Research Article

The use of CO_2 for reversible pH shifting, and the removal of succinic acid in a polymer-based two-phase partitioning bioreactor

Adam J. Hepburn and Andrew J. Daugulis*

Abstract

BACKGROUND: Succinic acid (SA) is an intermediate in the production of commodity chemicals, but SA bioproduction has not yet been commercialized due to end product inhibition and high product separation costs. Two-phase partitioning bioreactors (TPPBs) can increase volumetric productivity through *in situ* product removal, although SA uptake by polymers requires a pH below the pK_{A2} of SA (4.2). It was proposed to reversibly reduce the pH with CO_2 sparging for absorption of SA, followed by nitrogen stripping to allow continued bioproduction after returning to metabolic pH levels.

RESULTS: At 1 atm CO_2 sparging lowered the pH of RO water to 3.8 but only to 4.75 in medium, requiring acid/base pH adjustment in subsequent experiments. *Actinobacillus succinogenes* was temporarily exposed to pH 4.2 for between 5 min and 4 h to observe the effect on subsequent growth; cells could grow after up to 4 h of low pH exposure, sufficient time for SA uptake. Because atmospheric CO_2 could not adequately lower the pH of medium, a TPPB was operated with the pH being shifted using strong acid/base; SA was recovered *in situ*, however, the accumulation of salts hindered further cell growth.

CONCLUSION: Several key elements of this novel processing strategy were successfully demonstrated, and work is continuing with high pressure CO_2 to achieve the desired pH adjustment levels.
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Keywords: succinic acid; *Actinobacillus succinogenes*; two-phase partitioning bioreactor (TPPB); *in situ* product removal (ISPR)

INTRODUCTION

It is estimated that over 1 billion tons of waste biomass per year are available from the agricultural and forestry sectors in the USA which, if used as a feedstock for biotransformations, is enough to replace 30% of current US petroleum consumption in fuel, chemical production and energy.[1,2] While there are many chemicals that can be produced from biomass using microorganisms, one that can be used to make a particularly wide range of products is succinic acid (SA), a 1,4-diacid, that is a precursor to commodity chemicals such as 1,4-butanediol as well as specialty chemicals for the pharmaceutical industry.[3–6] Although biologically produced SA can provide an important precursor molecule leading to a wide array of value-added products, commercial bioproduction of SA does not currently exist.[7]

Reports in the literature list a maximum microbially produced SA concentration of approximately 50 g L^{-1}, suggesting that this upper limit is a result of end product inhibition (EPI), reducing volumetric productivity and leading to an inefficient process.[8] Moreover, a second drawback in developing a commercial process for SA bioproduction is product recovery, with pre-treatment steps and additional chemical inputs resulting in separation methods that constitute 60 to 70% of the total production cost.[9] Various methods of SA recovery have been proposed including ion exchange chromatography, precipitation with calcium hydroxide, liquid–liquid extraction with tri-*n*-octylamine, and electrodialysis, but all of these methods are constrained by high energy or chemical inputs.[10,11] An ideal recovery method for SA would have minimal chemical addition, require no additional energy inputs beyond normal operation and would leave the biomass unharmed to continue succinic acid production after reducing EPI.

Two-phase partitioning bioreactors (TPPB) are systems designed for *in situ* product removal, increasing volumetric productivity over single-phase bioproduction by using a second, immiscible phase to remove target molecules and eliminate EPI.[12–14] Although immiscible organic solvents were originally used as a sequestering phase for *in situ* product removal, low-cost commercial polymers have been shown to be equally effective in this capacity, while also possessing numerous advantages in terms of reduced cost, enhanced safety, and improved process operability.[12] One complication of SA separation using immiscible organic solvents or amorphous polymers, is that uptake requires the pH of the solution

* Correspondence to: Andrew J. Daugulis, Department of Chemical Engineering, Queen's University, Kingston Ontario Canada K7L3N6.
  E-mail: daugulis@chee.queensu.ca

Department of Chemical Engineering, Queen's University, Kingston Ontario Canada K7L3N6

Table 1. Polymer suppliers, glass transition temperature and structure for materials used in polymer screening

Trade Name	Grade	Source	T_g (°C)	Structure
Desmopan	453	Bayer Canada	−33.9	Polyester-based thermoplastic polyurethane
Elvax	650Q	DuPont Canada	N/A	Ethylene-vinyl acetate copolymer
Engage	8450, 8842	Dow Chemical	−32, −58	Ethylene octane copolymer
EVAL	27%, 38%	Sigma- Aldrich	46, −30	Ethylene vinyl acetate
Fusabond	N416D	DuPont Canada	N/A	MA modified nylon
	C190D, C250D	DuPont Canada	N/A	MA modified ethylene vinyl acetate
Hytrel	3548, 4078, 8206	DuPont Canada	−45, N/A, −59	PBT and Polyether block copolymer
Kraton	06175M, D1102K	Kraton Polymers	−80, −80	Styrene and butadiene triblock copolymer
Nucrel	925	DuPont Canada	N/A	Ethylene and Methacrylic acid copolymer
PEBAX	2533, 7033	Arkema Group	−65, −65	Polyether block amide copolymer
Si Rubber	N/A	N/A	−127	Silicone rubber

to be below the lower pK_A of succinic acid, a value of 4.2, in order for the molecule to be in the undissociated state, in a manner similar to the removal of butyric acid by liquid–liquid extraction.[15] Carbon dioxide gas dissolves in water forming carbonic acid, which dissociates and lowers the pH, and in theory could be used to lower the pH without the addition of strong acid solutions. This reduction in pH using CO_2 can be reversed by sparging nitrogen through the liquid, driving out the CO_2 and restoring the pH to its original value. Using CO_2 to adjust the pH of the system in a reversible way reduces the need for the addition of strong acids and bases and minimizes the mass of salts left in solution.

