

**THE SYNTHESIS OF SUCCINIC ACID AND ITS EXTRACTION  
FROM FERMENTATION BROTH USING A TWO-PHASE  
PARTITIONING BIOREACTOR**

by

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## Abstract

Succinic Acid (SA) is an intermediate in the production of fine and commodity chemicals. No commercial SA bioproduction process exists due to process limitations including end product inhibition and high product separation costs, which account for 70% of total production 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).

Sparging  $CO_2$  gas into the bioreactor was proposed to temporarily lower the pH of the medium, allowing for SA uptake. At 1atm  $CO_2$  sparging lowered the pH of Reverse Osmosis (RO) water to 3.8 but only to 4.75 in medium, requiring the use of  $H_2SO_4$  and KOH for pH adjustment in subsequent experiments. Polymers were screened for SA uptake and the effect of pH on uptake from 2.2 to 6.2 was also studied. Only Hytrel<sup>®</sup> 8206 showed non-zero uptake with a partition coefficient for SA of 1.3. Cell cultures of *Actinobacillus succinogenes* was exposed to pH 4.2 for times from 5 minutes to 4 hours to determine whether cells could grow after low pH exposure. *A. succinogenes* resumed growth after up to 4 hours of low pH exposure, giving a sufficient time span for SA uptake in the bioreactor. A single-phase run was operated as a benchmark for comparison to the TPPB system which removed SA from the fermentation broth by pH cycling; lowering the pH to 3.8 for uptake, then increasing it to 6.7 to continue bioproduction. Uptake from fermentation broth took 60 minutes, within the time causing no effect on cell growth from low pH exposure. The two-phase run yielded 1.39g/L-h, unchanged compared to the single-phase run which gave 39g/L of SA after 28 hours. Though pH cycling reduced the concentration of SA through polymer uptake, the salts added for pH adjustment hindered further cell growth. The TPPB system demonstrated that SA can be efficiently removed from solution without complex separation methods. Future work will use pressurized vessels to

increase the solubility of CO<sub>2</sub> and lower the pH of fermentation broth for SA uptake without the need for strong acids.

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# 1.0 Introduction

## 1.1 Background

The world in which we live relies heavily on the use of petroleum not only as a source of fuel but as a feedstock for the products we use and the containers that hold them. Currently, the chemical industry consumes over one billion barrels of oil per year and overall consumption of liquid fuels such as crude oil and natural gas amounts to over 30 billion barrels per year [Delhomme et al., 2008, Conti and Doman, 2010]. Of this current consumption, only 1.4% comes from biofuels. A current estimate of total accessible oil reserves is 1.3 trillion barrels, and with the reported consumption levels, world oil resources will be depleted by 2055 [Conti and Doman, 2010]. Given that oil reserves will eventually become scarce, more focus is being put on catalyst improvement to increase efficiency in traditional synthesis, particularly for the production of fine chemicals [Sheldon and van Rantwijk, 2004]. This still does not solve the problems associated with chemical production from dwindling oil reserves. Ideally, biochemical production of chemicals from fuels using renewable resources could replace a large portion of petroleum consumption.

In the United States alone, it is estimated that over 1 billion tons of biomass per year is available from the forestry and agriculture sectors without affecting food supplies; enough to replace 30% of current US petroleum consumption as fuel, chemical production and energy [Perlack et al., 2005]. This unused mass could act as a starting material for bioproduction. As biomass is plant and plant-derived material, it is produced through the removal of atmospheric carbon dioxide, a pollutant which has a large impact on the environment. Additionally, this large volume of biomass available on an annual basis allows for easier accessibility compared to oil extraction. The ability of biomass to act as a carbon dioxide sink as well as its abundance in agriculture and forestry makes it an ideal raw material for the bioproduction of commodity and

specialty chemicals [Sauer et al, 2009].

Bioproduction is defined as the bioconversion of organic feedstocks to energy or chemicals, either through the use of enzymes or entire microorganisms [Otero and Nielsen, 2010]. It is a preferred production method, as traditional methods of production face problems with diminishing sustainability and increasing negative environmental impact [Otero and Nielsen, 2010]. Bioproduction has the advantage that it takes place at temperatures between 30°C and 40°C, far below temperatures required for chemical synthesis [Sheldon and van Rantwijk, 2004]. Another advantage of bioproduction is the high selectivity of target molecules when stereoselectivity is important, particularly in the production of pharmaceuticals. L-phenylacetylcarbinol (L-PAC), a precursor to ephedrine, can be produced by the microorganism *Candida utilis* whereas chemical synthesis generates many by-products and does not have high selectivity [Khan and Daugulis, 2010].

While there are many chemicals that can be produced from renewable resources using microorganisms as biocatalysts, one that can be used to make a wide range of products is succinic acid. A 1,4-diacid, it is a precursor to commodity chemicals such as 1,4-butanediol as well as specialty chemicals for the pharmaceutical industry [Song and Lee, 2006]. This particular chemical falls into a group known as diacids, which have been highlighted in a 2004 US Department of Energy report as one of the top value-added chemicals produced from biomass [Werpy and Petersen, 2004]. There are several strains of bacteria available which can efficiently transform glucose and other sugars into succinic acid, but, overall, separation of the product from the fermentation broth is difficult. While some research suggests that succinic acid production can be as high as 100g/L, in most cases the maximum reported concentration of succinic acid produced is approximately 50g/L. This upper limit in the literature may indicate that end product inhibition is taking place. As the succinic acid production decreases near this 50g/L upper limit, the volumetric productivity of the system decreases, giving a system that is less efficient than if it were unaffected by end product inhibition. Overall, the bioproduction process has yet to be made

commercially viable and competitive with the traditional chemical production process.

Aside from end product inhibition, the main bottleneck in developing a commercial bioprocess for succinic acid is efficient product recovery. The fermentation broth must be filtered of impurities such as cells, substrate and other acids, as well as reduced in pH to generate the protonated form of the succinic acid. These pre-treatment steps result in a separation process that is 60 to 70% of the total production cost of succinic acid [Hong and Hong, 2005]. Various methods of succinic acid recovery have been proposed including ion exchange chromatography, precipitation with calcium hydroxide, liquid-liquid extraction with tri-*n*-octylamine, and electro dialysis, but all of these methods have drawbacks in terms of high energy or chemical inputs [Kurzrock and Weuster-Botz, 2010]. An ideal recovery method for succinic acid would have minimal chemical addition, require no additional energy inputs beyond normal operation and would leave the biomass unharmed to continue succinic acid production.

Two-Phase Partitioning Bioreactors (TPPB) are systems designed for substrate delivery or *in-situ* product removal depending on the biotransformation taking place. Previous work in the Daugulis group has helped reduce the effects of end product inhibition in the production of 2-phenylethanol from *Kluyveromyces marxianus* [Gao and Daugulis, 2009]. With a second phase chosen to effectively remove succinic acid from solution, a system can be created to perform *in-situ* product removal, allowing the system to continue without end product inhibition. While previous TPPB systems used an organic solvent as the second, immiscible phase, recent research has shifted to the use of polymer beads, the benefits of which include lower cost, re-use and nonbioavailability [Amsden et al., 2003]. One complication is that polymer uptake requires the pH of the solution to be below the lower  $pK_A$  of succinic acid, a value of 4.2 [Gao and Daugulis, 2010]. The addition of acid to lower the pH and base to raise it after succinic acid absorption is undesirable because this would increase costs and lead to an excess of salts in the system. Carbon dioxide gas, when dissolved in solution, reacts with water to form carbonic acid that dissociates and lowers the pH. This reaction is reversible by sparging nitrogen through the liquid, driving out

the carbon dioxide and restoring the higher pH. Using carbon dioxide to ‘temporarily’ adjust the pH of the system reduces the need for acids and bases and decreases the mass of salts left in solution.

## **1.2 Objectives**

The proposed process was to use a TPPB system to remove succinic acid from solution and reduce end product inhibition. The process produced succinic acid to inhibitory levels, then the pH of the system was adjusted below the  $pK_{A2}$  of succinic acid using dissolved carbon dioxide gas to create undissociated product. Polymers with an affinity for succinic acid absorbed it from solution. After removing the polymers from the system, the pH was returned to operational levels by removing dissolved  $CO_2$  and production continued without inhibition.

The first set of experiments examined the physical aspects of the system. The main advantage of this process was the use of carbon dioxide gas to lower the pH of the system below the  $pK_{A2}$  of succinic acid for absorption into the polymer. A set of experiments showed how the pH of the system can be adjusted using  $CO_2$  in ultrapure water as well as in the presence of growth medium components. A polymer was selected and absorbance at various pH levels was characterized. The goal was to determine the polymer to use for succinic acid absorption from the system and how effective the  $CO_2$  gas was at adjusting the pH.

Biological testing showed how the selected microorganism responded to exposure to a low pH and how it recovered based on the length of exposure time. The purpose of this test was to show the maximum time span possible for succinic acid absorbance before lasting negative effects were seen in the cells. A single-phase benchmark fermentation was also established against which TPPB tests was compared. Proof of concept showed through a combination of physical and biological testing that a system was developed which produced succinic acid in high concentrations and removed it from fermentation broth in a more efficient and less energy- and

resource-intensive method than current separation processes in the literature while lowering end-product inhibition.

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## **2.0 Literature Review**

### ***2.1 The use of Microorganisms in Biosynthesis***

The most basic form of a biocatalyst exists as a single enzyme transforming one compound to another. While a single step involving one enzyme is ideal, biotransformations are often complex and can have many intermediate steps and side-reactions. For more complex biotransformations, the ultimate form of a biocatalyst is a living cell that has enzymes capable of bioconverting a substrate, via multiple steps, into the desired product in one process [Sheldon and van Rantwijk, 2004]. The term industrial biotechnology was first used in the early 1980's, defined as the bioconversion of organic feedstocks to chemicals or energy [Pass, 1981]. The use of industrial biotechnology is becoming more popular as traditional chemical synthesis is characterized by decreasing sustainability, increasing material costs and environmental damage [Otero and Nielsen, 2010]. In comparison with traditional synthesis, bioproduction has a few distinct advantages. Because they are living, microorganisms prefer to operate at neutral pH levels and lower temperatures compared to other processes which can require reactor temperatures of over 300°C [Sheldon and van Rantwijk, 2004]. Additionally, the selectivity of the enzymes in biocatalysts results in less downstream processing and less waste [Sheldon and van Rantwijk, 2004]. These advantages have led to successful applications of biosynthesis in commercial settings.

### ***2.2 Industrial Applications of Biosynthesis***

Total global chemical production sales will be as high as \$1 600 billion USD per year within the next five years, 20% of which will come from industrial biotechnology [Otero and Nielsen, 2010]. Bioproduction will represent \$320 billion USD per year broken down into the



following areas: commodity, 15% polymers, 15%, specialty and fine chemicals, 70% [Kuhn et al., 2010]. Several examples of commercially established bioproduction in fine chemical markets are given below. The antibiotic Penicillin is produced by *Penicillium chrysogenum* with a market of \$1.5 billion USD and the amino acids L-glutamate and L-lysine are generated by *Cornybacterium glutamicum* with annual production exceeding 600 000 tons [Kuhn et al., 2010]. Additionally, over 1 600 000 tons per year of citric acid is produced commercially through *Aspergillus niger* [Sauer et al., 2008]. With increased focus on bioproduction, a wider range of chemicals will continue to be produced, including succinic acid.

## 2.3 Succinic Acid

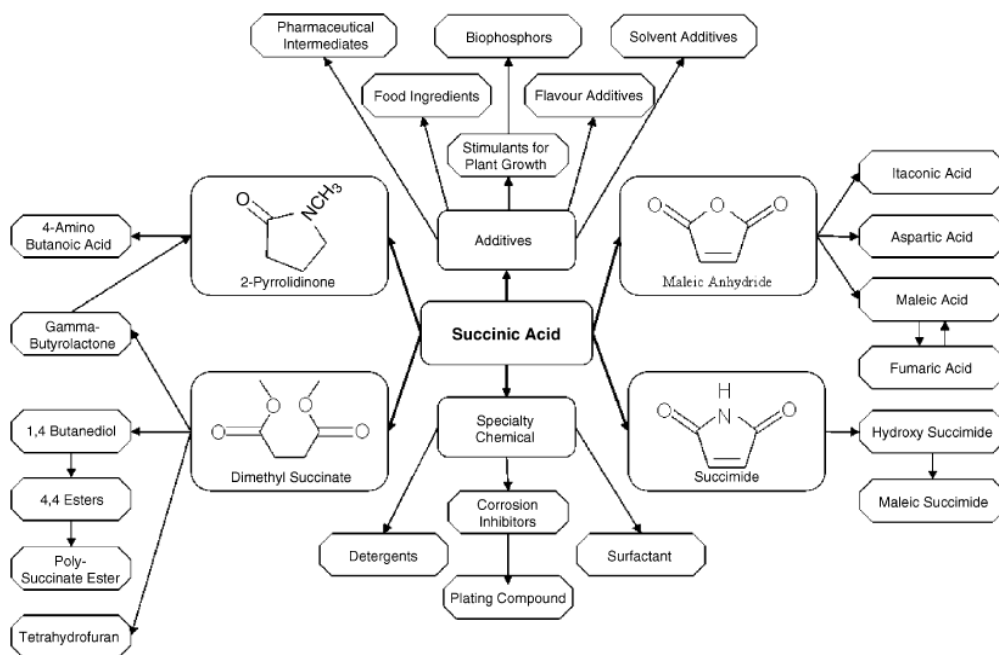
Succinic acid is a 1,4-diacid that has become a chemical of significance in recent years, and was identified as one of the top 12 value-added chemicals to be produced from biomass in a report released by the US Department of Energy [Werpy and Petersen, 2004]. The properties of succinic acid are listed in the table below [J.T. Baker, 2008]

**Table 2-1: Physical properties of succinic acid**

CAS Number	110-15-6
Molecular Formula	$(\text{CH}_2\text{COOH})_2$
Molecular Weight	118.09 g/mol
Melting Point	188°C
Boiling Point	Decomposes at 235°C
Specific Gravity	1.56
Solubility	100g/100ml at 100°C
	7.69g/100 ml at 25°C
pK <sub>A1</sub>	4.21
pK <sub>A2</sub>	5.64

Succinic acid can be used to create a large array of chemicals including resins and biodegradable polymers, as well as surfactants, detergents, pharmaceuticals and food additives [Lee et al., 2002, Lin et al., 2008]. It can also be used as an ingredient in animal feeds and used to stimulate growth in plants [Wan et al., 2008]. Another major area in which succinic acid can

serve as an intermediate chemical is in the creation of 1, 4-butanediol, a chemical that was expected to have a demand of 479 000 tons per year by 2008 and 1.5 million tons per year by 2011 [McKinlay et al., 2007, ChemWeek, 2008]. In addition to succinic acid itself, succinate salts were being consumed at a rate of 92 000 tons per year as of 2003, showing a very large demand for succinic acid and its derivatives [McKinlay et al., 2007]. An example of the chemical derivatives of succinic acid is shown in Figure 2-1.



**Figure 2-1: Succinic acid and its chemical derivatives [Song and Lee, 2006]**

Succinic acid can be produced anaerobically through fermentation by bacteria isolated from ruminant animals and is an intermediate of the reductive TCA cycle [Lee et al., 2002]. Through biochemical production, succinic acid exists in its dissociated form since the pH of fermentation is above its  $pK_A$  values of 4.20 and 5.61 [Lee et al., 2008]. When succinic acid is synthesized from petrochemical sources, it is labelled as chemically produced whereas succinic acid from bioproduction is considered naturally produced as it is sourced from biomass [Zeikus et al., 1999]. This distinction of chemical versus natural production can change the areas in which succinic acid can be used as well as affect the selling price. Industries such as foods and

pharmaceuticals will prefer to use succinic acid produced from renewable resources to avoid potential health hazards from production residues even though the product is identical through both production methods [Gascon et al., 2006]. As of early 2008, succinic acid was sold between \$6 and \$9 per kilogram with annual demand of 15 000 tons with growth at six to ten percent per year [Bechthold et al., 2008]. As fermentation becomes a more cost-efficient method and the number of chemicals synthesized from succinic acid increases, demand will rise [Song and Lee, 2006]. Current succinic acid production through petrochemical processing has raw material input costs of \$1.03 per kilogram whereas fermentation has higher costs, although the bioproduction cost estimate varies based on the scale of production [Song and Lee, 2006]. Currently there is no process for bioproduction on an industrial scale and all succinic acid produced today is done so through chemical synthesis.

## 2.4 Current Production of Succinic Acid

Succinic acid produced using petrochemical resources is derived from maleic anhydride, which is produced from *n*-butane through oxidation over vanadium-phosphorous oxide catalysts [Liu et al., 2008, Gascon et al., 2006]. A simplified reaction pathway of *n*-butane to maleic anhydride is shown below in Figure 2-2.

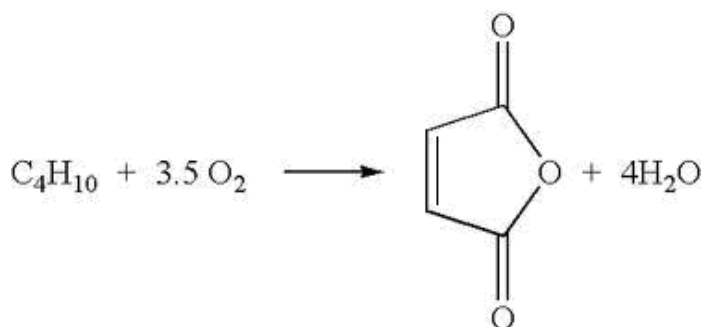


Figure 2-2: Reaction Pathway from *n*-butane to Maleic Anhydride [Sutton et al., 2003]

The reaction from maleic anhydride to succinic acid begins by hydrolysis, breaking one of the single bonds between carbon and oxygen, forming maleic acid. The addition of hydrogen breaks the carbon-carbon double bond and completes the reaction, forming succinic acid.

Succinic acid produced from fossil fuels is what gives it the distinction of not being a natural product [Song and Lee, 2006]. While this method of production is currently cheaper than processing by fermentation, there are some very large drawbacks [Wan et al., 2008]. As the term petrochemical processing implies, succinic acid is made using non-renewable resources such as natural gas which will become more difficult to find as time passes. As this raw material becomes harder to locate and demand continues to increase, it will become increasingly expensive [Isar et al., 2006]. In addition, the removal of oil and gas from deposits, transportation and processing require a lot of energy and generate a large amount of emissions [McKinlay et al, 2007]. This process is not sustainable in the long term and another solution is required if industries want to continue producing succinic acid with increasing demand [Wan et al, 2008].

