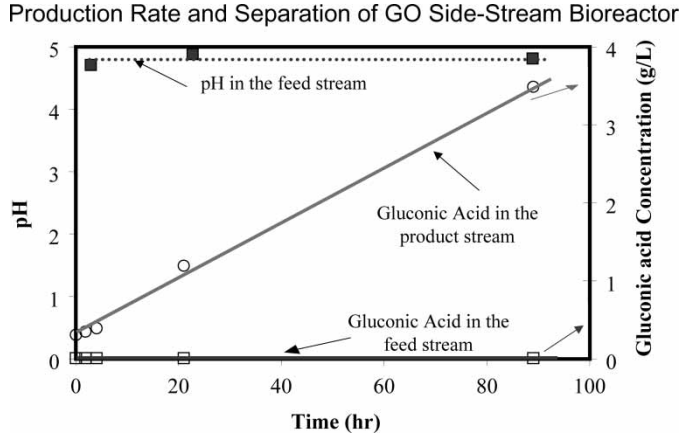

Figure 5. Enzymatic side-stream bioreactor for converting glucose to gluconic acid via GOx. No gluconic acid accumulated in the feed stream as demonstrated by the constant pH. Gluconic acid separation in the side-stream bioreactor reached ~100% as demonstrated by trace amounts of gluconic acid in the feed stream versus accumulation of gluconic acid in the product stream.

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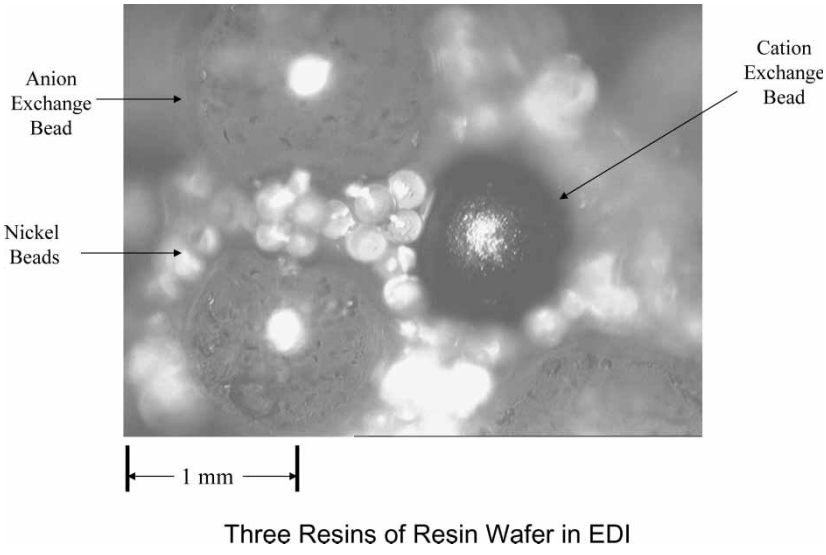

Figure 6. Microscopic view of a three resin wafer capable of ion exchange and specific enzyme attachment. Displayed are the anion exchange resins, cation exchange resins, and Ni-resins in the RW of the EDI.

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separated to the product stream, the poor product formation rate suggests that the separation rate was insufficient. Gluconic acid transport from the feed stream to the RW is probably the rate-limiting step to an overall efficient continuous separation.

Immobilized Separative Bioreactor

In the second separation scheme, the enzyme was immobilized in the RW affording simultaneous product formation and separation. Glucose fructose oxidoreductase (GFOR) was selected as the model enzyme to benchmark the separative bioreactor. GFOR couples two redox reactions, the conversion of glucose to gluconolactone (which is subsequently hydrolyzed to gluconic acid) and fructose to sorbitol (40–42). With a GFOR-based separative bioreactor, gluconic acid is transported across the ion exchange membrane while sorbitol is retained in the feed solution, thus GFOR was a good model enzyme to test whether the ionizable species (gluconic acid) is the major source of product inhibition.