The selection of the sequestering phase in TPPBs also requires the identification of candidate materials (here, polymers) and testing for the extent and rate of target molecule uptake. In addition, since polymer uptake of SA would be near or below the pK_A of SA, the organism used for the production of SA would need to be assessed in terms of its robustness in being exposed (temporarily) to reduced pH, with a return to metabolic pH values.

The objective of this work was to undertake an initial study of the achievability of the three interrelated components of this novel processing strategy: the feasibility of CO_2-induced pH shifting, screening of candidate polymers for SA uptake, and the effect of temporarily reduced pH on microbial activity. To this end, CO_2 at atmospheric pressure was used to determine the extent of pH reduction possible with RO water and with fermentation growth medium. In addition, the presence of individual salts on pH shifting by CO_2 was also determined. Eighteen polymers were tested in terms of their ability to absorb SA from solution, and the most effective one was further characterized in terms of uptake near the pK_A of SA. *Actinobacillus succinogenes*, the organism used in this work, was also exposed to pH 4.2 for various periods of time (corresponding to the time that would be required for polymer uptake of SA), and the pH was then returned to its optimal metabolic value (6.7) to determine the effect of exposure at low pH on the resumption of cell growth. Finally, a fermentation was operated with *Actinobacillus succinogenes*, and SA was absorbed by *in situ* product removal to provide a demonstration of concept for this processing strategy.

MATERIALS AND METHODS
Chemicals and polymers
Unless otherwise specified, all chemicals were purchased from Fisher Scientific Company, Ltd (Ottawa, ON) or Sigma-Aldrich Canada, Ltd (Oakville, ON). Medical grade carbon dioxide and

ultra-high purity nitrogen were supplied by Linde Canada, Ltd (Kingston, ON). Polymers were all of commercial grade with details regarding properties and source shown in Table 1. Prior to use, the polymers were washed, with agitation, three times with hot tap water on a stir plate, twice with RO water, and allowed to air dry overnight.

Organism and media formulation
Actinobacillus succinogenes (ATCC) 55 618 was purchased from the American Type Culture Collection. Cells were originally grown in a shake flask on sterile TSB medium at 30 °C for 48 h and 200 rpm, and cryopreserved with 20% glycerol at −75 °C until required.

The growth medium used in serum bottle experiments, referred to as the original medium, in RO water contained (g L^{-1}): yeast extract, 5; NaHCO$_3$, 12.6; NaH$_2$PO$_4$·H$_2$O, 8.5; K$_2$HPO$_4$, 5.5; NH$_4$Cl, 2; NaCl, 1. 1 mL L^{-1} of a trace elements mix was also added which contained (mg L^{-1}): nitrilotriacetic acid, 3000; MgSO$_4$·H$_2$O, 3000; MnSO$_4$·H$_2$O, 500; FeSO$_4$·7H$_2$O, 100; CaCl$_2$·2H$_2$O, 100; CoCl·6H$_2$O, 100; Na$_2$MoO$_4$, 25; NiCl$_2$·6H$_2$O, 25; Na$_2$WO$_4$·2H$_2$O, 25; ZnCl$_2$, 13; CuSO$_4$·5H$_2$O, 10; AlK(SO$_4$)$_2$·12H$_2$O, 10; H$_3$BO$_3$, 10; NaSeO$_3$, 10. A second growth medium, referred to as the minimal growth medium and containing fewer buffering salts, was used in fermentations and consisted of (g L^{-1}): yeast extract, 5; NH$_4$Cl, 2; NaCl, 1; K$_2$HPO$_4$, 0.5 and 1 mL L^{-1} of the mineral mix. A glucose concentration of 55g L^{-1} was used in the fermentations based on previous studies.[16,17]

Temporary pH shifting using carbon dioxide and nitrogen
pH shifting tests were conducted in a 5 L BioFlo III reactor (New Brunswick Scientific, Edison, NJ) with a 3 L working volume and equipped with two Rushton turbine impellers and a perforated steel tube sparger. Experiments were conducted over a range of temperatures (10 °C to 20 °C) and agitation speeds (200 rpm to 800 rpm). pH was measured using a Broadley-James pH probe and tracked using TracerDAQ data acquisition software (MicroDAQ.com, Ltd, Contoocook, NH). CO_2 and N_2 were sparged sequentially into the system at 1.6 vvm. The tests were performed in RO water, original medium and minimal growth medium. The pH was tracked for 60 s prior to sparging with CO_2 gas, followed by nitrogen sparging after the system had reached an equilibrium pH.