## ***2.5 Succinic Acid Production Though Fermentation***

The process of fermentation is receiving increasing attention as it can use renewable feedstocks as substrates and is seen as a more 'green' technology compared to chemical production because of its renewable resource consumption and its limited impact on the environment [McKinlay et al., 2007]. The direction in which succinic acid production is heading can be summarized in a quote from McKinlay et al (2007). "Considering the current political and economical climate of crude oil-based industries, bio-based chemical production is in a position to complement and compete with existing petrochemical markets."

Studies show that in the United States alone, 1 billion tons of biomass is available annually from the forestry and agricultural sectors without hindering food production [Perlack et al., 2005]. In a summary paper on the bio-based succinate industry in 2007, it was projected that

with all the chemicals that can be synthesized with succinic acid as an intermediate, there exists a market of \$15 billion US [McKinlay et al., 2007]. Estimates are that succinic acid could be produced through fermentation at \$2.20 per kilogram at a production level of 5,000 tons per year, but the price would drop to \$0.55 per kilogram if production levels reached 75,000 tons per year [Kang and Chang, 2005]. This upper production level may seem high, but this area of biochemical production will soon reduce the need for petrochemicals, so demand is expected to rise [Werpy and Petersen, 2004].

Succinic acid, when produced through fermentation, converts glucose to succinic acid along a portion of the reductive cycle of the tricarboxylic acid (TCA) cycle [Lee et al., 2002]. Figure 2-3 depicts the reactions and enzymes in a typical fermentation process that transforms glucose to succinic acid.

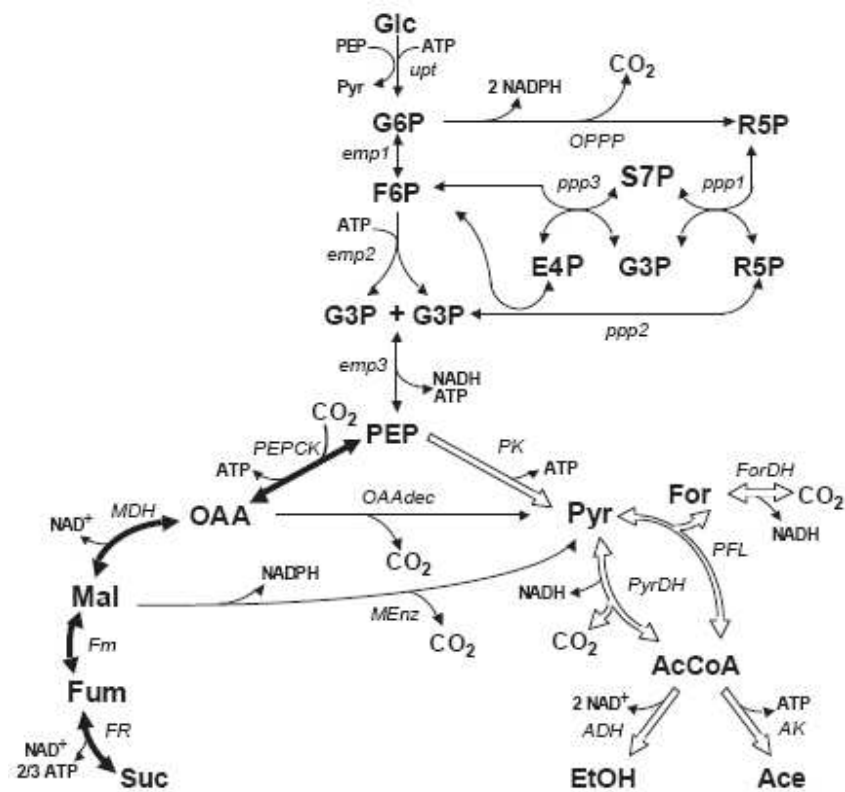


Figure 2-3: Metabolic pathway of a typical succinic acid producing microorganism [McKinlay and Vieille, 2008]

First, glucose is converted to glucose-6-phosphate by hexokinase, which also adds phosphate to the molecule [McKinlay and Vieille, 2008]. Next, three separate enzymes that are part of the Embden-Meyerhoff-Parnas glycolytic pathway lead to the production of phospho-enol-pyruvate (PEP) [McKinlay and Vieille, 2008]. From PEP, the metabolic pathway can take one of two paths depending on the level of carbon dioxide available to the system [McKinlay et al., 2007]. If there is not enough CO<sub>2</sub> present in the system, the preferred metabolic pathway creates end products of formate, ethanol and acetate, as shown on the right side of Figure 2-3. With ample supply of carbon dioxide to the system, the microorganism favours the production of succinic acid, the left half of Figure 2-3 [McKinlay et al., 2007]. Through this pathway, PEP is converted to oxaloacetate by PEP carboxykinase with the addition of CO<sub>2</sub>. This creates a 4-carbon chain, giving this series of reactions the name 'C4 pathway' [Lee et al., 2008]. The presence of high levels of carbon dioxide in the system strongly regulates the activity of PEP-carboxykinase [Zeikus et al., 1999]. The next reaction adds hydrogen to oxaloacetate to produce malate, which is converted to fumarate by fumarase with the removal of a water molecule [McKinlay and Vieille, 2008]. Finally, with the addition of hydrogen, succinate is formed in its ionic state, which is common as the pH range of production is above the pK<sub>A</sub> values for succinic acid [McKinlay and Vieille, 2008, Zeikus et al., 1999]. Succinic acid can be created by protonating the succinate ion and producing the undissociated acid, which can be done in a number of different processes as discussed later. The theoretical yield of succinic acid from glucose plus carbon dioxide should be 1.17 moles per mole of glucose based on stoichiometry [McKinlay and Vieille, 2007].

This production method is not without unwanted by-products, however. In a study on the fermentation of wheat by Du et al. (2007), acetic acid and formic acid were moderate by-products produced in concentrations of 9.2g/L and 6.1g/L, respectively. These values are lower than the final concentration of succinic acid at 27.2g/L, but still make the separation process more time consuming and costly. Another major problem, shown on the metabolic pathway in Figure 2-3, is

that the conversion of glucose to glucose-6-phosphate takes place with the side reaction of converting PEP to pyruvate, an irreversible step [McKinlay and Vieille, 2008].

Succinic acid is also a highly reduced product, so throughout the fermentation process from glucose, four electrons are required. Therefore, in addition to carbon dioxide gas, hydrogen has been suggested as an addition to act as a reducing agent since reductant levels can limit the reaction rate [Lee et al., 1999, McKinlay and Vieille, 2008]. In studies done by Lee et al. (1999) the addition of hydrogen often decreased fermentation time, making the process more efficient. However other studies make no mention of the addition of hydrogen gas and obtain comparable concentrations of succinic acid [Lee et al., 1999, Liu et al., 2008b]. Additionally, other reducing agents, including formate and Natural Red dye have been used with encouraging results [McKinlay and Vieille, 2008].

For bioproduction of succinic acid to be economical, major areas of biological process improvement need to be addressed, including limiting the use of low-cost amino acids, achieving high yield and concentration and using inexpensive carbon sources [Glassner and Datta, 1989]. Fermentation requires both substrate and media which contain the energy sources, nutrients and minerals needed to ensure optimal productivity rates [Lee et al., 2002]. Depending on the microorganism chosen for the fermentation process, there are many carbon sources available. In the case of succinic acid production, most of the major sugars present in biomass can be used effectively, including glucose, fructose, arabinose and xylose [McKinlay and Vieille, 2007]. While the cost of these sugars is relatively low, other carbon sources have been examined, in attempts to lower the cost of fermentation. These other sugars range from glycerol and wood hydrolysate to waste whey from milk and cheese production [Bechthold et al., 2008, Wan et al., 2008]. McKinlay and Vieille (2007) suggest that a succinate-based market through fermentation can be economical provided that the price of oil remains above \$40 per barrel and if corn remains above \$90 per ton. McKinlay et al. (2007) suggest that productivity goals of 150g/L and 5g/L·h are ideal production targets to compete with oil-based succinic acid production.

As succinic acid production from fermentation reaches a point where industrial facilities are being constructed, they will be located very near to supplies of biomass as well as carbon dioxide. An ideal location would be in close proximity to an ethanol plant. These production facilities could make their process more value-added by using their waste carbon dioxide and biomass, such as cane molasses and cheese whey for succinic acid production. Since fermentation of succinic acid is considered a CO<sub>2</sub> fixing process, this would turn a by-product of the first process into an input for the second [Urbance et al., 2004, McKinlay and Vieille, 2007]. This co-product system would also reduce greenhouse gases produced in the creation of ethanol and the waste plant material could also be used as a substrate for succinic acid bioproduction [Zeikus et al., 1999]. As with any bioproduction, a biocatalyst is required and there are many microorganisms in existence that can perform this transformation.

### **2.5.1 Bacteria for Producing Succinic Acid**

The rumen is the primary chamber of the stomach of a ruminant animal, and it is here that succinic acid-producing bacteria are found [Lee et al., 2002]. Among the 200 bacterial strains found in the rumen there are *Ruminococcus albus*, *Cellulolytic prevotella ruminicola*, *Bacteroides amylophilus*, and *Bacteroides fragilis*, but these organisms are not the most efficient succinic acid producers [Lee et al., 2002]. The bacteria that produce succinic acid in greatest concentrations and those that are the focus of the majority of research are *Actinobacillus succinogenes*, *Anaerobiospirillum succinoproducens*, and *Mannheimia succiniciproducens* [Corona-Gonzalez et al., 2008]. Several *Escherichia coli* strains have been genetically engineered for succinic acid production, but are not natural to ruminant animals [Corona-Gonzalez et al., 2008]. The succinic acid concentrations generated by these bacteria range from 45g/L for one of the variants of *E. coli* up to 60g/L for *Actinobacillus succinogenes* [Corona-Gonzalez et al., 2008]. Interestingly, the metabolic pathway shown in Figure 2-3 for *A. succinogenes* is very similar to that of *A.*



*succinoproducens* and the steps from PEP production to succinate formation are identical [Van der Werf et al., 1997].

The metabolic pathways in the mutant strains of *E. coli* were designed to mimic the reactions that produce succinic acid in *A. succinogenes* and *A. succinoproducens* with some alterations made to 'knock out' some of the genes that lead to the production of undesired products. This manipulation of the genes has led to a decrease in the overall succinic acid production of the bacteria and overall the production of succinic acid is limited to 12 moles per 100 moles of glucose whereas the typical production from *A. succinogenes* is 1.2 moles per mole of glucose [Van der Werf et al., 1997]. Wu et al. (2007) concluded that this slowed growth and production is caused by the inability of *E. coli* to regenerate  $\text{NAD}^+$  from NADH due to the disruption of the LDH enzyme. For this research project, manipulation of bacteria for succinic acid production was not a goal, so the only work done with regard to bacteria was organism selection. Several criteria were determined for bacteria selection, most importantly Biohazard Level 1 to eliminate any special handling procedures. The strain best suited to production was found to be *Actinobacillus succinogenes*.

### **2.5.2 *Actinobacillus succinogenes***

The main succinic acid producing bacteria that are the focus of much of the literature were evaluated and the microorganism selected was *Actinobacillus succinogenes*, in particular strain 130Z, American Type Culture Collection (ATCC) 55618. This bacterium was isolated from bovine rumen at the Michigan Biotechnology Institute (MBI) International in Lansing [Guettler et al., 1999]. This strain is facultatively anaerobic, capnophilic and gram-negative and is considered to have the most potential as a succinic acid producer in an industrial setting [Wan et al., 2008]. An alternate form of *A. succinogenes* has been isolated from cow rumen at the School of Biotechnology at Southern Yangtze University in China, but this strain is unavailable through

ATCC and has been labelled as Biohazard Level 2 [Liu et al., 2008]. Compared to the other major producers of succinic acid, there are several advantages of this bacterium including tolerance to high levels of substrate, high resistance to product inhibition, and high production rate. It also has a high tolerance of oxygen, low production of unwanted acid by-products and can use a number of substrates in comparison to other succinic acid producing bacteria.

Guettler et al. (1999) stated that compared to other succinic acid producing bacteria, *A. succinogenes* produced the highest quantities [Samuelov et al., 1991]. Typical production concentrations are reported between 30 to 60g/L after 48 hours [Liu et al., 2008a, Liu et al., 2008b]. Wan et al. (2008) stated that this bacteria had enormous potential as a succinic acid producer because of the high concentrations it can generate. Many sources state that *Actinobacillus succinogenes* is the microorganism of choice for creating an industrial process, having a high tolerance of succinic acid [Lin et al., 2008].

Another factor to consider is how product inhibition affects cell growth. As products accumulate in the aqueous phase of the fermentation, production rates decrease as the cells are forced to spend more energy maintaining the cell rather than fermentation and cell growth. Cells that have a higher tolerance of products can continue producing quickly and give a higher final concentration. The succinic acid production ranges mentioned earlier seem to indicate that there is an upper limit on the final concentration achievable from *A. succinogenes* possibly due to end product inhibition. A study by Wan et al. (2008) showed that *A. succinogenes* could withstand a succinic acid concentration of up to 66.4 grams per litre before production ceased after 84h, showing that production can continue up to high concentrations before the system reaches end product inhibition.

In addition to its high product tolerance, *Actinobacillus succinogenes* can grow from a wide variety of carbon sources. A study by Zeikus et al. (1999) showed that this organism can ferment L-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, manitol, mannose, sucrose, D-xylose and salacin. Another study performed similar tests and agrees with the previous

results, adding that xylose can be fermented into succinic acid as well [Wan et al., 2008]. For the scope of this thesis, the ability to use many feedstocks was not a criterion in bacterium selection. The only carbon source that will be used in this research is glucose, but should this production method be applied on an industrial scale, knowledge that a large number of feedstocks can be used is beneficial.

All of the bacteria that can ferment glucose to succinic acid require anaerobic conditions to function properly, but many of these different organisms require strict anaerobic conditions. This is not the case with *Actinobacillus succinogenes*, however, and a small amount of oxygen can be tolerated in the headspace of the fermentor [Urbance et al., 2003]. This tolerance adds to the list of desirable qualities in a bacterial strain for producing succinic acid as there is less preparation involved in removing oxygen from the system.

The use of *A. succinogenes* for the production of succinic acid yields far fewer by-products compared to other bacteria tested, making the separation process simpler and less costly. Experiments performed by Guettler et al. (1995) with this particular strain showed that the ratio of succinic acid to acetic acid was 85 to 1 and succinic acid to formic acid of 160 to 1. Another study showed that when succinic acid was produced from cane molasses, a succinic acid concentration of 39.4 grams per litre was achieved while only 4.4 grams per litre of acetic acid and 1.5 grams per litre of formic acid was formed, giving ratios of 8.9 to 1 and 26.2 to 1 [Liu et al., 2008]. These ratios are still quite large and further show that *Actinobacillus succinogenes* is an ideal choice for use in the production of succinic acid. Though there are many benefits of this microorganism, there are still limitations with bioproduction that need to be addressed.

## **2.6 Current Limitations with Fermentation**

The process of carbohydrate fermentation is an attractive alternative to the production of chemicals from crude oil because of the renewable nature of feedstocks, but it is not without

limitations. First, fermentation takes place in a defined pH range and for *A. succinogenes* this is between 6.0 and 7.2 with the optimal pH for fermentation at 6.8 [Wan et al., 2008]. A higher pH will lead to higher cell growth but will increase the amount of by-products formed [Lee et al., 2002]. Below a pH of 6.0, little cell growth occurs due to increased cell maintenance demand [Lee et al., 2002]. Although studies show that succinate is the major product formed as pH decreases, a point is reached at which the cells cease to ferment glucose [Van der Werf, 1997]. Unfortunately, the  $pK_A$  values of succinic acid are 4.20 and 5.61, so when succinic acid is produced it is as a dissociated molecule. This is a problem because most separation methods require the undissociated acid form of succinic acid so additional steps are needed to lower the pH to remove products.

Throughout the fermentation process, succinic acid is produced as well as the by-products formic and acetic acid. Because of this, base needs to be constantly added to the fermentation to keep the process operating at or near its optimal pH. This is usually done through the addition of sodium hydroxide, which raises the pH and gives the equivalent salt disodium succinate [McKinlay et al., 2007]. If too much sodium is added to the system the osmolarity of the solution changes and cells will flocculate; their productivity decreases as they spend energy on cell maintenance [Liu et al., 2008a].

Fermentation reactions also have problems because cells are living organisms and as production takes place, product inhibition can occur and cell growth and synthesis stop because more energy is required to maintain cells [Lee et al., 2002]. In a recent example, the fermentation of carvone from carveol was limited by end product inhibition because the product accumulated and became toxic for the cells in the fermentation broth [Morrish et al., 2008]. Studies have shown that end product inhibition is a limitation in succinic acid production using *A. succinogenes*, as well [Urbance et al., 2004]. To date, the highest productivity seen from this organism is 1.34 g/L, far below the target of 2.5g/L·h suggested by the US Department of Energy

[Yang, 2007, Werpy and Petersen, 2004]. Addressing end product inhibition would increase this low productivity by allowing increased production of succinic acid.

The formation of by-products can also hinder fermentation because it can have a similar effect to product inhibition at lower concentrations. As is the case with succinic acid fermentation, by-products such as acetate and formate can limit succinic acid production as well as take away from the carbon source used by the bacteria to make the main product, reducing the yield of the desired product [Huh et al., 2006]. An inhibition study performed by Lin et al (2008) found that formic acid was the strongest inhibitor to cell growth and succinic acid production. The inhibitory concentration of formic acid was 16g/L a concentration 10 fold lower than succinic acid inhibitory levels. The impact of by-product is also noticed in the downstream process since there is more unwanted material to be removed [Lee et al., 2008]. The idea of altering organisms in a way to reduce or eliminate by-products has been suggested and, as mentioned earlier, some work has been done to alter the genetic code of bacteria to ensure that only specific genes are expressed, limiting the number of unwanted products [Lee et al., 2008]. However, this can lead to decreases in growth and production rates, as shown in the examples of genetically engineered *E. coli* [Wu et al, 2007]. Because of these limitations, Lee et al. (2008) have said that as it stands, recovery volume and yield are not high enough to warrant fermentation in large-scale applications using these genetically modified strains.