His-GFOR was attached to the separative bioreactor by placing 9.8 mg in the 2 L feed stream. After 1/2 hour permeation at 300 mL/min, the feed pump was shut off and 100 g/L of both glucose and fructose were added to the feed stream. To provide enough conductivity at the beginning of the

experiment, the concentrate compartment was spiked with 2 L of 5.5 g/L gluconic acid and the electrode rinses were both 0.5 N Na_2SO_4. The separative bioreactor was run continuously for 72 hours (3 days) at constant current 0.3–0.4 A, while monitoring the pH and gluconic acid concentrations. The gluconic acid concentration in the product stream increased from 5.5 g/L to 18.5 g/L. The results from this experiment are displayed in Fig. 7 (time zero corresponds to the addition of the sugars and advent of solution flow through the separative bioreactor). When the sugars were added, the pH in the feed stream instantly dropped to pH 3 before the separative bioreactor stabilized at pH ~3.5. However, even under these conditions the enzymatic activity remained high at 32.1 mg gluconic acid/mg GFOR/hour. High activity was continuously maintained for 72 hours at room temperature, with 98.4% of the acid product captured, and a total productivity of 260 mg/L-feed/hr. In contrast, the reactivity of GFOR in free solution (not in the separative bioreactor) had an instantaneous rate at room temperature of 120, 15.7, and 0 mg gluconic acid/mg His-GFOR/hour for pH 6.4, 4.5, and 3.1, respectively. These performance results demonstrate that the accumulation of gluconic acid suppressed and eventually eliminated GFOR activity under conditions where the separative bioreactor was able to maintain high activity. Solution GFOR has no activity at pH 3.1, but the feed stream pH of the separative bioreactor was stabilized at pH 3.5, thus, the sustained activity of GFOR in the separative bioreactor demonstrated that the local pH of the enzyme is considerably higher than the bulk feed stream. The sustained catalytic activity of unbuffered GFOR for 72 hours in the separative bioreactor demonstrates two points:

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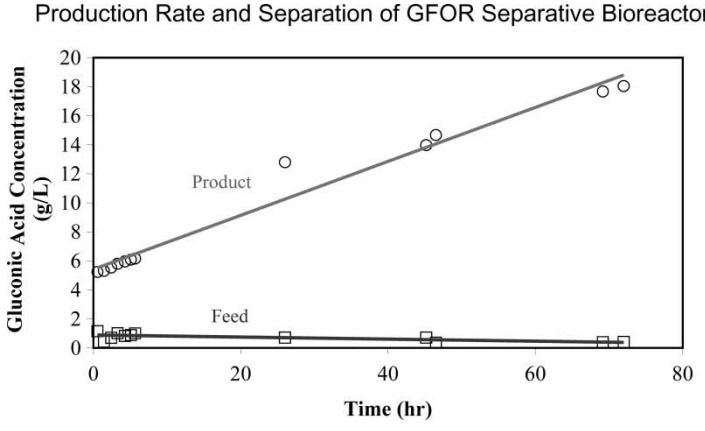

Figure 7. Enzymatic separative bioreactor for converting glucose and fructose to gluconic acid and sorbitol via GFOR immobilized in RW. The first proof-of-principle demonstration of the one-step separative bioreactor. Product formation was continuous over the course of the experiment and separation was ~98%.

1. the accumulation of sorbitol product did not inhibit the activity of GFOR and
2. product inhibition was alleviated by the simultaneous separation of the ionizable species, gluconic acid.

Separative Bioreactor Optimization and Regeneration

With process optimization, we were able to achieve a higher production rate at 1000 mg/L/hr and >99% separation efficiency with gluconic acid product concentration reaching ~16% in the product stream. With enzyme concentration, temperature, and the pH at constant values, enzymatic rates are dependent on substrate concentration, thus if the glucose/fructose feed concentrations were maintained then the GFOR activity would remain constant. But the GFOR reactivity dropped between time zero to 20 hours because glucose/fructose substrate concentrations were not renewed and thus the enzyme experienced a drop in the substrate concentrations (Fig. 8). Regardless of the initial drop in rate, the GFOR reactivity in the separative bioreactor was continuous well beyond its activity in solution (>20 hours).