A second set of tests was performed to determine which individual components of the minimal medium affected lowering of the pH by CO_2. Individual medium components were dissolved in 100 mL of RO water in a 250 mL Erlenmeyer flask, and the initial
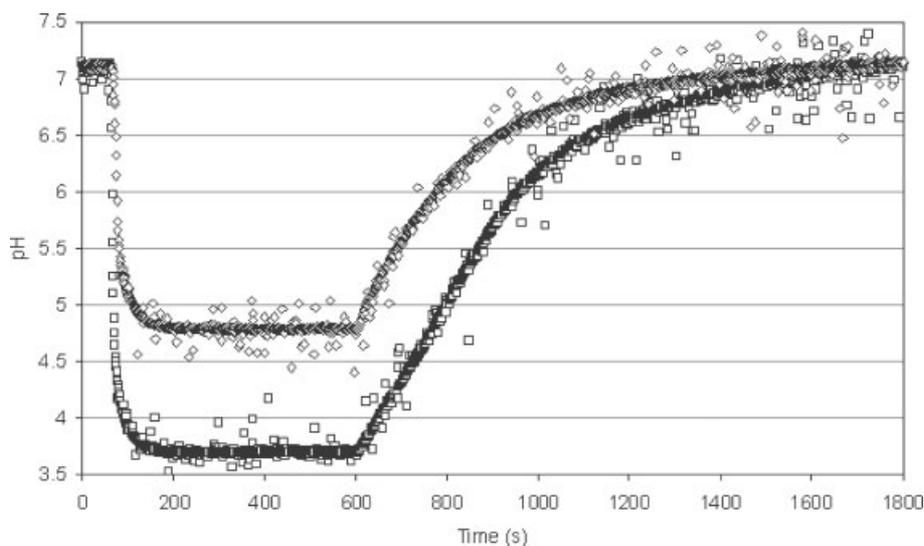
Figure 1. pH adjustment of RO water (open squares) and minimal medium (open diamonds) using carbon dioxide and nitrogen gas at atmospheric pressure, 20 °C and 500 rpm.

pH was measured. The flask was then placed on a stir plate and CO_2 was sparged with pH monitoring until an equilibrium pH was reached.

Partition coefficient testing

Uptake of SA by various polymers was determined by preparing a 10 g L^{-1} solution in RO water and placing 10 mL into 20 mL scintillation vials, to which 2.5 g of the individual polymers were added to give a polymer fraction of 25% (w/v). The vials were shaken overnight to reach equilibrium, and the equilibrium aqueous concentration was measured, giving the uptake of SA in the polymer phase by mass balance. The effect of pH on partition coefficient was determined by preparing a 20 g L^{-1} SA solution in RO water and dividing it into nine 100 mL aliquots. The pH was adjusted using 5 mol L^{-1} KOH or 5M H_2SO_4 to give a pH range of 2.2 to 4.3 (previous tests at pH 5.2 and 6.2 indicated no SA uptake and higher pH levels were excluded). 10 mL of each pH adjusted solution was placed into scintillation vials and polymer fractions ranging from 15% to 90% (w/v) were added. The vials were shaken overnight and the aqueous concentration was measured, allowing for calculation of the SA in the polymer. Based on the equation used by Rehmann and Daugulis, the partition coefficient was calculated as the ratio of polymer phase concentration and liquid phase concentration, as shown below.[18]

$$K_{S/W} = \frac{S_{sol.}}{S_{aq.}}$$

Serum bottle preparation for cell exposure to a low pH

A serum bottle (125 mL) containing 50 mL of the original growth medium and 10 g L^{-1} glucose were prepared, and the headspace was sparged for 30 s with carbon dioxide before adjusting the pH of each serum bottle to 6.7 using autoclaved 5 mol L^{-1} H_2SO_4 and sealing with a butyl rubber crimp cap. This bottle was inoculated with 200 µL of frozen cell stock and placed in an incubator at 37 °C and 200 rpm for 20 h. Eight additional serum bottles were prepared as described above, with one being adjusted to pH 4.2. A 3 mL sample was drawn from the 20 h culture bottle and injected into the pH 4.2 bottle; after 5, 10, 15, 30, 60, 120 and 240 min of low

pH exposure, 3 mL aliquots were removed from this low pH serum bottle and injected into pH 6.7 bottles. One serum bottle acted as the control for the study and had no exposure to pH 4.2. The optical density of the serum bottles was monitored at intervals of 2, 4, 8, 12 and 24 h after inoculation.

Bioreactor preparation

A 5 L BioFlo III (New Brunswick Scientific, Edison, NJ) with a 3 L working volume was used with the temperature, agitation and pH being maintained at 37 °C, 500 rpm and pH 6.7 (using 5 mol L^{-1} KOH), respectively. Minimal medium with 55 g L^{-1} glucose was sterilized and then added to the bioreactor aseptically, with CO_2 gas being sparged into the bioreactor at 0.4 vvm during operation. In the case of two-phase bioreactor runs, 1 kg of Hytrel® 8206 was added to the bioreactor prior to autoclaving.

Analytical methods

Aqueous samples were analyzed using HPLC (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 × 7.7 mm) at 60 °C with a 10 mmol L^{-1} H_2SO_4 mobile phase at 0.7 mL min^{-1}, and a UV-Vis detector (Varian Prostar, PS325) at 220 nm. Cell concentration was measured using optical density in a spectrophotometer at 660 nm (OD$_{660}$). Cell dry weight was calculated from a calibration curve where an absorbance of 1.0 equalled a cell dry weight of 401.2 mg L^{-1}. Glucose was measured as described by Miller with a spectrophotometer at 540 nm.[19]