Maintaining a proper level of substrate concentration is also a concern. In a patent by Datta (1989) it was noted that initial concentrations of glucose over 100g/L inhibited cell growth and succinate production, while concentrations lower than 20g/L produced so little product that it was not practical to perform product separation. Additional studies on optimal substrate levels showed similar results, indicating that the optimal initial glucose concentration was between 50g/L and 60g/L [Liu et al., 2008]. Another challenge is that fermentation reactions need to be constantly monitored to ensure ideal substrate concentrations for optimal growth since the glucose concentration decreases over time and must be replenished. While constant glucose

monitoring is not explicitly necessary for bioproduction, studies show that when the glucose is maintained at 15g/L production of succinic acid increases [Liu et al., 2008].

As it stands, current fermentation technology is not yet financially or operationally competitive with the production of succinic acid from butane. The major problem outlined by the US Department of Energy is the cost of fermentation, and significant improvement needs to occur if this process is to be used on an industrial scale [Werpy and Petersen, 2004]. A target productivity of at least 2.5g/L·h is required to be competitive with petrochemical production [Werpy and Petersen, 2004]. In addition to this production rate, purification stands as the largest expense of the succinic acid production process and is a major area for improvement [McKinlay et al., 2007].

Some sources suggest that separation and purification can account for up to 60% to 70% of production costs [Huh et al., 2006]. Based on these estimates of separation cost and the price of sugar at \$0.32 per kilogram as of December of 2010, a fermentation process with typical separation would have a total cost of over \$1.06 per kilogram with separation costs of at least \$0.74 per kilogram [Liu et al., 2008].

## ***2.7 Current Downstream Separation Methods***

Succinic acid production can be broken down into two major components, fermentation of carbohydrates to succinic acid and its separation and purification [Werpy and Petersen, 2004]. Most processes that use succinic acid as an input require it in its undissociated form, and this is where the majority of costs are incurred [Glassner and Datta, 1989, Werpy and Petersen, 2004]. The step of separation and purification poses the most challenges in the overall process due to the problems mentioned previously with fermentation, but it is also the best step to improve the economics of the process [Zeikus et al., 1999]. Product recovery, concentration, acidification and purification are the steps necessary to get succinic acid from succinate in the fermentation broth,

and currently, there is no single approach that meets all of these requirements [Jun et al., 2007]. There are a few methods that are currently in use at the laboratory scale that convert succinate to its undissociated form, remove impurities and increase the purity of the product. These include reactive extraction, ion exchange resins, electrodialysis, precipitation and nanofiltration [Lee et al., 2008].

Amine-based extraction is a method of reactive extraction that separates organic acids based on their  $pK_A$  values as it removes undissociated acids [Huh et al., 2006, Hong and Hong, 2005]. It is a promising method of separation because separation is possible *in-situ* at room temperature and pressure, so no pre-treatment is required for this method to function properly [Huh et al., 2004]. The focus of much literature in amine-based extraction is the use of tri-*n*-octylamine (ToA) because previous studies have shown that it extracts succinic acid very well [Huh et al., 2006]. Tri-*n*-octylamine for reactive extraction is toxic to cells, however. Because of this effect on cell growth and production, other methods of succinic acid extraction need to be investigated. There are additional steps that must take place to continue the process of separating and purifying succinic acid, such as vacuum distillation and crystallization, but given that the by-product organic acids have been removed, this step becomes easier, reaching a final purity of 99.8% with a yield of 73.1% [Lee et al., 2008, Bechthold et al., 2008].

Reactive extraction of succinate using tri-*n*-octylamine seemed to be the most promising separation method, a statement supported by Bechthold et al. (2008), but there are some problems that remain and would need to be addressed before further consideration is given to this method. The main factor is that the extraction process is sensitive to pH changes, so as the pH increases, there will be a decrease in the uptake in ToA [Jun et al., 2007]. Because only undissociated acids can be extracted using ToA, the pH needs to be kept low to ensure that acid is removed from solution [Huh et al., 2006]. Given that this separation method has no selectivity and removes acetic acid first, it should be considered more of a pre-treatment step rather than a process to remove succinic acid from solution. Further processing of fermentation broth is required after

acetic acid removal. Finally, the process of using amine-based extractants to remove succinic acid from the fermentation broth is relatively new and the possibility of long-term stability of this process needs to be studied to ensure there are no toxic or inhibitory effects on the cells, especially if extractant is used *in-situ* on an industrial scale [Bechthold et al., 2008]. Additional information is also required to determine the cost of this process on an industrial scale.

Ion-exchange resins have shown to be promising when used to separate lactic acid from fermentation broth [Patel et al., 2008]. Ion exchange technology involves using a resin that captures cations with an ionic resin. In the case of capturing lactate, a cationic resin is used, meaning that there is no need to alter the pH of the system to remove the molecule [Patel et al., 2008]. This method has also been suggested for use with succinic acid as a step prior to crystallization [Song et al., 2007]. There are, however, very limited research papers that discuss the use of ion-exchange resins with succinic acid purification. Datta et al. (1992) says that the use of a highly acidic ion exchange resin followed by a weak basic exchange resin can remove cations, anions and impurities, leaving behind a purified stream with low concentrations of nitrogenous impurities and sulphates. Using ion exchange resins would require a purification step to remove cells from the liquid and the selectivity of these resins is low, leading to additional purification steps after acid removal

Glassner and Datta (1989) suggested a conventional electrodialysis system to remove succinic acid from the fermentation broth after ultrafiltration. Electrodialysis is a process that incorporates ion exchange technology with membranes and electric potential difference to separate non-ionic or weakly ionic molecules from those that are ionized [Glassner and Datta, 1989]. In the fermentation broth, the dissociated succinate is ionic while other components, such as proteins, amino acids and carbohydrates, are either very weakly ionic or non-ionic. Electrodialysis targets the dissociated form of succinic acid and removes it while leaving behind other compounds. If electrodialysis is used while the fermentation is taking place, liquid from the fermentor could be run through the electrodialysis system and as the succinate ions are removed



the remaining fluid including cells can be recycled back to the fermentor [Lee et al., 2008]. Hong and Hong (2005) have stated that electrodialysis could be used in conjunction with other separation methods, notably amine-based extraction [Lee et al., 2008]. Succinate removal using ToA could be used as a pre-treatment, removing acetic acid before sending the succinate-rich stream onto the electrodialysis stack, improving the molar ratio of succinic acid to acetic acid [Lee et al., 2008].

Electrodialysis is a method that has significant potential, but there are also some shortcomings with using this system on an industrial scale. One of the most glaring problems is that electrodialysis requires energy to function. Given that energy costs are on the rise and electricity may come from non-renewable resources, it would seem counterintuitive to use this method since the intent of succinic acid production via fermentation is to reduce the use of fossil fuels and provide a more environmentally friendly process [Bechthold et al., 2008]. This is a sentiment also shared by Patel et al. (2008), pointing out that electrodialysis requires energy, while adding that fouling of electrodialysis membranes are potential downfalls with the process. The selectivity of electrodialysis is also an issue that raises concern since using this separation process, because acetate remains in the succinate-rich stream and still needs to be removed to achieve a high purity succinic acid product [Lee et al., 2008]. Glassner and Datta (1989) suggested the use of conventional electrodialysis, but then pointed out that the succinic acid purity was much lower than expected at 79.6% in addition to 19.9% of the acetic acid remaining from this process [Lee et al., 2008].

Precipitating succinic acid out of solution is a separation process that was first proposed by Datta et al. (1992). In this process, after the fermentation reaches completion, solids are centrifuged and separated out of the fermentation broth. This broth is then treated with calcium hydroxide, which creates calcium succinate; a solid that precipitates out of solution [Lee et al., 2008]. This solid is removed from the fermentation broth and washed three times with RO water to remove remnants from solution. In an acidification step, sulphuric acid is added to the solid,

which dissolves the calcium succinate and produces succinic acid. The calcium in solution reacts with sulphate to produce solid calcium sulphate, also known as gypsum [Datta et al., 1992]. This solid can be removed from the solution and the succinic acid, now dissolved in solution, can go on to be removed through other separation methods, such as vacuum distillation. This precipitation method can also take place *in-situ* through the addition of a calcium buffer where it helps maintain the pH of the system [Lee et al., 2008].

Precipitation appears to be the most common and simplest method for succinic acid separation, but it is also one of the most inefficient processes from an environmental and economic standpoint. During fermentation, the pH of the system is neutralized and buffered by the addition of chemicals such as lime, or calcium hydroxide. Then, when the slurry is treated to remove the succinic acid, large amounts of sulphuric acid are added to the solution, creating succinic acid from calcium succinate and generating calcium sulphate [Corona-Gonzalez et al., 2008, Davison et al., 2004]. Gypsum is unusable from this process as it can not be sold due to discolouration and smell [Kang and Chang, 2005]. Therefore, it must be disposed of in a landfill, which adds to the cost of separation. The amount of slurry and solid waste created from this process renders it unfit for commercial applications. Calcium succinate also needs to be washed after it is removed from the fermentor to ensure as few contaminants as possible are carried into the acidification step. This washing requires a large amount of water for the process as well, another environmental issue that makes precipitation an unappealing choice for succinic acid separation [Davison et al., 2004].

Nanofiltration is another relatively new method for separating out the different by-products of the fermentation broth from succinate. Kang and Chang (2005) studied how two different nanofiltration membranes separated the main products of glucose fermentation by *A. succinogenes*, including succinate, formate, and acetate. The results indicated that the filters had a very high retention of succinate, the only divalent ion product [Lee et al., 2008]. Tests were completed on individual components, with increasing numbers of components up to a quaternary

ion solution, and it was in this final test where the highest retention of succinate was seen [Lee et al., 2008]. This method can be improved upon as Kang and Chang (2005) suggested that the succinate retention and the passing of other ions could be improved by reducing the feed volume or increasing the area of the membrane. While nanofiltration shows promise there are still some aspects of the process that are not addressed, such as the price of membranes, membrane fouling and the application of this separation method in real fermentor broth. Tests in this study have only used simulated, abiotic fermentation medium and the impact of intermediate compounds such as fumarate and malate on separation is unknown.

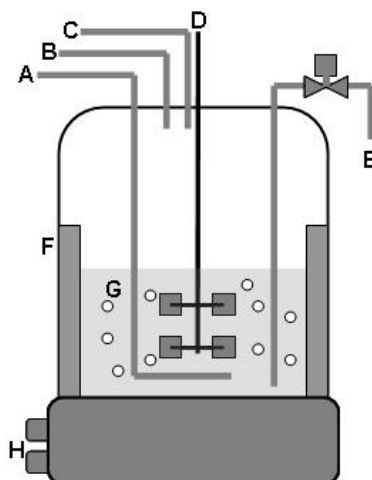
These five different separation methods each have positive aspects which can be compounded by combining steps to generate a higher succinic acid recovery than individual processes, but there are some drawbacks associated with them that require a new solution which reduces or eliminates the problems mentioned above. An ideal process is one that does not require removal or destruction of cells, requires a minimal amount of additional chemicals and can be done *in-situ*. A system that addresses all three of these needs is a Two-Phase Partitioning Bioreactor, or TPPB.

## **2.8 Two-Phase Partitioning Bioreactors**

A Two-Phase Partitioning Bioreactor is a bioreactor system that contains an aqueous phase in which a bioreaction takes place and a second phase which can either supply substrate to or remove products from the aqueous phase. This ensures the microorganisms used in the process are not inhibited by toxic levels of substrate or product while at the same time maximizing the amount of substrate present. When the process was first created and implemented, immiscible organic solvents were used as the sequestering phase, as tested for the extraction of ethanol from fermentation broth [Kollerup and Daugulis, 1985]. However, as certain solvents could potentially affect the organisms adversely, the selection process became troublesome [Amsden et al., 2003,

Morrish et al., 2008]. Certain criteria had to be met with regard to solvent choice, including biocompatibility, bioavailability, volatility and cost, among others [Amsden et al., 2003]

More recently, this solvent phase has been replaced by polymer beads, making TPPBs easier to operate because polymers are not bioavailable to microorganisms and can be separated more efficiently than a second liquid phase [Littlejohns and Daugulis, 2008]. In addition, the cost of the polymer is relatively low and the polymer can be reused after desorption of the target molecules, and this longevity helps keep costs low [Amsden et al., 2003]. A comparative study performed by Amsden et al. (2003) showed that polymer beads had no reduction in their performance, regardless of whether they were fresh beads or recycled beads that had been used in prior experiments. These results were confirmed in later research by Prpich and Daugulis (2007). Compared to solvents, polymer beads are already quite inexpensive given that a polymer like poly (ethylene-co-vinyl acetate), or EVA, costs \$3.40 per kilogram whereas the solvent 2-undecanone can cost up to \$80 per kilogram [Amsden et al., 2003]. Polymers can also be formed into many different shapes and sizes as the situation requires, adding to their versatility and the only change that occurs in this alteration is that of the diffusional length through the polymer [Prpich and Daugulis, 2004]. Polymers are also capable of absorbing chemicals with small molecular weights as observed in polymers used for drug delivery systems. Since the polymers are solid particles in an aqueous solution, separation of the polymer from the fermentation broth is much easier compared to removal of a second, immiscible phase and complete removal of the polymer is possible [Amsden et al., 2003]. A labelled schematic of a Two-Phase Partition Bioreactor making use of polymer beads is shown below in Figure 2-4.

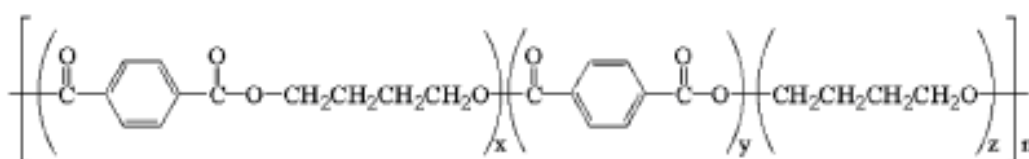


**Figure 2-4: Diagram of a TPPB system using polymer beads with aeration tube (A), acid input (B), base input (C), agitator with 2 Rushton impellers (D), sampling port (E), 4 steel baffles (F), polymers in fermentation broth (G) and heating/cooling jacket (H)**

TPPBs have been used for the degradation of toxic substances, such as benzene, toluene, ethylbenzene and *o*-xylene (BTEX) and phenol with excellent results, but these systems can also be used for the formation of important products [Littlejohns and Daugulis, 2008, Prpich and Daugulis, 2004]. Carvone is a flavour and fragrance compound that is used in a number of food products, and it was produced from carveol using a Two-Liquid-Phase Partitioning Bioreactor with an immiscible solvent as the second phase [Morrish et al., 2008]. This reaction was limited by end product inhibition as the accumulation of carvone became toxic to the cells [Morrish et al., 2008]. The use of a polymer phase instead of a second liquid phase increased the volumetric productivity by almost 4 fold [Morrish and Daugulis, 2008]. TPPB systems have also been used in the production of 2-phenylethanol and L-phenylacetylcarbinol to eliminate end product and substrate inhibition, respectively [Gao and Daugulis, 2009, Khan and Daugulis, 2010].

Initial research into polymer absorbance of succinic acid was performed by Gao and Daugulis (2010). In this study, various polymers were tested to determine what chemical features of the polymers led to succinic acid uptake. Various grades of Zytel<sup>®</sup>, a type of polyamide provided by DuPont Canada were tested because it was believed that hydrogen bonding

interactions between the polymer and succinic acid would lead to uptake. One polymer, Hytrel<sup>®</sup> 8206, yielded a partition coefficient at 1.3 which was the highest measured value in the study. Hytrel<sup>®</sup> is a block copolymer of polybutylene terephthalate, terephthalate and polytetramethylene glycol ether and its structure is shown below in Figure 2-5. The figure indicates that the second block in the copolymer is terephthalate but in the case of 8206, it is believed that this block is actually isophthalate, leading to a decrease in crystallinity and greater uptake compared to other Hytrel<sup>®</sup> grades.



**Figure 2-5: Chemical structure of Hytrel<sup>®</sup> showing terephthalate as the second block instead of isophthalate as in the structure of 8206**

All Zytel<sup>®</sup> polymer grades showed no uptake of succinic acid due to the high crystallinity of the polymers and correspondingly high glass transition temperatures of 70°C [Gao and Daugulis, 2010]. The hydrogen bonding sites within the polyamides were likely internally bound, limiting the number available for succinic acid uptake. The recommendations from Gao and Daugulis (2010) were to use more amorphous polymers with lower T<sub>g</sub> values as well as bulk polymers with chemically similar functional groups grafted to their backbone. In particular, the use of polymers containing maleic anhydride was suggested as it is a precursor to succinic acid through chemical synthesis.

Two-Phase Partitioning Bioreactors are ideal for reactions that are end product limited because they can remove final products from the fermentation broth as soon as they are generated, ensuring that the reaction rate does not decrease as the reaction proceeds. In the case of succinic acid production, the pH of fermentation is well above the pK<sub>A</sub> values of succinic acid, so it exists in solution as succinate. Interaction with the polymer is pH dependent and requires that the succinic acid be in its undissociated form or else it cannot be absorbed by the polymer

[Davison et al., 2004]. This difference between operating pH and polymer absorbance pH means that succinic acid cannot be removed as it is produced and, instead, a separate operational step is required to lower the pH of the system below the  $pK_{A2}$  of succinic acid. As mentioned earlier, pH adjustment has been shown to remove succinic acid from solution with the use of CaOH and  $H_2SO_4$ , but the waste generated makes this separation method infeasible. An ideal method to adjust the pH would be temporary, reversible and generate little to no waste. This can be accomplished through the use of carbon dioxide gas dissolved in solution to produce carbonic acid.

## **2.9 Polymer-Solute Interactions**

As all recent TPPB projects in the Daugulis research group make use of polymers for ISPR or substrate delivery, an understanding of the interaction between polymers and target molecules is required. Diffusion of molecules into the polymer phase is governed by the chemical potential gradient and, in many cases, the concentration gradient, as well. As changes in concentration can be measured and chemical potential cannot, concentration gradients are used to measure diffusion in to the polymer phase. This difference in potential is affected by the nature of the polymer, solute and the interaction between the two. An interaction parameter, used in Flory-Huggins solution theory and the Benesi-Hildebrand method for determining equilibrium constants, assuming regular solutions, is shown in Equation 2-1 below.

$$\chi_{12} = \frac{V_1(\delta_1 - \delta_2)}{RT} = \frac{z\Delta\omega}{kT} \quad (2-1)$$

In Equation 2-1, the most important variables are the solubility parameters,  $\delta_1$  and  $\delta_2$ , which are a representation of the energy density of a material. A polymer will absorb only target molecules which share similar energy densities. In the equation above, polymers will uptake

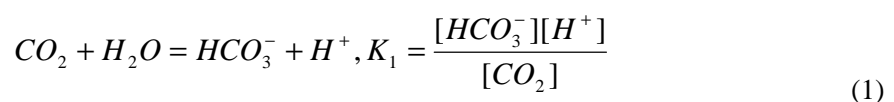
target molecules when the difference between  $\delta_1$  and  $\delta_2$  is less than  $2.045\text{MPa}^{1/2}$ . The requirement for similar energy densities comes from the process of diffusion itself. For a molecule to move into the polymer phase, the molecule must break the bonds it has in the solution and the polymer needs to effectively make space within itself for the target molecule to occupy and form new bonds. This movement of the polymer chain to create space for the solute occurs through random thermal motion within the polymer. In the case of succinic acid, this requires breaking the interactions between the molecule and water so it can move into the polymer.