We also developed procedures regenerate enzyme in situ in the separative bioreactor. Inactive His-GFOR was stripped from the resin wafer with 1 M imidazole and replaced with new fresh enzyme. After flushing out the imidazole, a fresh batch of the enzyme was reattached with the same methods previously described. When the enzyme was regenerated, similar reactivity was retained (Fig. 8). The results demonstrate that the separative

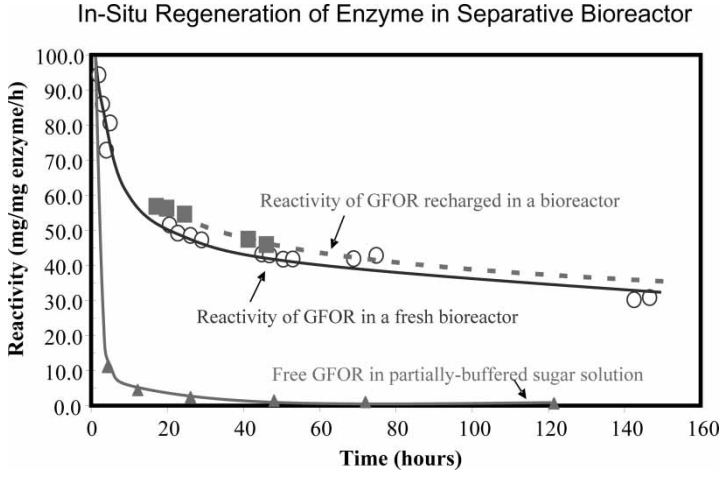

Figure 8. *In situ* regeneration of GFOR in the separative bioreactor. GFOR reactivity in the separative bioreactor was maintained beyond 100 hours whereas in solution GFOR activity only lasted 40 hours. The in situ regenerated GFOR maintained normal separative bioreactor activity.

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bioreactor could be used to provide a nearly optimal environment for sustained enzyme activity producing organic acids at high concentrations. In addition, the system could be operated for extended periods, with in situ enzyme regeneration as activity declines, avoiding the need for major servicing of the bioreactor.

Cell-Based Separative Bioreactor

In the second application we evaluated fermentative reactions with bacterial cells, such as E. coli. The charged surface of ion exchange resins provide a means to localize bacterial cells through electrostatic interactions, thus we predicted that the RW would attract the cells. Cellular conversions provide a much larger repertoire for producing charged biobased chemicals and also avoid the need to isolate enzymes. Complex cell growth media could complicate bioreactor operation and maintenance, because nutrients from the media may interfere with separation efficiency and product purity and possibly result in the salt rather than acid formation. This barrier was addressed with an E. coli strain engineered with obligate product formation of lactic acid in minimal media. The engineered strain uncoupled the anabolic reactions from the catabolic reactions of the cell to prevent growth while allowing the metabolism of glucose to lactic acid. In the absence of growth media (in sugar only), it was believed that the engineered E. coli strain would produce only lactic acid in the separative bioreactor avoiding cell division.

One g dry weight of CSM1 cells were added to 1 L of 10 g/L glucose in unbuffered water and the feed was run through the system at 200 mL/min at a constant current 0.1–0.3 A for $>$24 hours. The flow rate was reduced in comparison to enzyme experiments because the system exhibited a significantly greater pressure drop (i.e., operating pressure near our maximum threshold of 20 psig). The initial lactic acid concentration in the product stream was 100 mg/L and the total volume was 2 L (Fig. 9). The total reactivity was 0.223 g lactic acid/g dry cells/hour. These results revealed:

1. as predicted the bacterial cells immobilized on the RW surface and continued to produce lactic acid and
2. calculating from the unbuffered feed pH (5.8–7), we achieved a separation factor approaching $\sim$99.9%.