RESULTS AND DISCUSSION
pH adjustment using CO_2 and N_2 gas

With CO_2 sparging the pH of RO water was reduced from 7.0 to approximately 3.8 in less than 2 min, as shown in Fig. 1. This lower limit was reached with every RO water test, although as the temperature was decreased from 20 °C to 10 °C, the lower limit on pH decreased slightly from 3.85 to 3.75 due to the slight increase in CO_2 solubility at lower temperatures. As the CO_2 dissolved into solution, it reacted with water to form carbonic acid, which dissociated and caused the pH to drop. Because the
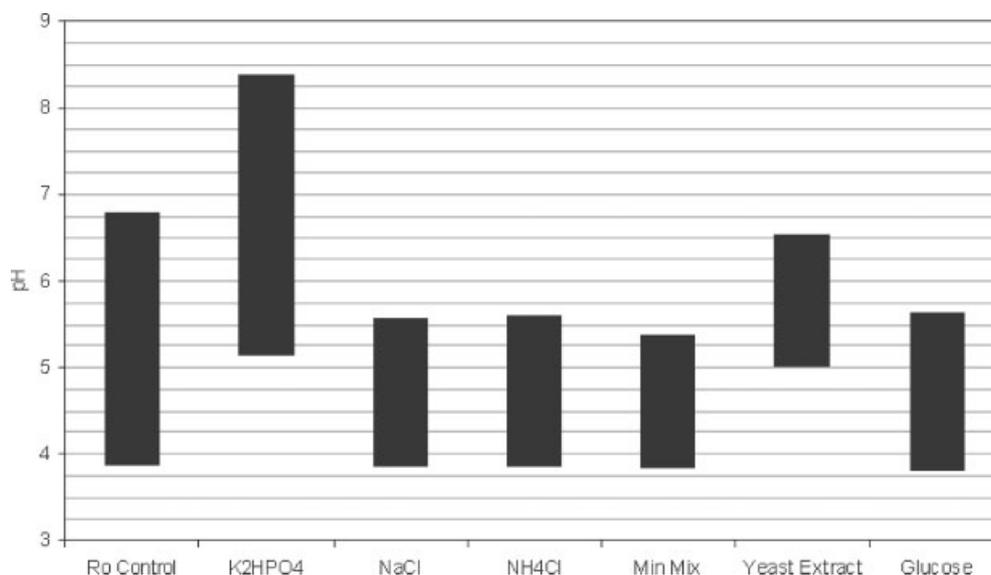
Figure 2. pH adjustment of individual growth medium components using CO_2 gas. Top of each bar represents initial pH, and bottom of bar the final equilibrium pH after CO_2 sparging.

K_{A2} for carbonic acid is 4.69×10^{-11}, there was no appreciable change in pH from the second proton dissociating.

Nitrogen sparging raised the pH of the system, although the time to reach the starting pH was much longer, as seen in Fig. 1. The rate of pH increase with the addition of nitrogen gas changed with the agitation rate of the system (data not shown) up to 500 rpm at which point the pH rate of change remained constant at this, and higher rotational speeds. The pH increase rate at 500 rpm and above was more than double that at 200 rpm (rates not shown). The trend of pH increase rates reaching a plateau at 500 rpm was similar in all tests in RO water regardless of temperature.

The minimal growth medium was tested similarly and an example of the results is also shown in Fig. 1 as the open diamonds. The shift in pH showed similar trends, however, the pH did not reach the same, low value as with RO water, plateauing at a pH of 4.75, slightly above the lower pK_A value of SA. Unless resolved, this situation presents a challenge, as SA uptake using polymer materials requires that the pH of the solution be at or below pH 4.2, the pK_{A2} of succinic acid. This was the reason that RO water was used in serum bottle and bioreactor experiments, as the presence of salts found in tap water could also hinder pH adjustment by CO_2. One factor that may facilitate CO_2 pH adjustment in growth medium is the consumption of medium components during fermentation, which would be expected to decrease the buffering effect of the salts as SA production progressed. Since some components of the growth medium obviously hindered pH reduction using CO_2 they were tested on their own to determine their individual effect on causing resistance to pH reduction. Each growth medium component was tested at its initial concentration in the minimal growth medium. The initial pH for each component started with no prior adjustment and is the pH of the solution obtained from dissolving each growth medium component. The results from the minimal growth medium are presented in Fig. 2. The top of the bar indicates the starting pH and the bottom shows the equilibrium pH with CO_2 sparging.

Figure 2 shows that the greatest impediment to pH reduction with CO_2 came from K_2HPO_4. Given that the concentration of K_2HPO_4 was only 0.5 g L^{-1}, the sensitivity of pH to this buffering

components is apparent. Again, the effect of growth medium was considered a 'worst-case scenario' as the bacteria will consume some of these salts during growth and reduce the impact they have on pH reduction using CO_2.

There are two solutions to the challenge of adequately reducing the pH using CO_2 gas. The first solution is to change the solubility of carbon dioxide in the system. This can be accomplished by increasing the pressure of CO_2 and/or reducing the temperature of the system. Elevated pressures are clearly a promising method of increasing the CO_2 solubility and thus further decreasing the pH.[20] Dodds *et al*. also showed that the solubility of carbon dioxide in water is greatly enhanced at reduced temperatures as the pressure is increased above 5 atm.[20] The second approach to reducing the pH below the pK_A of SA is to use a strong acid, although this is undesirable due to the accumulation of salts, exacerbated if the pH has to be subsequently raised with a strong base. One of the problems with current SA production methods is the extent of chemical addition used in the separation process, so the addition of acid to adjust the pH would be counterproductive to developing a more efficient separation method for SA. However, because the proposed CO_2 pH adjustment system for this biotransformation could not be performed above 1 atm with existing fermentation equipment, for the remainder of this research, strong acids were used to lower the pH, allowing polymer uptake to show proof of concept for the overall research goal of ISPR and reducing EPI.