As the target molecule for a given process is fixed, increasing the uptake of a target molecule into the polymer require alterations of the polymer. In the case of diffusion of a solute into a polymer, the target molecule will take the path of least resistance to internally bond with the polymer. Decreasing the resistance of diffusion through the polymer occurs through changing the crystallinity of the polymer, either by increasing the number of amorphous regions within the polymer or by selecting a polymer with a glass transition temperature below the operational temperature range of the bioreactor. In a block copolymer, for example, increasing the fraction of amorphous monomer will disrupt the crystallinity, seen as interactions of the rigid sections, and give a softer polymer with a lower  $T_g$ . Alteration of the  $T_g$  for a polymer can occur by incorporating larger pendant groups on polymers which hinder alignment of segments of the polymer chain and increase the flexibility of the polymer structure. The incorporation of plasticizers such as phthalates can also lower the  $T_g$  by interfering with intermolecular bonding within the crystalline regions of the polymer. As the  $T_g$  of a polymer decreases, molecules are more likely to leave the liquid phase provided that the monomers that make up the polymer share similar solubility parameters.



## 2.10 Carbon Dioxide and Carbonic Acid

Carbon dioxide is a key component in the production of succinic acid as it is fixed to phospho-enol-pyruvate (PEP) to create a four-carbon molecule. It is also a critical component in producing the feedstocks that will be used in biotransformation, adding more to the environmental benefits of bioproduction. The enzyme responsible for the reaction to produce oxaloacetate from PEP is PEP carboxykinase, which is strongly regulated by the amount of CO<sub>2</sub> in the system [Song et al., 2007]. Production levels increase as carbon dioxide is added to saturation in the system as it can also act as an electron acceptor [Song et al., 2007]. The addition of carbon dioxide for succinic acid production has been equivalently performed with the addition of MgCO<sub>3</sub> which can also act as a buffer for the pH, but this has the risk of inhibiting production and cell growth with the accumulation of magnesium salts [Lin et al., 2007, Samuelov et al., 1991]. Because of the risk of salt inhibition from the use of carbonates in the reactor and due to the fact that this reaction is carried out anaerobically, it is better to supply carbon dioxide gas to the system. When carbon dioxide is dissolved in water, it forms carbonic acid, which dissociates and can lower the pH of the system. When carbon dioxide is dissolved in a liquid phase it forms carbonic acid and dissociates through the reaction shown in Equation 1 [Song et al., 2007].



In the above equation, carbonic acid (H<sub>2</sub>CO<sub>3</sub>) dissociated once, contributing a proton to the solution. Carbonic acid is a diacid, but the equilibrium coefficient is such a small value for the second dissociation of carbonic acid that the effect on pH is negligible. The reaction between carbon dioxide and water and the dissociation of carbonic acid are reversible through the removal of the CO<sub>2</sub> headspace. As an example, if the carbon dioxide headspace of a reactor is replaced with nitrogen, the carbonic acid reverts back to water and dissolved CO<sub>2</sub>, the latter of which

desorbs from solution. It is possible with Henry's Law and work done by Song et al. (2007) to accurately predict the pH of a system given the pressure of CO<sub>2</sub> in the gas phase. Through calculations, a target pH can be attained by altering the pressure of the gas phase in the reactor.

The novel idea proposed in this work is to use CO<sub>2</sub> in the reactor to help lower the pH and protonate succinic acid, allowing it to be removed from the aqueous phase. If the pH of the system cannot be lowered with carbon dioxide at atmospheric conditions to below the lower pK<sub>A</sub> of succinic acid, pressurized CO<sub>2</sub> may be required. This inhibition of CO<sub>2</sub> at 1atm may be due to the buffering effects of the growth medium or products in their dissociated form. A pH just below the pK<sub>A2</sub> value of succinic acid can be set with a specific pressure to allow for succinic acid uptake. By lowering the pH with carbonic acid, no harsh chemicals such as sulphuric acid are required to change the pH and generate undissociated succinic acid. Not having to use acid for pH adjustment will lower the cost of materials needed for the process as well as reduce the amount of waste products created in the process. The use of carbon dioxide will become a large focus of the work done in this project, because if it is feasible without having an adverse effect on the bacteria, succinic acid can be removed from solution with a decrease in chemicals, additional energy and processing steps. It will help by lowering the pH of the solution, which will protonate succinate and give succinic acid to be removed from solution, reducing end product inhibition. It will also eliminate the need for complex separation steps such as electrodialysis or amine-based extraction.

## **2.11 Scope of Project**

The overall goal of this project is to use a bioreactor system with *Actinobacillus succinogenes* to generate succinic acid to the point of end product inhibition, remove this product using polymer beads, allowing continued bioproduction. Product removal will take place by adjusting the pH of the system below the pK<sub>A2</sub> of succinic acid using carbon dioxide gas, protonating the succinate molecule for uptake into the polymer. Once absorption is finished and

the polymers are removed from the system, the carbon dioxide gas will be replaced with nitrogen so the pH of the system is raised to the operating pH of the bioreactor, allowing bioproduction to continue without end product inhibition.

The first phase of this research will examine the physical aspects of the system, with separate focus on how carbon dioxide affects the pH of the system as well as which polymers show the highest absorbance of succinic acid. The tests with carbon dioxide will determine if it can be used to lower the pH below the  $pK_{A2}$  of succinic acid and how temperature and agitation rate affect this pH change. These tests will be conducted in water and growth medium to see how the presence of nutrients affects pH adjustment. If the growth medium impedes carbon dioxide lowering the pH below 4.2, then an alternative growth medium is required. This will not consist of experiments in the lab, but rather determination of a growth medium will be from a compilation of the various mediums currently in use in literature. The growth medium chosen may need modification if results show that the salts used, which may contain many buffers, adversely affect the ability of carbon dioxide to alter the pH of the system.

Experiments will also be carried out to determine which polymer will allow for the greatest uptake of succinic acid from the system. Results from Gao and Daugulis (2010) has examined what factors led to an effective polymer for succinic acid absorption, but will be expanded upon with this research. Because pH changes the protonation of succinic acid, tests be performed to see how changes in the pH will change polymer uptake and as well as the effect of nutrients on succinic acid absorption.

The second phase of this research will focus on the biology of the project. The first test will find how the length of exposure to a low pH affects growth of the organism. The maximum exposure length the cells can withstand and survive will give a maximum time frame for succinic acid absorption into the polymer phase. Next, a single phase biotransformation of succinic acid will take place to produce a high product concentration in the shortest time possible. This single-phase run will be optimized in terms of growth medium, agitation and pH control and the

optimized run will serve as a benchmark against which two-phase bioreactor runs will be compared. A system will be designed for contacting the polymer beads with the fermentation broth at a low pH while ensuring that the beads are easily removable from solution to allow bioproduction to continue. This system will be used with the results from the low pH tolerance test of the cells to produce and remove succinic acid from solution, allowing the system to continue producing product. Desorption tests will determine how much water is required to completely wash succinic acid from solution and how many washes are required.

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## **3.0 The use of carbon dioxide for pH adjustment in a Two-Phase Partitioning Bioreactor and the effect of pH on succinic acid absorption in polymer materials**

### **3.1 Preface**

Based on the information presented in Chapter 2, bioproduction of succinic acid is preferred to chemical synthesis because it uses renewable resources and the reaction does not require high temperatures or expensive catalysts. A number of bacterial strains exist which can produce succinic acid, but *Actinobacillus succinogenes* has been chosen for this research as it is the most robust and, therefore, most likely microorganism to be used in industrial-scale bioproduction. The major problem with bioproduction is the separation process. There is currently no separation method that can be applied on an industrial scale without excess waste generation or high energy requirements. Another hindrance to industrial-scale bioproduction of succinic acid is end product inhibition, a limitation that can be reduced through a system incorporating *in-situ* product removal. Two-Phase Partitioning Bioreactors (TPPBs) can separate succinic acid from solution and allow bioproduction to continue without end product inhibition.

While using a TPPB system to absorb succinic acid is an effective way to improve separation, there are interactions between polymers, succinic acid and pH that must be characterized and understood before this process can be implemented in a bioreactor. Carbon dioxide gas will be used to lower the pH of the liquid below the  $pK_{A2}$  of succinic acid, but the effect of agitation, temperature and presence of growth medium must be determined to elucidate their effect on pH adjustment. No project in the Daugulis lab to date has been this complicated by the need to change the pH of the system to effect product removal. A polymer must be chosen for use in the system which can effectively absorb succinic acid. While polymer selection is part of any TPPB protocol, the hydrophilic nature of succinic acid complicates this selection process as all other projects in the Daugulis group have dealt with the removal of generally hydrophobic compounds. Once a polymer is chosen, tests must determine how the pH of the solution affects

uptake. While the effect of pH on succinic acid absorption will be tested initially in water, the bioreactor will have growth medium, cells and products, so tests on these materials must also be undertaken. Chapter 3 hopes to develop an understanding of pH adjustment using carbon dioxide and how polymer uptake of succinic acid will be affected by growth medium components and the pH of solution.

### **3.2 Abstract**

Succinic acid is a 1,4-diacid that can act as a precursor for pharmaceuticals and detergents as well as commodity chemicals, including polyesters. Currently, no commercial bioproduction process exists for succinic acid because of low volumetric productivity from end product inhibition. A Two-Phase Partitioning Bioreactor can reduce end product inhibition through *in-situ* product removal although the pH needs to be lowered for polymer uptake to occur. Additionally, the pH of the system will affect uptake, so its impact was also studied.

A study was conducted to determine whether the bioreactor pH could be lowered to the  $pK_{A2}$  of succinic acid with carbon dioxide gas and raised back to the operational pH with nitrogen gas stripping. The effects of temperature, agitation rate and growth medium on pH adjustment were also tested. The salts of the growth medium and products were tested individually to determine which one(s) buffered the pH. Screening of 50 polymers found only one which had a partition coefficient of 1.3 for succinic acid uptake, Hytrel<sup>®</sup> 8206. The partition coefficient was also tested against changes in initial pH and to determine the impact of growth medium on the partition coefficient.

While carbon dioxide reduced the pH of RO water to 3.8, pH reduction was limited to 4.75 in a dilute medium solution. The succinate ion had the greatest negative effect on pH adjustment. No succinic acid uptake occurred above the  $pK_{A2}$  and the only change to uptake in

growth medium was an increase in sample equilibrium pH, indicating stronger pH control is required during absorbance.

Keywords: Succinic acid, carbon dioxide, Two-Phase Partitioning Bioreactor (TPPB), *in-situ* product removal (ISPR)

### **3.3 Introduction**

A report released by the US Department of Energy outlined twelve value-added chemicals which can be produced from biomass, one of which was succinic acid [Werpy and Petersen, 2004]. Succinic acid is a 1,4-diacid which can be used as a derivative for specialty chemicals in the food and pharmaceutical industry as well as commodity chemicals including  $\gamma$ -butyrolactone and 1,4-butanediol [Zeikus et al., 1999]. Current chemical annual production of succinic acid is approximately 16,000 tons [Sauer et al, 2009]. Chemical synthesis begins with n-butane converted to maleic anhydride, which is then hydrated and hydrogenated to produce succinic acid [Kurzrock and Weuster-Botz, 2010]. The conversion of n-butane to succinic acid is a chemical synthesis process which consumes petroleum and requires both a high temperature and expensive catalysts for the reaction [Li et al., 2010].

Bioproduction is becoming a more favourable process as microorganisms assume the role of catalysts for reactions. Biotransformations take place at 37°C and neutral pH conditions, greatly reducing energy inputs compared to chemical synthesis [Sheldon and van Rantwijk, 2004]. The added benefit of biochemical production is that a compound produced naturally without the risk of contamination from harsh chemicals can be used in industries which require high purity such as pharmaceuticals [Song and Lee, 2006]. Additionally, consumers are developing a preference for naturally produced products, so biological production will increase [Gao and Daugulis, 2009].

While a process to create succinic acid from renewable resources has been established and is preferred to chemical synthesis, no commercial process exists. Due to end product inhibition, the current volumetric productivity of bioproduction is lower than the target set by the US Department of Energy as the minimum value required for an industrially viable process [Werpy and Petersen, 2004]. Additionally, the separation process for succinic acid production can account for as much as 70% of the overall costs, which has prevented the process from becoming commercially realized [Huh et al., 2006]. Current separation methods include precipitation, electro dialysis, crystallization by temperature-based and reactive extraction, each of which are not without limitations. Precipitation produces large volumes of waste and uses many additional chemicals [Corona-Gonzalez et al., 2008]. Electro dialysis and temperature crystallization are energy intensive in terms of electricity or cooling and reactive extraction has a low selectivity for succinic acid [Bechthold et al., 2008, Huh et al., 2006]. A new production and separation method is required if commercial bioproduction of succinic acid is to be realized.

Two-Phase Partitioning Bioreactors (TPPBs) have been used in the synthesis of specialty chemicals including carvone and L-phenylacetylcarbinol (L-PAC) [Morrish et al., 2008, Khan and Daugulis, 2010]. A TPPB system increases volumetric productivity over single-phase bioproduction by using a second, immiscible phase to remove target molecules and eliminate end product inhibition. Extending the use of TPPB systems to succinic acid production will allow for more efficient downstream processing of the compound without the need for extra energy and chemical inputs compared to current separation methods. Carbon dioxide gas must be supplied to the system for anaerobic growth, but can also be used as a source of carbonic acid to lower the pH of the system. pH adjustment is critical for succinic acid absorption as polymers will only remove the molecule in its undissociated form [Gao and Daugulis, 2010].

If carbon dioxide is used to lower the pH of a bioreactor below the  $pK_{A2}$  of succinic acid, the effects of temperature, agitation and growth medium must be determined. Polymer selection will be critical as a polymer must be found to remove succinic acid, a very hydrophilic

compound. Previous Daugulis group research focused on hydrophobic compounds including phenol and polycyclic aromatic hydrocarbons (PAHs) [Rehmann and Daugulis, 2006, Rehmann and Daugulis, 2007]. Polymer uptake of succinic acid is also affected greatly by the pH of solution as this can change the ratio of the undissociated acid to its dissociated anion. An understanding of how various pH levels on polymers absorbing succinic acid will be developed.

### **3.4 Materials and Methods**

#### **3.4.1 Chemicals and Polymers**

Unless otherwise specified, all chemicals were purchased from Fisher Scientific Company, Ltd. (Ottawa, ON) or Sigma-Aldrich Canada, Ltd. (Oakville, ON). Monosodium succinate was purchased from Tokyo Kasei Kogyo Company, Ltd. (Tokyo, Japan). Medical grade carbon dioxide and ultra-high purity nitrogen were supplied by Linde Canada, Ltd. (Kingston, ON). Polymer samples were all of commercial grade and donated by various companies as listed in Table 3-1. In general, polymers were in the shapes of spheres or ellipsoids with diameters of approximately 3mm to 5mm. Prior to use, polymers were washed three times with hot tap water on a stir plate and twice with RO water. After a final, overnight wash in RO water, they were allowed to air dry.

**Table 3-1: General information for polymers tested for succinic acid absorption**

Trade Name	Grade	Source	T <sub>g</sub> (°C)	Structure
Co-acrylic acid	N/A	Sigma- Aldrich	102	poly(ethylene-co-acrylic acid)
Co-methacrylic acid	N/A	Sigma- Aldrich	61	poly(ethylene-co-methacrylic acid)
Desmopan	453, 9370A	Bayer Canada	-33.9, -70	Polyester-based thermoplastic polyurethane
Elvax	40W, 650Q, 770	DuPont Canada	N/A	ethylene-vinyl acetate copolymer
Engage	8003, 8100, 8200, 8407, 8450, 8480, 8842	Down Chemical	-46, -52, -53, -54, -32, -31, -58	Ethylene octane copolymer
EVAL	27%, 38%, 44%	Sigma- Aldrich	46, -30, -45	Ethylene vinyl acetate
Eval	H-101BD, LC-E151B	EVAL America	62, 55	Ethylene vinyl alcohol
Exxelor	PP1042	Exxon Mobil	N/A	MA modified polypropylene
	PP-g-MA	Exxon Mobil	N/A	Polypropylene
	N416D	DuPont Canada	N/A	MA modified nylon
Fusabond	C190D, C250D	DuPont Canada	N/A	MA modified ethylene vinyl acetate
Hytrel	3548, 4078, 5544, 6108, 8206, 8238, 8532	DuPont Canada	-45, N/A, -55, N/A, -59, -50, N/A	PBT and Polyether block copolymer
Kraton	6175M, D1102K	Kraton Polymers	-80, -80	styrene and butadiene triblock copolymer
Makrolon	2658	Bayer	145	Polycarbonate
Nucrel	925	DuPont Canada	N/A	Ethylene and Methacrylic acid copolymer
Nylon	50DB 66, 6-6	N/A	47, 60	Polyamide
PD	702, 1274	LyondellBasell	N/A	Polypropylene homopolymer
PEBAX	2533, 7033	Arkema Group	-65, -65	Polyether block amide copolymer
Polyethylene	LDPE, LLDPE, HDPE	Sigma- Aldrich	-110, -70, -110	Various densities of polyethylene
Pro FAX	PF611, Isotactic, 6253	LyondellBasell	N/A	Polypropylene
SBR Pillows	N/A	N/A	-55	Styrene butadiene rubber
Si Rubber	N/A	N/A	-127	Silicone rubber

### 3.4.2 Analytical Methods

Succinic acid concentrations were measured using High-Performance Liquid Chromatography (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 x 7.7 mm) at 60°C with a 10mM H<sub>2</sub>SO<sub>4</sub> mobile phase at 0.7mL/min. Absorbance was measured in a UV-Vis detector (Varian Prostar, PS325) at 220nm. Additionally, a UV spectrophotometer was used (Ultrospec 3000, Pharmacia Biotech, Sweden) at 205nm with quartz cuvettes as a secondary method of measuring liquid concentrations of succinic acid.