The data demonstrated that the separative bioreactor is a favorable platform for organic acid fermentation, because typically the accumulation of high acid levels in a continuous fermentation shut down the metabolic pathways that produce the desired acid product, inhibiting fermentation. Although lactic acid product formation and separation was operating simultaneously and continuously, the experiment was halted after 24 hours because of

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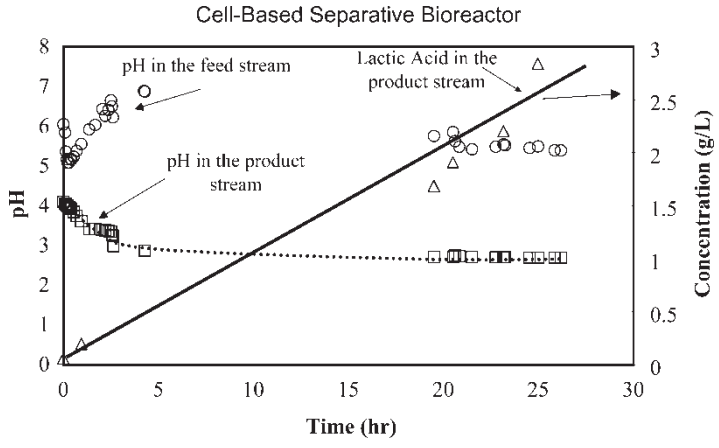

Figure 9. Cell-based separative bioreactor for converting glucose to lactic acid via genetically engineered *E. coli.* At the left axis are the pH of the feed streams and product streams. The pH in the product stream remained lower than the feed stream demonstrating separation of the organic acid. At the right axis the concentration of lactic acid in the product stream was determined over the course of the experiment. Lactic acid production was continuous.

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increasing pressure. Microscopic investigation of the RW at the end of the experiment indicated that the pressure increase was due to cells aggregating at the entrance of the RW, hindering flow. Future work will include optimizing and engineering of the cell lines to reduce aggregation and/or to specifically attach cells to the RW surface.

CONCLUSIONS AND FUTURE WORK

The goal of the separative bioreactor is to mimic the cell management of organic acids. The separative bioreactor overcame conventional bioreactor limitations including

1. inefficient batch processing
2. product inhibition
3. acidification and
4. inefficient pure product separation/capture.

The separative bioreactor provides a platform for a continuous process, with the integrated and simultaneous product formation and capture of organic acids. The one-step separative bioreactor avoided product accumulation, avoided product inhibition, controlled pH, enhanced transport rates,

enhanced separation, and directly captured the product without salt waste. Specifically we were able to show:

- Separation of the pure gluconic acid with the side-stream bioreactor at ~100% when GOx was used as the model enzyme with production at 72.5 mg/L-hr.
- Separation of the pure gluconic acid (~16% in the product stream) in the separative bioreactor at ≥99% with continuous, high enzymatic reactivity at 32.1 mg gluconic acid/mg GFOR/hour at ~pH 3.5 and production reaching 1000 mg/L-hr with unbuffered immobilized GFOR.
- Separation of the pure lactic acid in the separative bioreactor at 99.9% with immobilized E. coli unbuffered in sugar water with reactivity at 0.223 g lactic acid/g dry cells/hour.

In this work, we demonstrated that the separative bioreactor has the capability to perform separations at very low equilibrium ion concentrations with continuous product formation due to simultaneous product formation and capture as engineered by biocatalyst immobilization within the RW-EDI. We continue to optimize parameters such as pH-tuning, reactor design, biocatalyst attachment efficiency, wafer design, and alleviation of pressure drop.

As the economy shifts from petroleum based fuels to biobased fuels, the corresponding biobased chemicals industry will develop. By addressing some of the critical technical barriers to organic acid production, we believe that the separative bioreactor could accelerate the transition of the chemical and materials industry from fossil feedstocks to biomass feedstocks.

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ACKNOWLEDGEMENTS

The authors thank the Department of Energy for funding under Contract No. W-31-109-Eng-38 with the Argonne National Laboratory, which made this work possible. The work was partially funded by the DOE Office of the Biomass Program. The authors are grateful to Kimberly Baier for purifying His-GFOR and Dr. Joyce Sutcliffe for the generous gift of the prototrophic Escherichia coli strain.

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