Polymer screening

An initial group of polymers was chosen to determine their uptake of succinic acid. Polymer selection followed the work of Gao and Daugulis, which showed that Hytrel® 8206 gave the highest partition coefficient while the other polymers tested showed little or no uptake of SA.[21] The polymers chosen for the screening process are shown in Fig. 3. Several grades of Hytrel® were selected to determine the effect of polymer hardness within a single polymer type on uptake. PEBAX® was selected based on the polyamide groups in its structure and the belief that the main factor affecting uptake molecules was hydrogen bonding between polymer and SA, which the polyamide group provided.
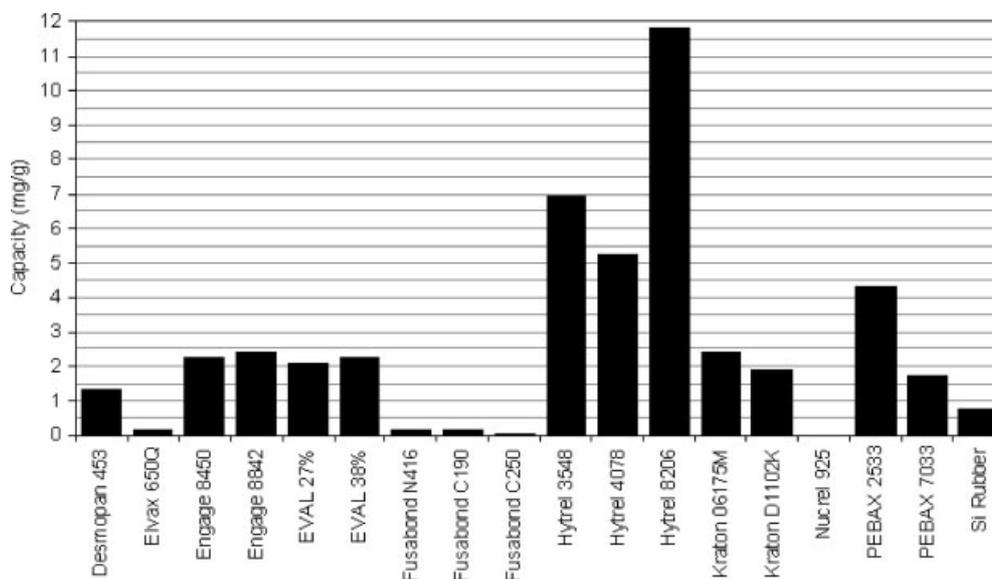
Figure 3. Succinic acid capacity of various polymers with 25% polymer fractions.

Fusabond® was chosen based on Gao and Daugulis suggesting that polymers grafted with molecules chemically similar to SA would aid uptake.[21] Given that Fusabond® polymers have been modified with maleic anhydride, they met the recommendations of Gao and Daugulis.[21] Nucrel® 925 and Elvax 650Q were chosen based on recommendations from DuPont Canada that the methacrylic acid and vinyl acetate may help SA uptake by the polymers. High percentages of these components increase the size and number of amorphous regions within the polymer, the areas where sorption occurs.

Because of the large number of polymers tested, only one polymer-to-aqueous ratio was tested, 25% (w/v). The pH of the samples was left unadjusted at approximately 2.64, well below the pK_A of SA to avoid any changes in acid–salt equilibrium as succinic acid was absorbed and the pH changed. The results of this test are presented in Fig. 3. Samples were duplicated and the average values are reported.

Of the 18 polymers tested, five showed almost no uptake of SA. Several others showed a small affinity, and Hytrel® 8206 had the highest removal of succinic acid from solution. All three Hytrel® polymers showed an SA capacity higher than all the other polymers tested, in agreement with the work of Gao and Daugulis.[21] New tests to estimate the partition coefficient were performed with all three Hytrel® polymers at various polymer ratios but only 8206 yielded a non-zero partition coefficient value of 1.3 (data not shown).

In spite of the suggestion that maleic anhydride grafted to polymers would increase polymer uptake, all three Fusabond® polymers showed almost no sorption. The lack of uptake in these polymers may be due to the hardness of the material. The hardness of Fusabond® polymers is due to a large portion of the bead being crystalline, not allowing for penetration of solutes into the amorphous regions. The capacity tests performed on PEBAX® polymers showed that as the hardness increased, succinic acid uptake decreased. PEBAX® 2533 was softer than 7033 and, as such, had a higher succinic acid capacity. Following the recommendations from DuPont Canada with regard to increasing the fraction of vinyl alcohol in a polymer to aid uptake, the capacity of succinic acid increased from EVAL® 27% to EVAL® 38%.

Alternatively, Nucrel® 925 and ELVAX® Q650 showed very little uptake, but these polymers are comprised of vinyl acetate rather than vinyl alcohol. Compared with vinyl acetate, vinyl alcohol polymer showed superior uptake due to increased hydrogen bonding, believed to be the main attractive force for SA uptake in polymers. The conclusion from the polymer screening experiments was that Hytrel® 8206 would be used for all future work with this research, including tests to determine the effect of pH on uptake.

Partition coefficient as a function of pH

The pK_{A2} of succinic acid is 4.2, the point at which 50% of the succinic acid in solution is in its undissociated form and, therefore, only half of the succinic acid in solution is available for uptake by amorphous polymers at this pH. While decreasing the pH of the system below pH 4.2 would ensure that more succinic acid is undissociated, the lower pH may require the addition of more acid or higher carbon dioxide pressure. A decrease in the pH of solution far below 4.2 would also put greater strain on the microorganisms if they were present during the pH shift/polymer uptake phase of the ISPR cycle. To determine which pH level was required to ensure high levels of succinic acid uptake, a series of experiments was undertaken to test how the partition coefficient varied with respect to pH. The results for the partition coefficient are shown in Fig. 4(a) and (b).

In Fig. 4(a), the partition coefficient is represented by the slope of the line generated from the equilibrium concentration values for each pH, the values of which are shown in Fig. 4(b). The data at pH 2.53 gave a partition coefficient of approximately 1.3, comparable with previous studies on the partition coefficient of Hytrel® 8206.[21] In data sets for pH 2.2 and 3.2, initial liquid concentrations were lowered, arising from dilution by the acid and base addition for adjusting the initial pH. As a result, the concentration in the polymer was lowered due to a slight decrease in the concentration gradient. Most importantly, however, was that the change in liquid concentration had no effect on the partition coefficient and all three sets of data below pH 4.1 gave values near 1.3, as shown in Fig. 4(b).