### 3.4.3 Temporary pH Shifting using Carbon Dioxide and Nitrogen

Tests to determine the effect of bioreactor agitation and temperature on pH shifting were carried out in a 5L BioFlo III reactor (New Brunswick Scientific, Edison, NJ) with a 3L working volume. Two Rushton turbine impellers were fixed to the agitator shaft above a perforated steel tube which acted as the gas sparger. Experiments were conducted at three temperatures from 10°C to 20°C and at each temperature the agitation rate was varied from 200rpm to 800rpm, a typical range of agitation for lab-scale bioreactors. pH was measured using the reactor's pH probe and tracked using TracerDAQ data acquisition software (MicroDAQ.com, Ltd., Contoocook, NH). CO<sub>2</sub> and N<sub>2</sub> were sparged into the system at 1.6vvm. The pressure of the system remained at atmospheric conditions and tests were first performed in RO water. The tests were repeated using the following growth medium (in g/L): yeast extract, 5; glucose, 55; NaHCO<sub>3</sub>, 12.6; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 8.5; K<sub>2</sub>HPO<sub>4</sub>, 5.5; NH<sub>4</sub>Cl, 2; NaCl, 1. 1mL/L of a trace elements mix was also added which contained the following compounds (in mg/L): nitrilotriacetic acid, 3 000; MgSO<sub>4</sub>·H<sub>2</sub>O, 3 000; MnSO<sub>4</sub>·H<sub>2</sub>O, 500; FeSO<sub>4</sub>·7H<sub>2</sub>O, 100; CaCl<sub>2</sub>·2H<sub>2</sub>O, 100; CoCl<sub>2</sub>·6H<sub>2</sub>O, 100; Na<sub>2</sub>MoO<sub>4</sub>, 25; NiCl<sub>2</sub>·6H<sub>2</sub>O, 25; Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O, 25; ZnCl<sub>2</sub>, 13; CuSO<sub>4</sub>·5H<sub>2</sub>O, 10; AlK(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O, 10; H<sub>3</sub>BO<sub>3</sub>, 10; NaSeO<sub>3</sub>, 10. This medium, referred to as the original medium, was created from a compilation of several growth media since there seemed to be no standard in previous research. A second growth medium, referred to as the minimized medium, was created by decreasing the mass of buffers present to facilitate the pH adjustment in the system using CO<sub>2</sub>. It contained the following components (in g/L): yeast extract, 5; glucose, 55; NH<sub>4</sub>Cl, 2; NaCl, 1; K<sub>2</sub>HPO<sub>4</sub>, 0.5 as well as 1 mL/L of the mineral mix shown above. This growth medium was also tested using the same methods as tests in RO water and the original growth medium. The test started by reaching the set temperature and agitation. The pH was tracked for 60 seconds prior to sparging to show initial pH conditions, then CO<sub>2</sub> gas was sparged into the system. After



equilibrium conditions were reached, the gas was switched to nitrogen and left sparging for up to 20 minutes, long enough for the pH to return to its original level.

A second set of tests was performed to determine which individual growth medium components affected lowering of the pH by CO<sub>2</sub>. Samples containing only one of each of the growth medium components were prepared in 100mL of RO water in a 250 mL Erlenmeyer flask. These salts in solution were at the same concentrations listed above in both the original and minimal growth medium. CO<sub>2</sub> was sparged into the samples through a 27ga. needle and the pH change was monitored. Samples were left sparging until no pH change was seen for 10 minutes. In addition to these tests on growth medium, salts of the bioreaction products were subjected to the same testing in the following concentrations (in g/L): monosodium succinate, 35.58; disodium succinate, 41.16; ammonium formate, 6.85; sodium acetate, 13.63. These concentrations were used to give succinate, formate and acetate concentrations of 30, 5, and 10g/L, respectively. The target concentrations were chosen as they are representative of the lower range of concentrations attained in bioreactor runs from other researchers [Liu et al., 2008a, Liu et al., 2008b].

### **3.4.4 Partition Coefficient Testing**

Samples for partition coefficient testing were prepared by creating a solution of 20g/L of succinic acid in RO water and placing 10mL into 20mL scintillation vials. Polymer beads were weighed and put into these vials to give polymer fractions ranging from 5% to 25% (w/v) for initial polymer screening and 15% to 90% for all subsequent tests. The pH was adjusted up using 5M KOH and down using 5M H<sub>2</sub>SO<sub>4</sub> for tests of the partition coefficient at different pH values from 2.2 to 6.2, a range of 2 full pH units above and below the pK<sub>A2</sub>. Tests involving growth medium contained the minimal medium as outlined above. Scintillation vials were sealed and placed in an open air shaker overnight at room temperature and the liquid concentration was then measured, allowing for calculation of the succinic acid in the polymer. Based on the equation

used by Rehmann and Daugulis (2006), the partition coefficient was calculated as the ratio of polymer phase concentration and liquid phase concentration, as shown below in Equation 3-1.

$$K_{S/W} = \frac{S_{sol.}}{S_{aq.}} \quad (3-1)$$

The partition coefficient in this form is a simplification of the equation of Gibb's free energy describing a mixture of chemicals and their chemical potentials. Equation 3-2 shows the simplified form of this equation relating the standard free energy change for the reaction to the equilibrium constant. Isolating for the equilibrium constant and replacing the activities with the concentration and activity coefficient gives Equation 3-3, assuming a hypothetical reaction of  $\alpha A + \beta B \leftrightarrow \sigma S + \tau T$ . While this equation shows the true equilibrium constant as it includes activities, the Equation 3-1 presented above is a more practical calculation and is suitable for this research.

$$\Delta_r G^\theta = -RT \ln K \quad (3-2)$$

$$K = \frac{[S]^\sigma [T]^\tau}{[A]^\alpha [B]^\beta} \times \frac{\gamma_S^\sigma \gamma_T^\tau}{\gamma_A^\alpha \gamma_B^\beta} \quad (3-3)$$

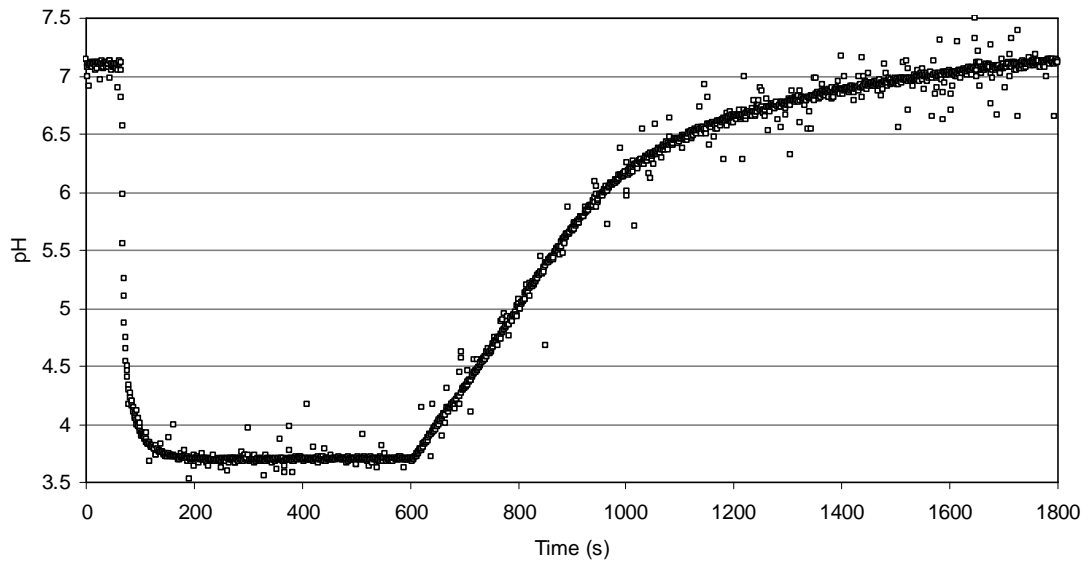
$$K = \frac{[S]^\sigma [T]^\tau}{[A]^\alpha [B]^\beta} \times \frac{\gamma_S^\sigma \gamma_T^\tau}{\gamma_A^\alpha \gamma_B^\beta} \quad (3-3)$$

## **3.5 Results and Discussion**

### **3.5.1 pH Adjustment using Carbon Dioxide**

With the flowrate of CO<sub>2</sub> into the reactor at 1.6vvm, the pH of the system was reduced to approximately 3.8 in less than 2 minutes, as shown in Figure 3-1. This lower limit was reached with every RO water test, though sample groups varied slightly with temperature. As the

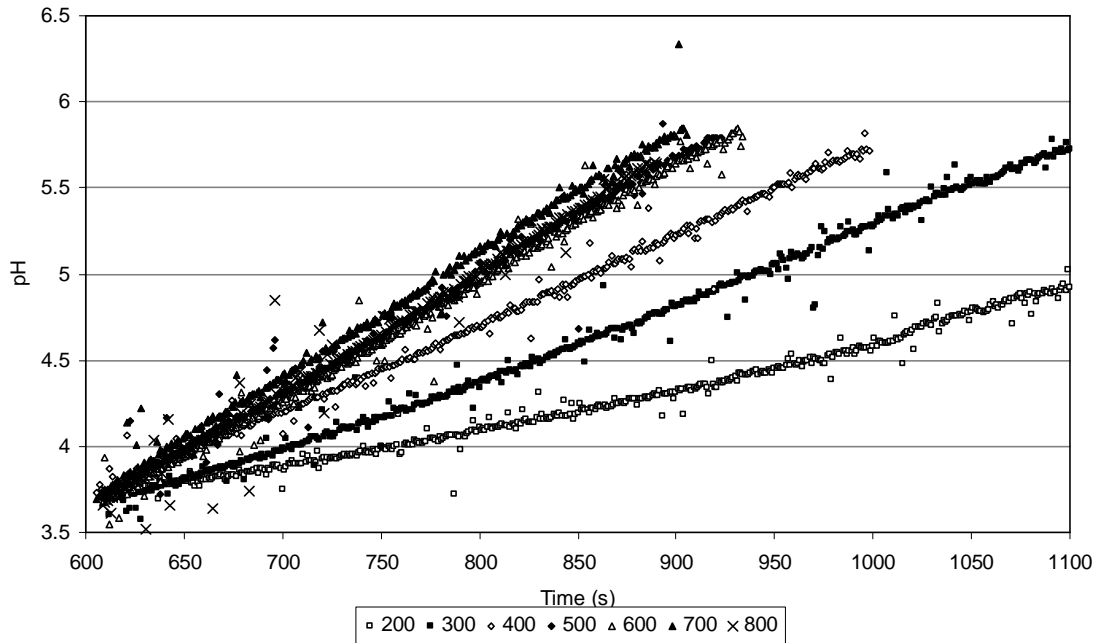
temperature was decreased from 20°C to 10°C, the lower limit on pH decreased slightly from 3.85 to 3.75 due to the slight changes in CO<sub>2</sub> solubility at lower temperatures.



**Figure 3-1: pH change in RO water using CO<sub>2</sub> and nitrogen, 15°C, 500rpm**

The gases were sparged in from the bottom of the bioreactor and allowed to bubble through the liquid before reaching the headspace of the system. CO<sub>2</sub> is denser than other components of air and the gas filled the headspace after bubbling through the liquid with excess venting through an open port in the headplate. At the gas-liquid interface as the bubbles passed through the liquid in the bioreactor, the gas was pure carbon dioxide and equilibrium was established quickly. As the CO<sub>2</sub> dissolved into solution, it reacted with water to form carbonic acid, which dissociated and caused the pH to drop. Because the  $K_{A2}$  for carbonic acid is  $4.69 \times 10^{-11}$ , there was no appreciable change in pH from the second proton dissociating. Given the rapid change to pH 3.8, the pH of the system was raised using nitrogen.

Nitrogen sparging raised the pH of the system, although the time to reach the starting pH was much longer, as seen in Figure 3-1. The rate of pH increase with the addition of nitrogen gas changed with the agitation rate of the system, visible in Figure 3-2.



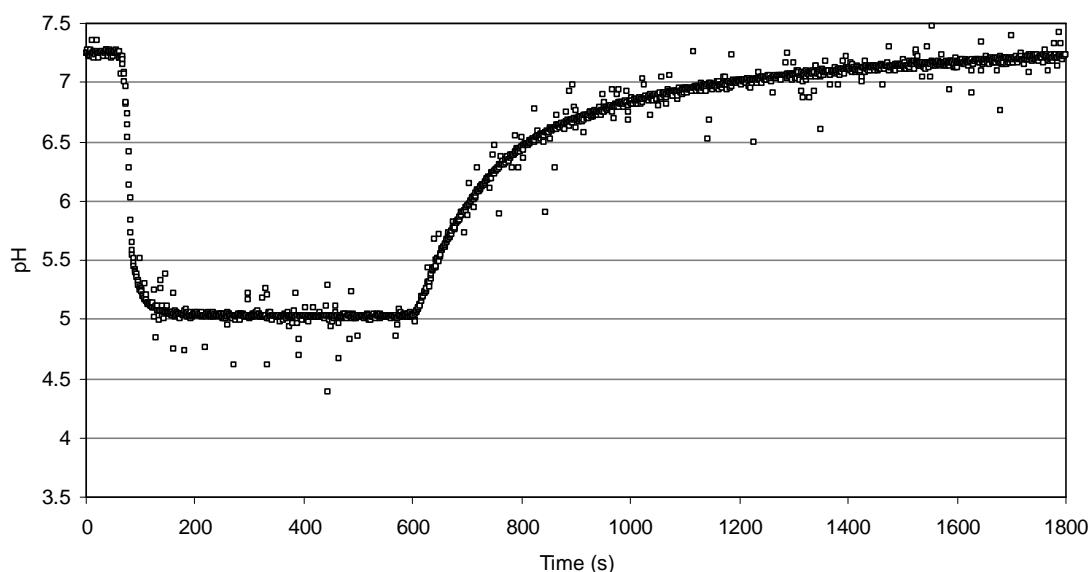
**Figure 3-2: The effect of agitation rate on increasing the pH of the system using N<sub>2</sub> gas**

This figure shows only the linear portion of the curve, but the test was continued until the system approached the initial pH of approximately 7.0. The linear portion of these graphs was used because the slopes of these lines could be used as a method of comparison for the effect of agitation rate on pH increase while sparging nitrogen gas. As the agitation rate increased, the rate of change increased up to 500rpm where the pH rate of change reached a plateau. Regression performed on the linear portion of these curves confirmed that above 500rpm there was no difference in how fast the pH increased. The pH increase rate at 500rpm and above was more than double that at 200rpm (rates not shown). The trends shown in Figure 3-2 were similar in all test groups regardless of temperature.

The difference in rate of pH change between carbon dioxide and nitrogen gas was due to the difference in solubility between the two gases and the effect of agitation on the gas-liquid interface in the bioreactor. When the sparging gas was changed to nitrogen it began to bubble through the liquid and reach the headspace, where it displaced the carbon dioxide gas that had filled the headspace. The nitrogen gas in the bioreactor began to strip carbon dioxide from the liquid, causing the pH to increase, but the solubility of nitrogen in water, 0.019g/kg at 20°C, is lower than CO<sub>2</sub>, 1.7g/kg at 20°C. The removal of carbon dioxide was slower because of this difference in solubility, which led to a longer time to increase the pH of the system. As the agitation rate increased, the shear force of the impeller caused the nitrogen from the aerator to form smaller bubbles, allowing for faster dissolution into the liquid, causing the pH to rise faster. Above 500rpm this shear effect peaked and the diffusion of nitrogen into the liquid became the rate limiting factor.

From these experiments it was concluded that it was possible to quickly decrease the pH of liquid to below the pK<sub>A2</sub> of succinic acid using carbon dioxide gas in the bioreactor. When using CO<sub>2</sub> and nitrogen gas to adjust the pH of the system, an agitation rate of 500rpm to 800rpm allowed for fastest pH change to the system. 500rpm was used as this agitation rate would use the least amount of energy while still achieving the same effect as a higher rate. Using this set of tests as a 'best case scenario' for adjusting the pH of a bioreactor, focus shifted to the growth medium proposed for this process and its impact on the pH adjustment process.

As different temperatures were not a large factor in the rate of pH change, tests across temperature were not made when using both the original and minimal growth media. Figure 3-3 shows the effects of CO<sub>2</sub> on water with growth medium present under the same conditions as Figure 3-1.



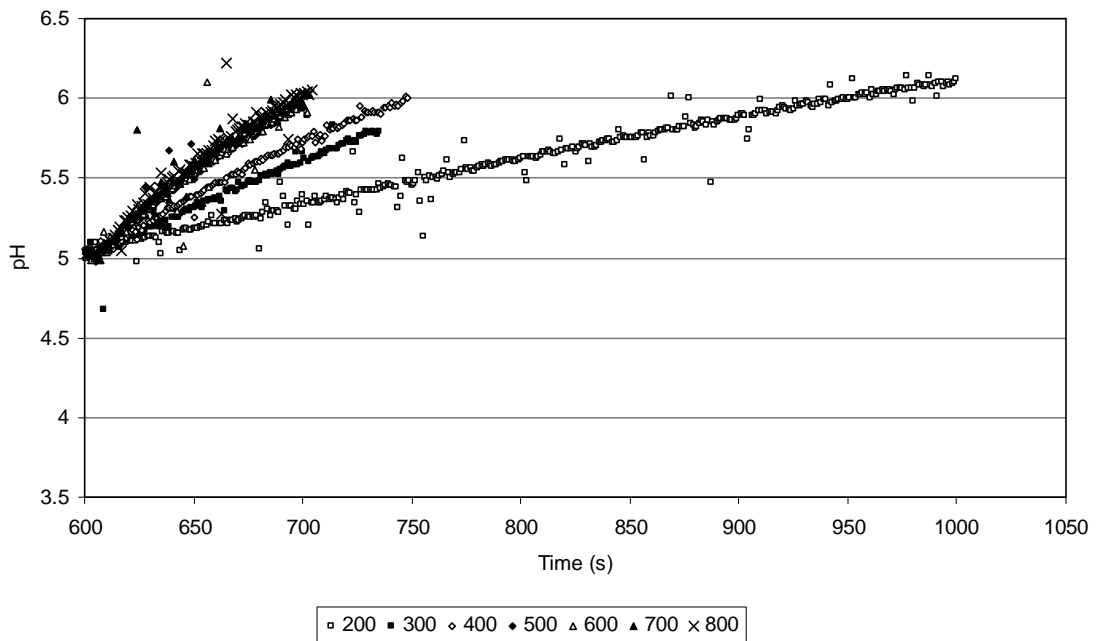
**Figure 3-3: pH change in growth medium using CO<sub>2</sub> and nitrogen, 20°C, 500rpm**

Figure 3-3 shows the lower limit of pH was only 5.0 compared to the previous value of 3.8. The pH of the system still decreased quickly as with tests in RO water, but the increase in pH when sparging with nitrogen differed considerably, as confirmed by observing the slopes shown in Figure 3-4. At 200rpm, the system responded almost identically to tests performed with RO water, but all other agitation rates showed faster pH increases. In particular, tests in the range of 500rpm to 800rpm showed an almost 66% increase in the pH rate of change with nitrogen sparging in medium. In biotransformations when the pH needs to be adjusted back to operating conditions, the faster increase in pH means the cells are exposed to a low pH for a shorter period of time.

The inability of the carbon dioxide gas to decrease the pH to the previous lower limit was due to the addition of buffering salts in the growth medium. In water without growth medium, as CO<sub>2</sub> dissolved in solution it reacted with the water molecules and formed carbonic acid, a diacid which immediately dissociates once and lowers the pH leaving a carbonate ion HCO<sub>3</sub><sup>-</sup> in solution.

The salts added as part of the growth medium prevented the dissociation of carbonic acid by acting as buffers, reacting with the protons released from the dissociation and limiting the impact on pH adjustment.

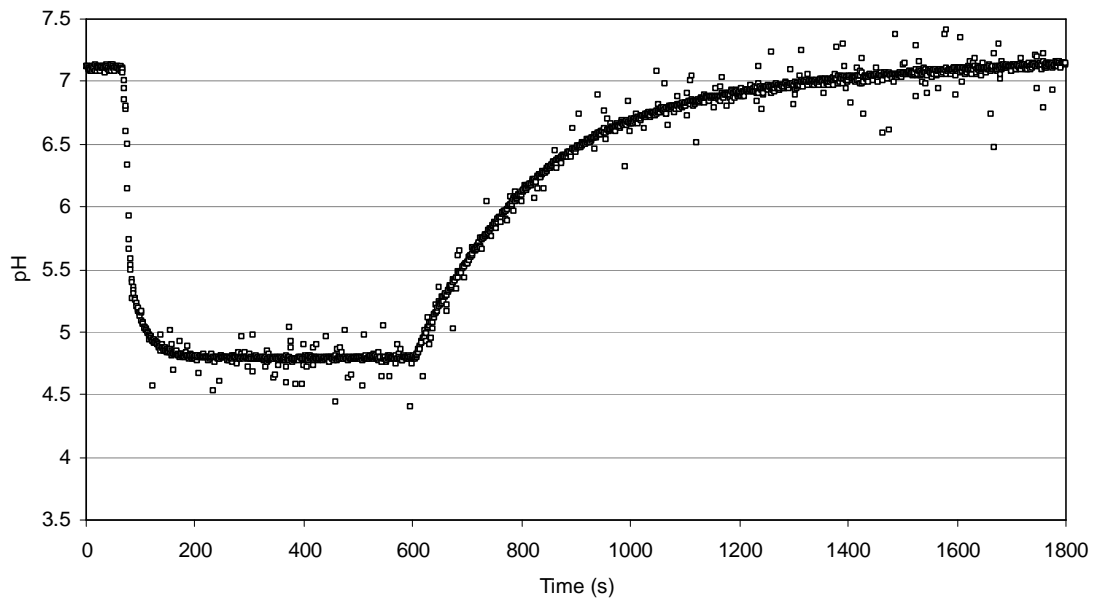
The rate of pH increase with the addition of nitrogen in growth medium, as shown in Figure 3-4, can also be explained by the presence of buffering salts in solution. As nitrogen passed through the system, it stripped out carbon dioxide, which caused the carbonic acid to revert back to carbon dioxide. Because of the buffering agents present in the growth medium, there was also less carbonic acid in solution, leading to a faster pH change over time. Additionally, the buffers in solution hastened the process of removing carbonic acid by forcing the dissociating carbonic acid to the products.



**Figure 3-4: Effect of agitation rate on increasing the pH of the system using N<sub>2</sub> gas in growth medium**

The studies that were used to determine a growth medium for this work were performed in serum bottles rather than bioreactors. As such, the sealed bottles did not allow for external pH control and relied on buffers in solution to maintain the pH near a desired value of approximately

6.5. Therefore, the original medium was used for growing cells in serum bottles, but a new growth medium was created when buffering was not necessary in the presence of external pH control. This new medium, referred to as the minimal growth medium, used fewer buffering compounds but retained salts which would be required by the cells in a bioreactor. The  $\text{NaHCO}_3$  was completely removed and the phosphates were greatly reduced because these components were believed to have the greatest buffering affect. The minimal growth medium was tested in the same way as before and an example of the results is shown in Figure 3-5.



**Figure 3-5: pH change in minimal growth medium using  $\text{CO}_2$  and nitrogen,  $20^\circ\text{C}$ , 500rpm**

The figure above showed similar trends with the previous two groups of experiments; when nitrogen was used to raise the pH, the results were comparable to the previous group of tests with the original growth medium. The pH was not as low as the tests performed using RO water but reached a pH of 4.75 compared to the original growth medium which only reached pH 5.0. While the minimal growth medium reached a lower pH, it was not lowered to the  $\text{pK}_{\text{A}2}$  of succinic acid (4.2). However, the medium components will be consumed during fermentation and



are expected to have a smaller impact on pH adjustment as succinic acid production progresses. Since some components of the growth medium still hindered pH reduction using CO<sub>2</sub> they were tested individually to determine if they should be removed from the system.

### 3.5.2 pH Adjustment in the Presence of Individual Growth Medium Components

Each growth medium component was tested at the concentrations found in both the original and minimal growth media. The initial pH for each component started with no prior adjustment and is the pH of solution from dissolving each growth medium component. The results from the original growth medium are presented in Figure 3-6. The top of the bar indicates the starting pH and the bottom shows the equilibrium pH with CO<sub>2</sub> sparging.

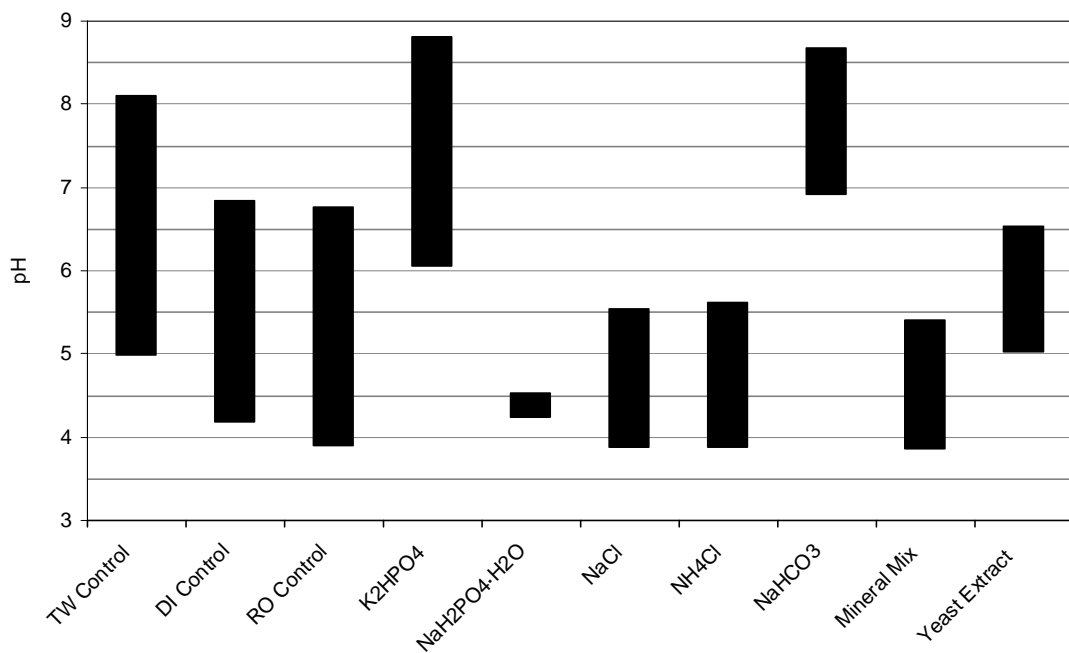
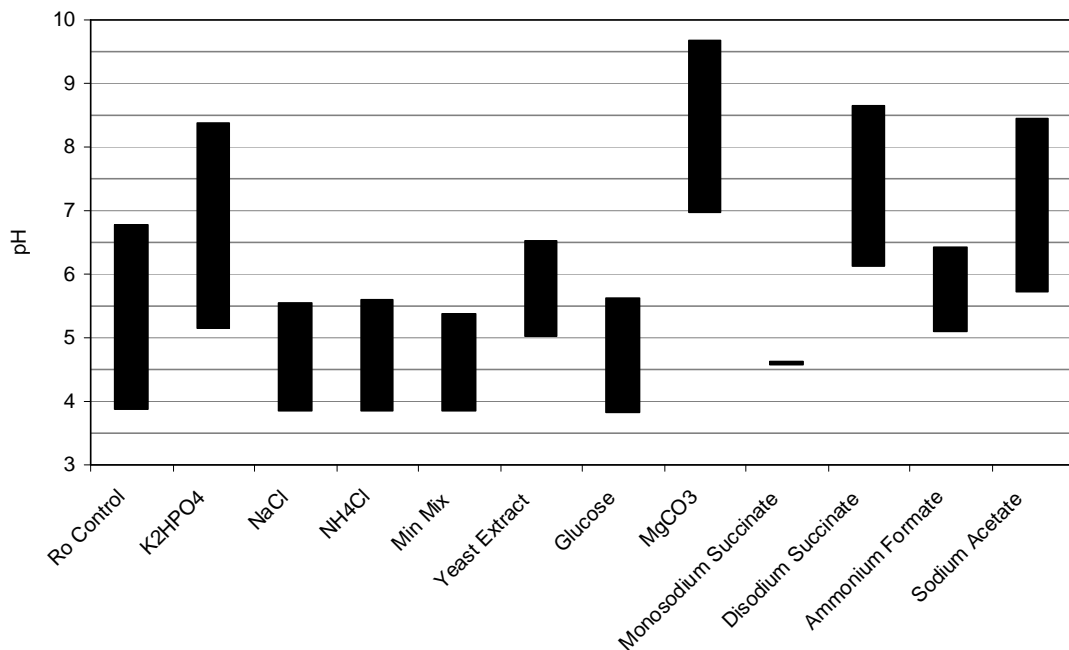


Figure 3-6: Individual component pH adjustment for the original growth medium

The figure above shows that, prior to assessing the growth medium components, the water used in the bioreactor could have had an impact on pH adjustment. In most industrial circumstances, tap water is preferred because it requires no additional processing. Kingston's water contains large amounts of dissolved calcium carbonate which would have hindered the pH adjustment process. RO water was used for all future work to remove this confounding aspect from the results. With regard to the original growth medium,  $K_2HPO_4$  and  $NaHCO_3$  clearly prevented the pH from being lowered using  $CO_2$ , justifying their removal when the minimal growth medium was formulated. The next major hindrance to pH adjustment came from yeast extract, but it was deemed essential to cell growth, a fact that agrees with the work of Liu et al. (2008b) which stated that yeast extract gave one of the highest succinic acid concentrations compared to other nitrogen sources. A repeat test using minimal growth medium components and product salts of the bioproduction process, succinate, acetate and formate, are shown in Figure 3-7.



**Figure 3-7: Individual component pH adjustment for the minimal growth medium and product salts**

The figure above shows the decrease in concentration of  $K_2HPO_4$  reduced the lower pH limit almost one full pH unit from 6.1 to 5.17. Given that the concentration of  $K_2HPO_4$  was only 0.5g/L, the sensitivity of pH to buffering components was apparent. 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 by  $CO_2$ . Another compound that was tested in this group was  $MgCO_3$  which had been suggested for use in pH control rather than NaOH or KOH [Liu et al., 2008a]. While a concentration of 55g/L was used in this test, chosen to match the amount of glucose added, it had a low solubility and existed mostly as a solid slurry. It caused the most hindrance to pH reduction of any compound, even at its aqueous concentration of 266.6mg/L at room temperature. The use of  $MgCO_3$  for pH control would seriously undermine any attempt to alter the pH using  $CO_2$  if it was not completely consumed.

One concerning feature of Figure 3-7 was how succinate, acetate and formate salts hindered pH reduction using  $CO_2$  sparging. In a bioreactor where succinic, acetic and formic acid are produced, the pH is controlled using a base such as KOH. As such, the products are in solution in their ionic form. As succinic, acetic and formic acid are weak acids, their salts act as strong conjugate bases. Therefore, the compounds which cause the greatest impediment to pH adjustment are the products of the biotransformation even though they will be removed using *in-situ* product removal.

There were two solutions to the problem of succinate, acetate and formate hindering pH reduction using  $CO_2$  gas. The first solution to the problem was to change the solubility of carbon dioxide in the system. Data presented by Dodds et al. (1956) in Figure 3-8 show the solubility of carbon dioxide in water at 1atm shows little change with respect to temperature. It is not until the pressure is increased above 5atm that any appreciable change in solubility is seen. The second solution was to use a strong acid to lower the pH to 4.2, though this was undesirable. One of the problems with current succinic acid production methods is the number of chemicals that are added in the separation process, so the addition of acid to adjust the pH is counterproductive to

developing a more efficient separation method for succinic acid. However, because the proposed pH adjustment system for this biotransformation could not be performed above 1atm without damaging the equipment, the conclusion of this set of experiments was that for the remainder of research, strong acids would be used to lower the pH, allowing product uptake to show proof of concept for the overall research goal of ISPR and reducing EPI.

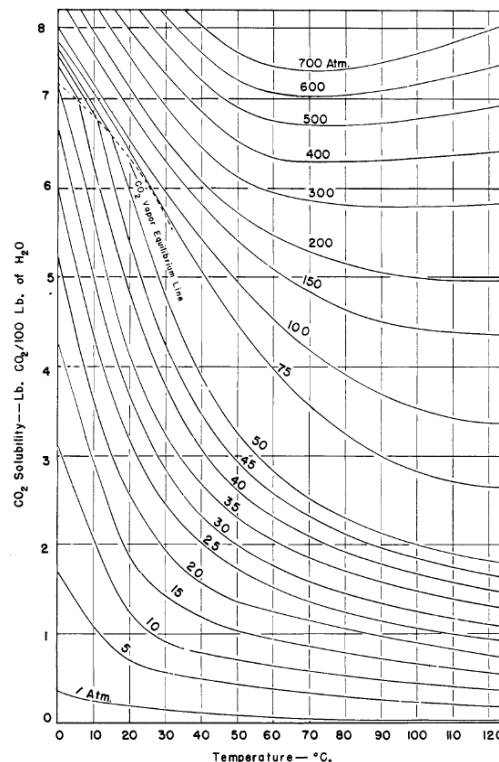
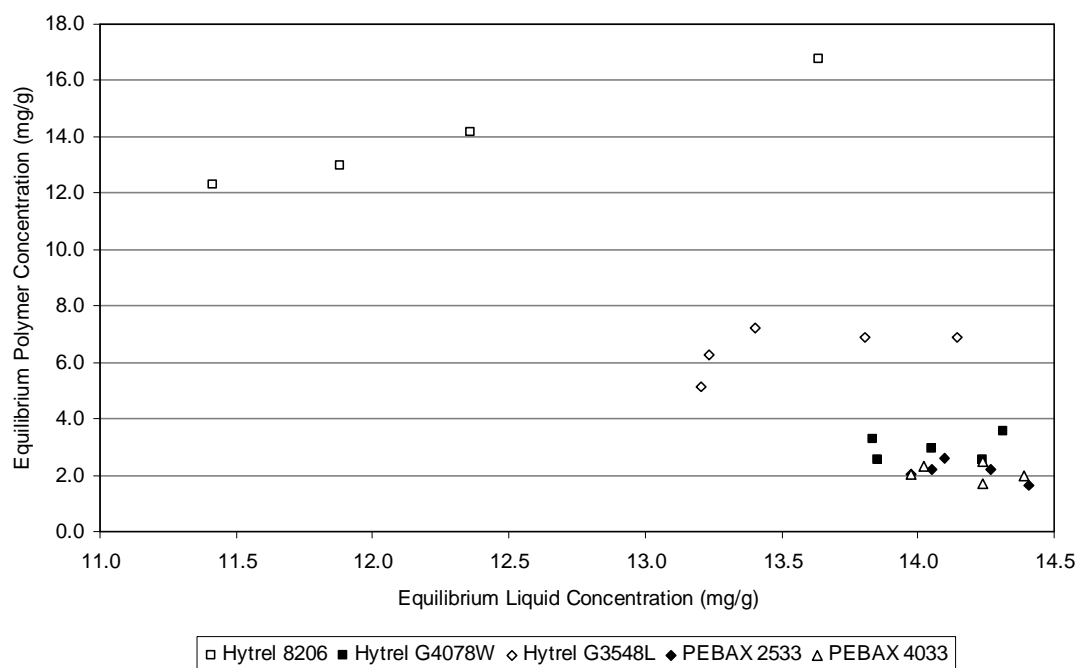


Figure 3-8: Graph from Dodds et al. (1956) showing carbon dioxide solubility as a function of temperature and pressure

### 3.5.3 Polymer Screening

An initial group of ten polymers was chosen to determine which would give the highest partition coefficient for succinic acid. Polymer selection followed the work of Gao and Daugulis (2010) which showed that Hytrel<sup>®</sup> 8206 gave the highest partition coefficient while the other polymers tested showed little or no uptake of succinic acid. For initial polymer screening, the following polymers were selected: Hytrel<sup>®</sup> 8206, G4078W, G3548L; PEBAX<sup>®</sup> 2533, 4033;

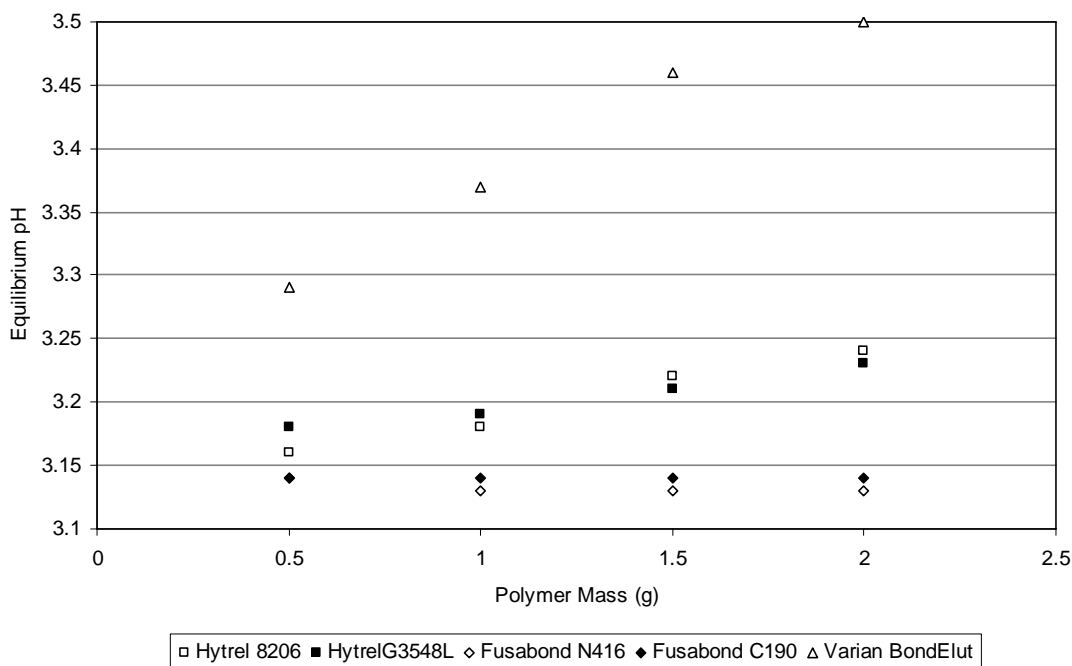
Fusabond<sup>®</sup> N416, C190, C250; Nucrel<sup>®</sup> 925, Elvax<sup>®</sup> 650Q. Varied grades of Hytrel<sup>®</sup> were selected to determine how the measured hardness of the material altered the partition coefficient, though the exact composition of Hytrel<sup>®</sup> 8206 was unknown for proprietary reasons. PEBAX<sup>®</sup> was selected based on the polyamide groups in its structure and the belief that the main force between polymer and succinic acid molecules is hydrogen bonding, which the polyamide provided. Fusabond<sup>®</sup> was used based on recommendations from Gao and Daugulis (2010) that polymers with molecules chemically similar to succinic acid grafted to them would aid in uptake. Given that Fusabond<sup>®</sup> polymers have been modified with maleic anhydride, they met the recommendations of Gao and Daugulis (2010). Nucrel<sup>®</sup> 925 and Elvax 650Q were chosen based on recommendations from DuPont that the methacrylic acid and vinyl acetate may help succinic acid uptake. High percentages of these components increase softness in the polymer and allow for a higher availability of amorphous regions within the polymer to absorb succinic acid. For initial screening, the pH of the samples was left unadjusted at approximately 2.64, well below the pK<sub>A</sub> to avoid any changes in acid-salt equilibrium as succinic acid was absorbed and the pH changed.