Trends for the experiments conducted near the pK_{A2} of SA (4.1–4.3) were difficult to discern. While the data in Fig. 4(a)
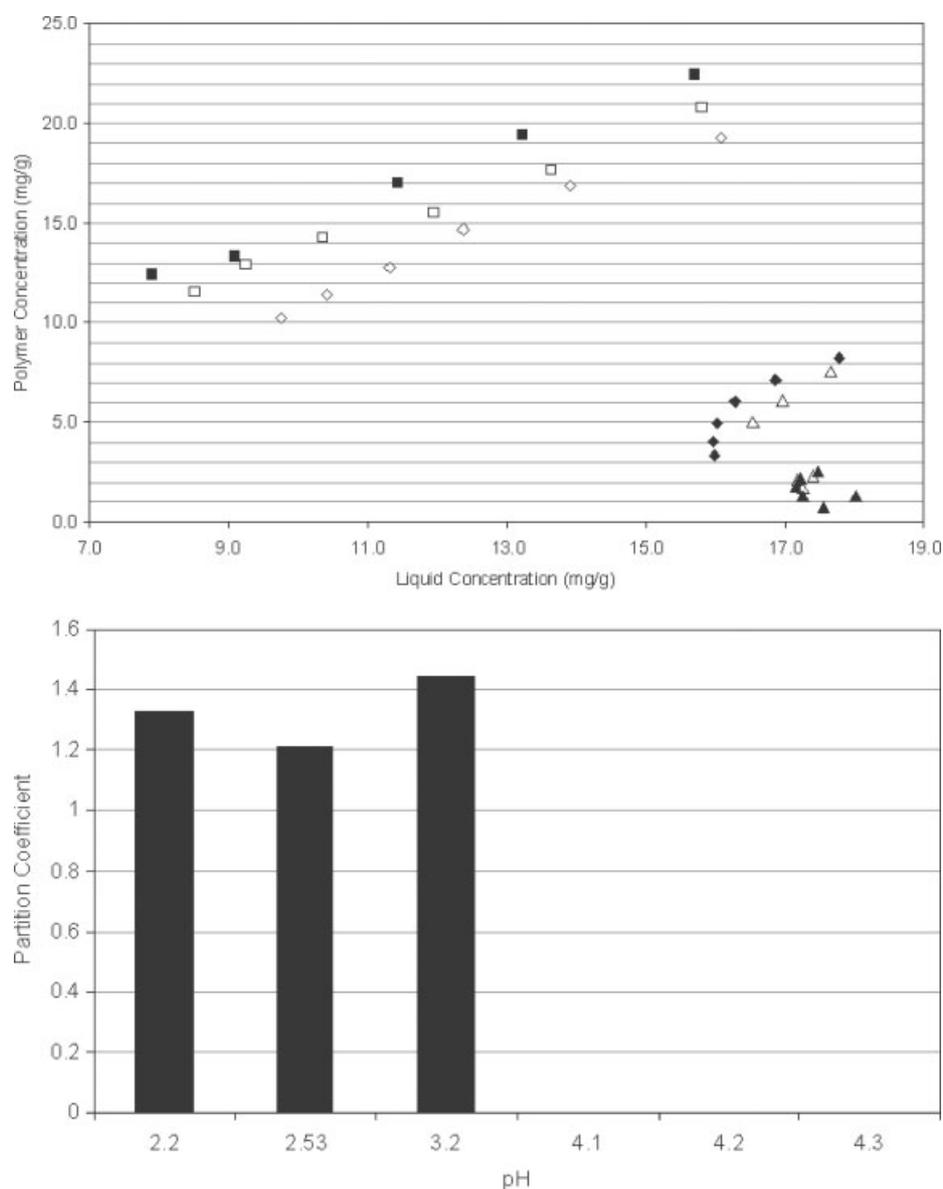
Figure 4. (a) The effect of pH on partition coefficient at pH 2.20 (closed squares), 2.53 (open squares), 3.20 (open diamonds), 4.10 (closed diamonds), 4.20 (open triangles) and 4.30 (closed triangles), and (b) partition coefficient as a function of initial pH.

indicated a partition coefficient of approximately 2.3 at pH 4.1, these data were considered unreliable due to the shape of the curve. Because the data at pH 4.1 to 4.3 showed no linear trend, a partition coefficient could not be calculated, as shown in Fig. 4(b). To shed additional light on the samples at pH 4.1 to 4.3, the equilibrium pH (after contacting with polymers) was measured in all samples to determine if the final pH value could be correlated with SA uptake. These samples showed that pH increases as the polymer masses increased, but the pH plateaued at approximately 4.65, another indicator that succinic acid uptake from pH 4.1 to 4.3 did not increase with an increasing polymer fraction. An equilibrium may have been established in which the tendency of the system to maintain succinate ions in solution equalled the activity of polymers to remove succinic acid and uptake ceased. Ideally, a pH of 3.2 to 3.8 should be used for succinic acid absorbance as it shows no ill effects on uptake compared with pH 4.2.

In addition to testing how the pH of the system affected succinic acid uptake, a test was performed to ensure there was no change in the partition coefficient as concentration increased for a fixed mass of polymer used. In tests ranging from 20 g L^{-1} to 60 g L^{-1}, the partition coefficient remained at 1.3 (data not shown).