**Figure 3-9: Equilibrium concentrations of polymer samples contacted with succinic acid solutions**

Of the ten polymers tested, five showed no signs of succinic acid uptake as the liquid concentration of those samples were left unchanged. The remaining polymers are shown in Figure 3-9. Of the results in Figure 3-9, only two polymers showed any non-zero partition coefficient - Hytrel<sup>®</sup> 8206 and G3548L - which is consistent with the results of Gao and Daugulis (2010). The partition coefficient and equilibrium polymer concentration of succinic acid did appear to increase as polymer hardness decreased in the set of Hytrel<sup>®</sup> samples. The comparison amongst Hytrel<sup>®</sup> samples did not include 8206 because it is unclear how similar its structure is compared to the other two Hytrel<sup>®</sup> polymers. The three remaining polymer samples showed slight uptake of succinic acid, but there was no appreciable trend. While uptake of succinic acid from solution was observed for both Hytrel<sup>®</sup> and PEBAX<sup>®</sup> polymers, it was a fraction of that shown for 8206. The slope of the line from the data points in Figure 3-9 would yield a partition coefficient but in the case of all polymers except Hytrel<sup>®</sup> 8206, the slope was either zero or had a negative value.

Some polymers from the initial screening were subjected to another one using pH change as an indicator of uptake. Changes in pH can indicate that succinic acid is leaving the liquid phase, thereby raising the pH of the aqueous solution. Samples were prepared with a succinic acid solution of 5g/L and the polymer beads were in the same fractions as the previous test. The initial pH of all samples was 3.14 and the pH of the system was measured after each sample reached equilibrium. The results are presented in Figure 3-10 and show that in the case of Hytrel<sup>®</sup> 8206, the pH rose, which agreed with Figure 3-9 showing that succinic acid was leaving the liquid phase. In addition, Fusabond<sup>®</sup> showed no uptake of succinic acid and the pH remained unchanged. For the sake of comparison, an adsorbent, Varian BondElut<sup>®</sup>, was tested knowing that succinic acid uptake would occur and be reflected in a pH change. This adsorbent has the same structure as the resin inside the HPLC column used for analysis, which confirmed succinic acid removal from solution.



**Figure 3-10: Equilibrium pH after succinic acid uptake**

Hytrel<sup>®</sup> 8206 showed the best uptake, though the partition coefficient was not a large value, as shown in Figure 3-9. It was the only polymer of ten selected to give a consistent, non-zero partition coefficient value. A wider range of polymers was tested with a 25% (w/v) polymer fraction to determine if any performed as well as Hytrel<sup>®</sup> 8206. Because of the large number of polymers involved only one polymer fraction was tested and the spectrophotometer was used to measure the liquid concentration of succinic acid. The results of this larger test are presented in Figure 3-11. The results show a similar result for Hytrel<sup>®</sup> 8206 although there were slight differences for G4078W and G3548L. These tests were duplicated to ensure that the difference in the results was not the result of equipment used. Again, from all the polymers tested, Hytrel<sup>®</sup> 8206 performed far better than any other polymer. Though the results here indicated that Hytrel<sup>®</sup> 3548 performed moderately well, the initial polymer screening determined that there was no discernible trend for a partition coefficient.

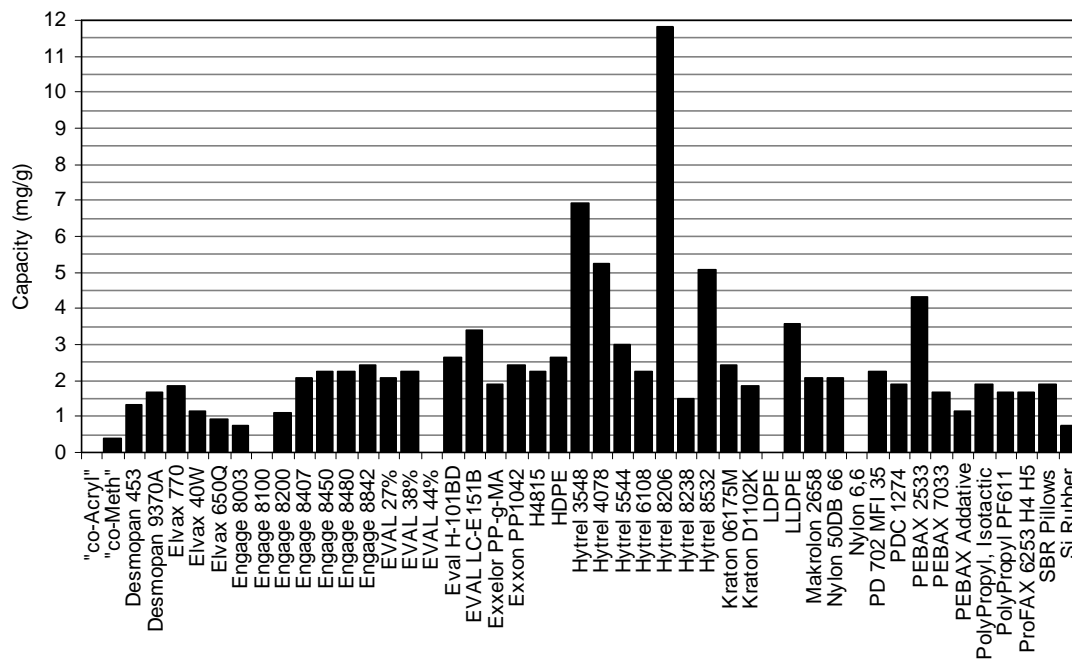
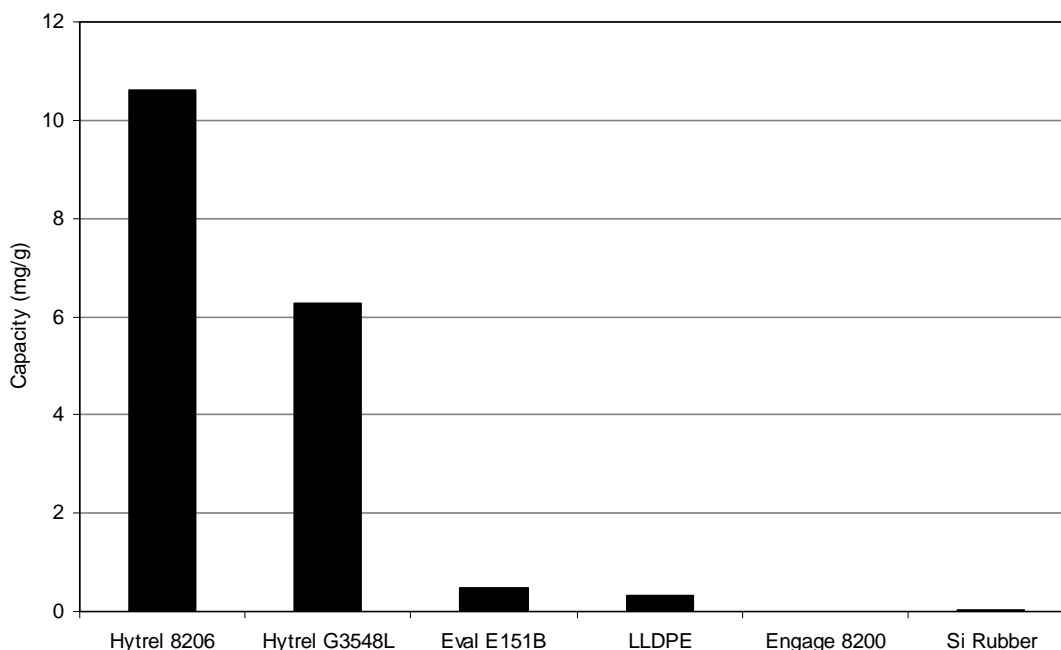


Figure 3-11: Succinic acid capacity in 25% polymer fraction samples

To ensure the secondary screening was reliable, six polymers from secondary screening were tested using the HPLC, shown in Figure 3-12. The test confirmed the reliability of work using the spectrophotometer as the results for Hytreil<sup>®</sup> 8206 and G3548L remained the same.





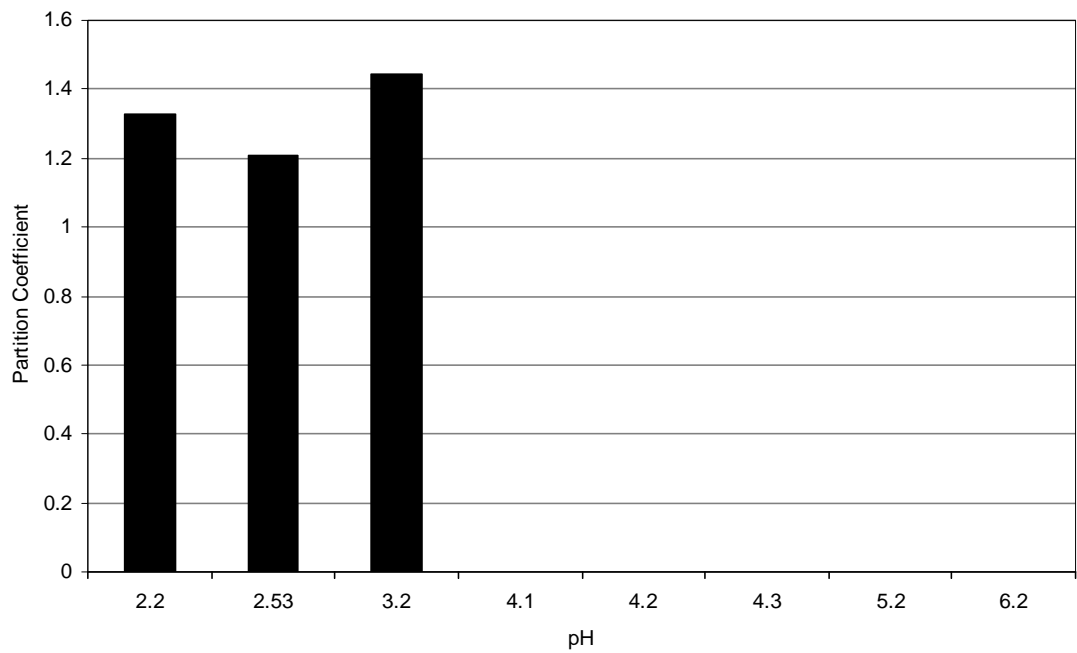
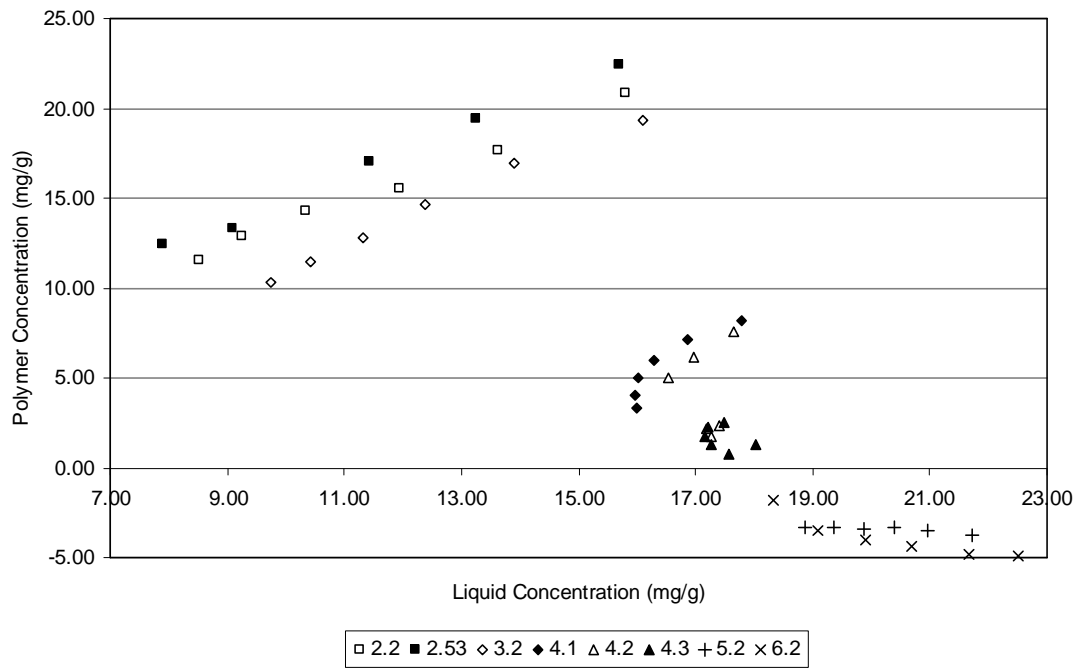
**Figure 3-12: Capacity of select polymers using HPLC analysis**

Through all polymer selection and screening, the best performing polymer was Hytrel<sup>®</sup> 8206, with a partition coefficient of approximately 1.3, which was used for all future research. Given these polymer beads will be used in a system in which pH plays a significant role, tests were performed to understand how variations in pH affect succinic acid uptake by the polymer.

### 3.5.5 Partition Coefficient as a Function of pH and Concentration

The  $pK_{A2}$  of succinic acid is 4.2, a 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. 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 during the pH shift/polymer uptake phase of the ISPR

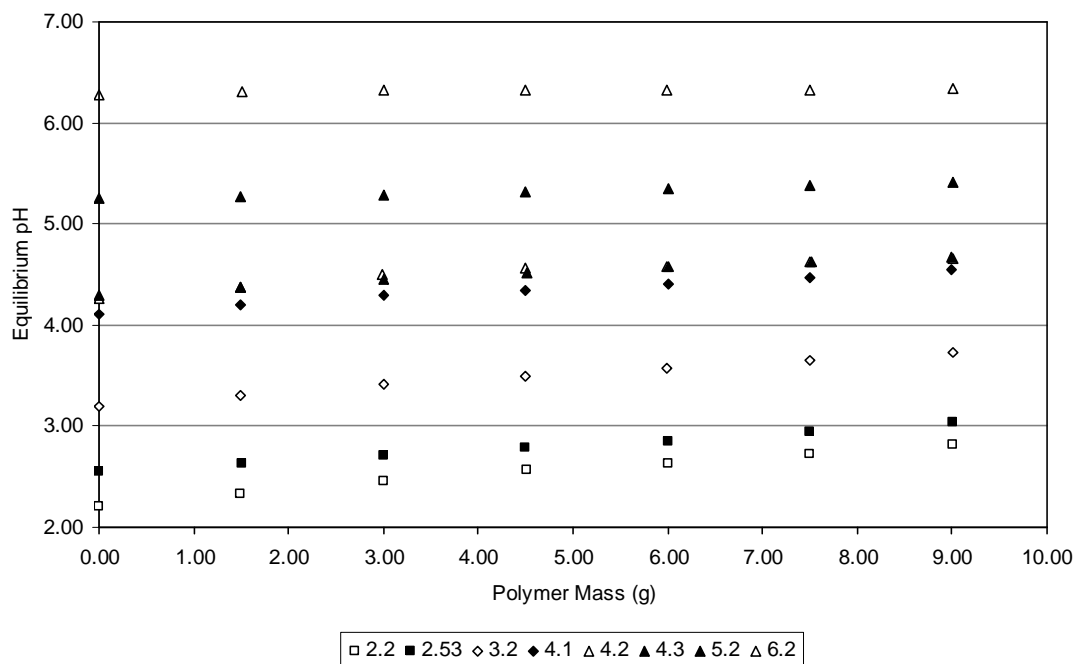
cycle. To determine which pH level was required to ensure high levels of succinic acid uptake, a series of experiments was prepared to test how partition coefficient varied with respect to pH. The results for the equilibrium concentrations in the liquid and polymer phase are shown in Figure 3-13a and the calculated partition coefficient values are shown in Figure 3-13b.