Cell exposure to a low pH

Given that the pH of the SA medium during polymer uptake would be below pH 4.2, tests were required to determine whether the microorganisms could withstand low pH conditions, allowing for continued production after succinic acid was removed from the bioreactor during ISPR operation. Figure 5 shows the optical density of the cells after exposure to low pH conditions, ranging from 5 min to 4 h. Also included in this figure is the optical density of the original serum bottle from which all samples were drawn, which acted as a control to compare against all other samples.
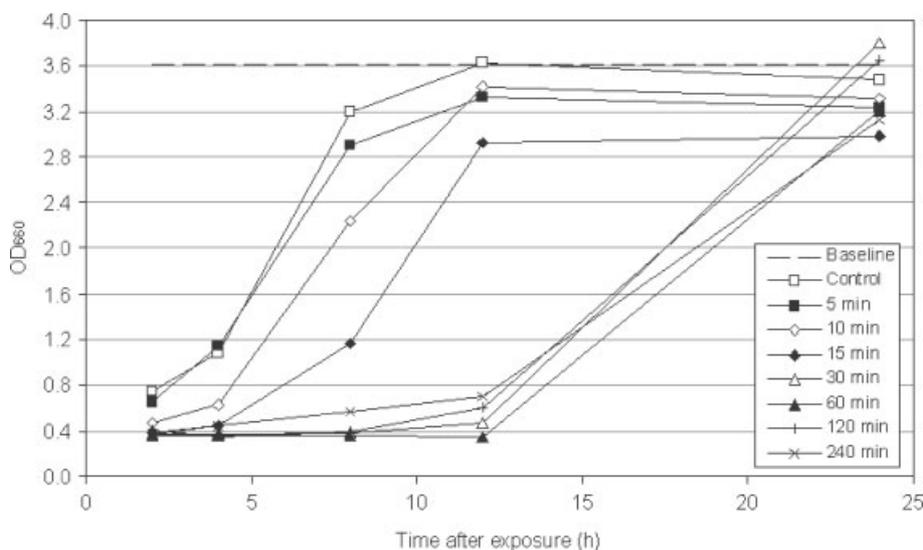
Figure 5. Optical density of samples after low pH exposure for between 5 and 240 min, also including the control sample and the OD_{660} of the original serum bottle.
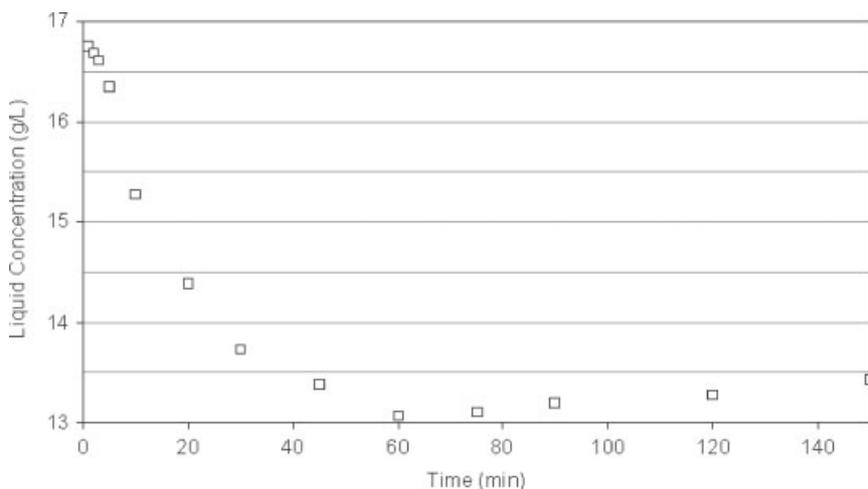

Figure 6. Liquid succinic acid concentration during polymer uptake.

Figure 5 shows that the control test grew quickly and reached a final OD within 12 h. This was expected as the inoculum for the control sample was not exposed to a low pH and showed unhindered growth. Samples with exposure times up to 15 min had the same trend and reached a maximum OD after 12 h at biological pH conditions. Samples with exposure times of 30 min to 4 h had a distinct lag phase which lasted for a minimum of 12 h. At some point after 12 h, the OD_{660} of the serum bottles increased and rose to levels comparable with the control and samples showing no lag phase.

These results show that a longer cell exposure time to low pH led to a longer cell recovery time. However, the most important information from this test was that *Actinobacullis succinogenes* withstood up to 4 h of low pH conditions and the biomass recovered to values similar to cells not exposed to low pH. This, therefore, demonstrates that removing succinic acid using polymer beads can take place by temporarily lowering the pH to 4.2 and raising it to the normal bioreactor operating pH without permanently damaging the cells. With regard to the effect of achieving reduced pH by operating at higher CO_2 pressures, high

pressure CO_2 impact varies by species, and some species such as *S. cerevisiae* have demonstrated good tolerance.[22] Bearing in mind that *Actinobacillus succinogenes* originates from the bovine rumen, a high CO_2 environment, and is both capnophilic and carbon-fixing, it is possible that this organism may not only tolerate, but thrive in elevated CO_2 conditions.[23] Previous research into polymer uptake of target molecules indicated that polymer uptake times were on the order of 1 h which should minimize the effect on cells.[24,25]

Polymer uptake from fermentation broth
New partition coefficient tests were undertaken in fermentation broth to confirm the uptake results relative to the abiotic study. For this test of uptake over time, 1 kg of Hytrel® 8206 was placed directly into a bioreactor containing 3.7 L of broth at the end of an SA fermentation. The polymer fraction in the bioreactor for this test was approximately 26% (w/v). The pH of the system was lowered to, and maintained at 3.8 using 5 mol L^{-1} H_2SO_4. The agitation rate was set to 500 rpm and the liquid SA concentration was monitored for 3 h. The results are presented in Fig. 6 and show
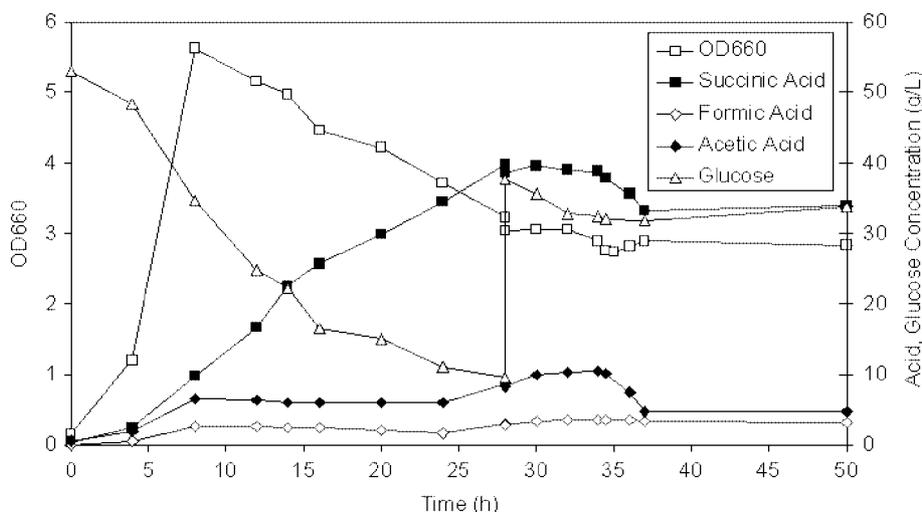
Figure 7. Biotransformation of succinic acid.

that the liquid concentration decreased quickly through the first hour, then levelled off for the remainder of the experiment. At the agitation rate used, the time required for succinic acid uptake into the polymer phase was approximately 60 min, as has been observed in previous research for other target molecules.[24,25] This suggests that exposing the cells for 1 h at pH 3.8 for SA uptake may indeed be feasible. The succinic acid uptake from the fermentation broth gave a polymer capacity of 13.1 mg g^{-1}, comparable with earlier studies where the capacity was 12 mg g^{-1} in abiotic capacity tests. The reason for this difference may have been due to the slightly different pH employed and/or the presence of salts.

Using the equilibrium concentration in the liquid and polymer phases, a partition coefficient was calculated to be 1.00. This partition coefficient was lower than previous values, but the difference may be due to the various salts in solution and possibly the other products and intermediates of the bioreaction; formate, malate and fumarate, whose pK_A values are below 3.8 Nevertheless, succinic acid was successfully removed from the liquid phase using a polymer, which was easily separated from the fermentation broth. Use of such a separation method is a first step in improving on other extraction methods in that it does not require large inputs of other chemicals or electricity and no pre-treatment of the system is required to filter out cells.

Bioreactor pH cycling to remove succinic acid

Using all previous information a bioreactor run was now undertaken, with the results shown in Fig. 7. At 28 h into the biotransformation, a 300 mL autoclaved bolus of nutrients was added to the bioreactor containing the initial mass of substrate and nutrients normally added to the bioreactor. It was because of this bolus that the product concentrations and optical density decreased due to dilution. The glucose concentration increased from the bolus addition, but due to fermentation broth dilution from pH control and bolus addition, the glucose concentration did not return to 55 g L^{-1}. Prior to bolus addition, the concentration of succinic acid was at its highest, reaching 40 g L^{-1} at 28 h, giving a volumetric productivity of 1.42 g L^{-1} h^{-1}. The succinic acid concentration achieved was comparable with bioproduction shown in the literature.[16,17]

At 34 h, to allow uptake of the SA by the added Hytrel®, the pH of the system was lowered to 3.8 using 5 mol L^{-1} H$_2$SO$_4$, taking

30 min. While a more concentrated acid would have decreased the pH faster, it may have caused cell damage as it entered the bioreactor. After the pH was adjusted to 3.8, the bioreactor was maintained for 60 min to allow for SA uptake. The decrease in succinic acid concentration from 34.5 h to 35.5 h was due to polymer uptake from the fermentation broth. The decrease in succinic acid over this time resulted in a polymer concentration of approximately 10 g L^{-1}, a value close to that found in the previous uptake study. After 1 h, the polymer beads were separated from the fermentation broth and the pH of the fermentation broth was then raised to 6.7 using 5 mol L^{-1} KOH, requiring 30 min.

The bioreactor then continued to run after 36 h but cell growth and SA production did not resume, glucose was not consumed and no additional by-products were formed. This lack of cell growth is seemingly contrary to the results of the low pH exposure study, but the difference may be due to the large amount of salts, both from pH adjustment and nutrient addition.

CONCLUSION

In our view, the use of CO$_2$ gas to temporarily lower the pH of fermentation broth to below the pK_a of an organic acid is an attractive alternative to the use of strong acids and bases, as it is rapidly and readily reversible and leaves no residual salts that can affect subsequent fermentation performance, and that must be removed during product purification. Although we were not (barely) successful here in lowering the pH of fermentation broth adequately, higher pressures hold promise to achieve the desired result, and elevated pressure work is currently underway. pH shifting is very fast and, along with the relatively rapid uptake of SA by the polymers (requiring less than 1 h) it is anticipated that no lasting deleterious effects on the cells would be seen, allowing for resumption of SA production in subsequent cycles. Although the affinity for SA by the polymers tested in this work was relatively modest, current work is focusing on the testing of additional commercial polymers (another grade of Hytrel has already shown improved uptake) as well as rational polymer fabrication for enhanced SA uptake. If the work underway on SA production using CO$_2$ pH shifting and polymer uptake is successful, the ISPR production of other organic acids are the next targets.

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