**Figure 3-13: (a) Equilibrium liquid concentration of succinic acid versus equilibrium polymer concentration at various initial pH conditions and (b) partition coefficient as a function of initial pH**

In the Figure 3-13a, the data at pH 2.53 showed a partition coefficient where the pH remained unchanged from the 20g/L succinic acid solution. In data sets for pH 2.2 and 3.2, initial liquid concentrations were lowered, showing dilution from 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, this change in liquid concentration had no effect on the partition coefficient and all three sets of data below pH 4.1 gave values of approximately 1.3, as shown in Figure 3-13b. Again, this is comparable with the results of Gao and Daugulis (2010).

In the case where the pH was set to 4.1, the data points followed a predictable trend in Figure 3-13a until the polymer fraction of the system reached its upper values where the polymer concentration sharply declined. A similar phenomenon was noticed with the data at pH 4.2 and started at a lower polymer fraction than at pH 4.1. When the initial pH was set to 4.3, the data showed no trend at all, remaining in the same region as the outlying points from the pH 4.2 data. While the data in Figure 3-13a indicated a partition coefficient of approximately 2.3, these data were considered unreliable due to the shape of the curve. Because of the deviations from a predictable trend in the data at pH 4.1 to 4.3, no partition coefficient was determined, as shown in Figure 3-13b. Additionally, while Figure 3-13a showed an increase in polymer concentration as polymer fraction increased for pH 5.2 and 6.2, this was believed to be caused by water absorption by the polymer, the effect of which is expanded upon in Appendix A. To help explain the problems of absorbance at pH 4.1 to 4.3, the equilibrium pH was measured in all samples to determine if correlation to succinic acid absorbance was present. The results are shown in Figure 3-14.



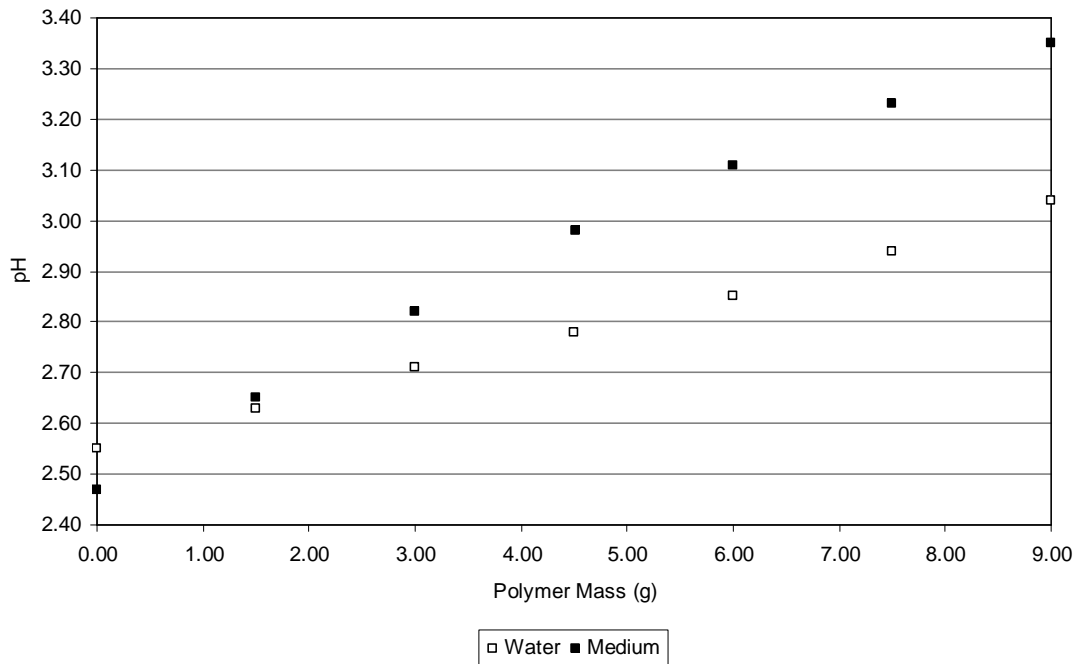
**Figure 3-14: Equilibrium pH values for partition coefficient samples**

The samples from 4.1 to 4.3 showed pH increases as the polymer masses increased, but plateaued at approximately 4.65, another indicator that less succinic acid was being absorbed by the system. An equilibrium was established where the drive of equilibrium to maintain succinate ions in solution equalled the drive of polymers to absorb succinic acid and absorption ceased. In tests performed at a pH well above the  $pK_{A2}$ , the results showed no change in the liquid succinic acid concentration with increasing polymer fractions. Ideally, a pH of 3.2 to 3.8 should be used for succinic acid absorbance as it shows no ill effects for absorption compared to 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 20g/L to 60g/L, the partition coefficient remained at 1.3 (data not shown).

### 3.5.6 The Effect of Medium Components on Partition Coefficient

The impact of medium salts on succinic acid absorption is important given their inevitable presence in the bioreactor. The results indicated that the growth medium had no effect on succinic acid uptake by Hytrel 8206 as previously demonstrated by Khan and Daugulis (2010) for another target molecule, benzaldehyde (data not shown). Similar to previous results in which the pH of the system was higher than the  $pK_{A2}$  of succinic acid, the polymers did not absorb any succinic acid. The partition coefficient was, again, calculated to be 1.3, which is comparable to the results above. The only difference with the test performed in the presence of growth medium components was the equilibrium pH after equilibrium had been reached, which showed a much higher increase than samples in only RO water. Figure 3-17 shows that the equilibrium pH rose by almost one full unit, indicating that the presence of salts acting as buffers caused a greater rise in the pH of the samples. This increased pH could require a larger mass of acid added to maintain the pH at 3.8 given that the pH of the system will rise faster in growth medium as succinic acid is removed. As shown in Figure 3-16, when the pH of the system rose, the mass of succinic acid removed from the system decreased. Lowering the pH to 3.8 will ensure succinic acid uptake while minimizing acid added to the system, however the pH must be controlled or the increase in pH arising from the presence of salts could hinder the absorbance process.



**Figure 3-15: Changes in equilibrium pH with changes in polymer fraction and the effect of uptake in RO water vs. minimal medium**

### 3.6 Conclusion

Carbon dioxide at atmospheric pressure has the ability to lower the pH of aqueous solutions and, in the case of RO water, achieve a pH below the  $pK_{A2}$  of succinic acid. While temperature had a limited affect on the system overall, an agitation rate up to 500 rpm greatly affected the rate at which the pH rose while sparging nitrogen into the system. The rate of pH increase from nitrogen was slower compared to the pH decrease using carbon dioxide due to the lower solubility of nitrogen gas in solution. Unfortunately, carbon dioxide is a weak acid and cannot overcome the effects of growth medium components which acted as buffers in the system. Even a minimal growth medium with greatly reduced salt concentrations impeded the pH from being reduced by sparging with carbon dioxide gas. If carbon dioxide gas is to be used to temporarily lower the pH of a bioreactor, a higher pressure is needed to increase the mass of  $CO_2$  dissolved and carbonic acid formed.

The individual growth medium components tested showed that most compounds did not have a significant impact on the ability to adjust the pH using CO<sub>2</sub> except for K<sub>2</sub>HPO<sub>4</sub>. This impediment was seen as a worst-case scenario in terms of growth medium components as their concentrations would decrease as the fermentation proceeded. The main problem seen with pH adjustment came from the succinate ion itself as it is a weak acid which forms a strong conjugate base in solution. The problem of succinate hindering pH reduction using CO<sub>2</sub> sparging was unavoidable as biotransformation occurs at approximately pH 6.7 where succinic acid would be present as a dissociated ion.

Of the 50 polymers tested, only Hytrel<sup>®</sup> 8206 showed a partition coefficient with a value of approximately 1.3. The partition coefficient and the mass of succinic acid absorbed were greatly affected by the pH of the system. Samples at least one unit below pH 4.2 showed no change in partition coefficient, but samples at the pK<sub>A2</sub> had lower succinic acid concentrations in the polymer and absorbance ceased above pH 4.65. This indicated that only the undissociated form of succinic acid was removed from solution. Overall, a pH lower than 4.2 is required in the bioreactor to ensure succinic acid is effectively removed

Succinic acid absorption from a bioreactor which has been affected by end product inhibition is possible. Until a system can be designed to allow for pressurization with carbon dioxide, however, an alternative method to lower and raise the pH must be used.



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## 4.0 Succinic Acid Bioproduction and Removal Using a Two-Phase Partitioning Bioreactor

### 4.1 Preface

The results from Chapter 3 indicated that carbon dioxide was not effective at atmospheric pressure to lower the pH of the bioreactor to below the  $pK_{A2}$  of succinic acid. As the reactor used for this research does not allow for pressurization beyond 1 atmosphere, strong acids and bases are required to lower the pH until a means is devised to operate at high  $CO_2$  pressures.

The physical testing also showed that, of the polymers considered, only Hytrel<sup>®</sup> 8206 was able to effectively take up succinic acid and yielded the highest partition coefficient. When the effect of pH on uptake was tested, samples with an initial pH of 4.1 or higher showed little or no uptake. All subsequent tests took place at pH 3.8 to give elevated polymer concentrations and avoid low partitioning at pH levels approaching 4.2. Additionally, in future testing, the pH in the bioreactor will be maintained at 3.8 during polymer uptake using 5M  $H_2SO_4$ . pH control ensures there are no changes in pH and shifts from undissociated to dissociated succinic acid as the product is taken up.

Having established the physical characteristics of the system, Chapter 4 will turn to the biological components of the system. The most important test is to determine the impact of low pH on cell growth. If the cells cannot recover from exposure to a low pH in a bioreactor then a new system design is needed to remove the cells from solution and recycle them to allow for continued biotransformation.

Succinic acid production can benefit from TPPB operation by eliminating end product inhibition, but a single-phase bioreactor run must be performed to determine a benchmark against which a two-phase system can be compared. While a growth medium has already been decided upon, small salt additions may be required if the medium does not result in efficient production of

succinic acid. Inefficiencies would include incomplete glucose consumption, a final product concentration lower than other reported values under similar conditions.

Once the base case is established, tests will be performed to determine whether the mass of succinic acid removed from fermentation broth is similar to uptake in abiotic tests performed in Chapter 3. The goal will be to lower the pH to 3.8 and remove succinic acid using Hytrel<sup>®</sup> 8206 using 5M H<sub>2</sub>SO<sub>4</sub>, then remove the polymers and raise the pH of the fermentation broth with 5M KOH to 6.7, allowing bioproduction to continue. The polymers will also be desorbed to determine what volume of RO water is required to remove at least 90% of succinic acid and how many wash cycles are required.

## **4.2 Abstract**

Succinic acid is a 1,4-diacid and an intermediate in the production of pharmaceuticals and commodity chemicals. Although bioproduction is possible no industrial processes exist, limited by end product inhibition (EPI) and high separation costs which amount to 70% of total production costs. Two-Phase Partitioning Bioreactors (TPPBs) can reduce EPI through *in-situ* product removal from the liquid phase. The pH of the fermentation broth must be adjusted below the pK<sub>A2</sub> of succinic acid for polymer uptake to occur.

To ensure cell growth was unaffected after exposure to low pH conditions, serum bottles containing cells were lowered to pH 4.2 for various times. Cell growth was measured after raising the pH to 6.7. All samples exposed for up to 4 hours had similar cell density increases after 24 hours at pH 6.7. Therefore, lowering the pH to 4.2 would not hinder cell growth after succinic acid uptake occurred in the bioreactor as long as the uptake period did not exceed 4 hours.

To determine the time required for succinic acid uptake from fermentation broth, 1kg of polymer beads was added to 3.5L of fermentation broth with pH control at 3.8. The succinic acid

concentration in the liquid phase was reduced by 3.7g/L after 1h, within the acceptable pH exposure time of 4h.

A single-phase biotransformation yielded 39g/L of succinic acid after 28 hours. The two-phase run performed similarly but after pH cycling using H<sub>2</sub>SO<sub>4</sub> and KOH to remove succinic acid, cell growth did not resume due to the excess salts from pH adjustment. This work has provided a preliminary demonstration of concept that polymers can reduce EPI and separate succinic acid from solution without requiring complex downstream separation methods.

Keywords: Succinic acid, Two-Phase Partitioning Bioreactor (TPPB), *in-situ* product removal (ISPR)

### **4.3 Introduction**

Over 1 billion tons of renewable resources are available annually in the United States from the forestry and agriculture sectors [Perlack et al., 2007]. With abundant renewable resources available that do not affect food supplies, processes are being developed which can use these materials as feedstocks for biosynthesis. One compound of interest which can be created from biomass is succinic acid. A 1,4-diacid, succinic acid serves as an intermediate in the production of food additives, biodegradable polymers and commodity chemicals including 1,4-butanediol [Zeikus et al., 1999]. Succinic acid is also mentioned by the US Department of Energy as one of the top twelve value-added chemicals produced from biomass [Werpy and Petersen, 2004]. Succinic acid bioproduction requires a volumetric productivity of 2.5 g/L·h to be competitive with chemical synthesis from *n*-butane through maleic anhydride [Werpy and Petersen, 2004]. The suggested volumetric productivity target has not been reached in research due to end product inhibition (EPI). Additionally, separation of succinic acid from the fermentation broth can account for up to 70% of total production costs, hindering bioproduction

on an industrial scale [Huh et al., 2006]. Current methods of succinic acid separation include precipitation, electrodialysis and reactive extraction using tri-*n*-octylamine, each of which has distinct advantages [Kurzock and Weuster-Botz, 2010]. However, crystallization suffers from excess waste generated, and electrodialysis requires additional energy inputs [Corona-Gonzalez et al., 2008, Bechthold et al., 2008]. Reactive extraction primarily removes acetic acid and becomes a pre-treatment step rather than a succinic acid separation method [Huh et al., 2006].

Two-Phase Partitioning Bioreactors (TPPBs) have been shown to alleviate EPI by removing products from the fermentation broth, as demonstrated recently in the production of 2-phenylethanol and benzaldehyde [Gao and Daugulis, 2009, Jain et al., 2010]. The sequestering phase can be a polymer material which targets products and removes them from solution by absorption. Previous work done by Gao and Daugulis (2010) showed that succinic acid can be taken up from the liquid phase using polymer beads, although the research in Chapter 3 indicated that this uptake was dependent on the pH of the solution. Because the pH increases as succinic acid is removed, the pH of a bioreactor must be maintained at least below the  $pK_{A2}$  of succinic acid for polymer uptake to be possible.

The requirement of a low pH for polymer uptake presents problems for the cells in a fermentation broth. Research has shown that as the pH of fermentation broth approaches pH 6.0, cell growth decreases and below 6.0, no cell growth occurs [Van der Werf et al., 1997, Liu et al., 2008b]. Given that the pH must be brought below 4.2 for polymer uptake, *Actinobacillus succinogenes* must be tested to determine how long the microorganism can be exposed to a low pH and continue growth after raising the pH to operating levels. Additionally, a system is needed to contact the polymer beads with the fermentation broth during uptake but allow for easy polymer bead removal. Otherwise, as the pH is raised, the polymer beads would leach succinic acid back into the fermentation broth.

The purpose of this research is to show that *A. succinogenes* can withstand exposure to low pH conditions long enough to allow for succinic acid removal from fermentation broth. A set

of single-phase biotransformations will determine the highest succinic acid concentration possible in our lab. A bioreactor configuration will be employed for polymer uptake from solution that allows for ease of polymer removal before the pH of the solution is increased and bioproduction continues. Succinic acid uptake from fermentation broth will be tested and a desorption study will determine the volume of water and number of washes to remove over 90% of the absorbed acid. Finally, a two-phase biotransformation will look to produce a high concentration of succinic acid, reduce the pH to 3.8 for product removal using polymer beads and return the pH to operational conditions for continued bioproduction.

## ***4.4 Materials and Methods***

### **4.4.1 Chemicals and Polymers**

Unless otherwise specified, all chemicals were purchased from either Fischer 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). Samples of commercial grade Hytrel<sup>®</sup> 8206 were donated to the research group by DuPont Canada (Kingston, ON). In general, the polymers beads were in the shape of ellipsoids with diameters of approximately 2mm to 5mm. Polymers were prepared by washing three times with hot tap water on a stir plate, then twice with RO water. After a final overnight wash in RO water, the polymers were left to air dry. The washing process was required to remove residue from the polymer beads remaining from their manufacturing process.

#### 4.4.2 Organism and Media Formulation

*Actinobacillus succinogenes* was purchased from the American Type Culture Collection (ATCC), 55618. As per ATCC instructions, cells were first grown in a 250mL solution of 40g/L TSB which was autoclaved at 121°C for 15 minutes prior to inoculation. Cells in solution were incubated at 30°C and 200rpm for 48 hours, then 0.5mL of the cell solution was mixed in a 2mL cryo vial with 0.5mL of a 20% glycerol storage solution. Cryo vials were stored at -75°C until required.

The growth medium used in serum bottle preparation, referred to as the original medium, contained the following (in g/L): yeast extract, 5; NaHCO<sub>3</sub>, 12.6; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 8.5; K<sub>2</sub>HPO<sub>4</sub>, 5.5; NH<sub>4</sub>Cl, 2; NaCl, 1. 1mL/L of a trace elements mix was also added which contained the following compounds (in mg/L): nitrilotriacetic acid, 3 000; MgSO<sub>4</sub>·H<sub>2</sub>O, 3 000; MnSO<sub>4</sub>·H<sub>2</sub>O, 500; FeSO<sub>4</sub>·7H<sub>2</sub>O, 100; CaCl<sub>2</sub>·2H<sub>2</sub>O, 100; CoCl<sub>2</sub>·6H<sub>2</sub>O, 100; Na<sub>2</sub>MoO<sub>4</sub>, 25; NiCl<sub>2</sub>·6H<sub>2</sub>O, 25; Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O, 25; ZnCl<sub>2</sub>, 13; CuSO<sub>4</sub>·5H<sub>2</sub>O, 10; AlK(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O, 10; H<sub>3</sub>BO<sub>3</sub>, 10; NaSeO<sub>3</sub>, 10. This growth medium was created after reviewing existing formulations in the literature and incorporating the most consistently used components. A second growth medium, referred to as the minimal growth medium, was used in fermentations and contained fewer buffering salts. This minimal growth medium contained the following components (in g/L): yeast extract, 5; NH<sub>4</sub>Cl, 2; NaCl, 1; K<sub>2</sub>HPO<sub>4</sub>, 0.5 as well as 1 mL/L of the mineral mix shown above.

A glucose concentration of 55g/L was chosen for biotransformations based on studies from Lin et al. (2008) and Liu et al. (2008b). These papers showed that at this glucose level succinic acid production and optical density were at their highest. Also, this was the highest concentration which showed no residual glucose after fermentation in a bioreactor [Liu et al., 2008a]. Acetic acid production was also higher using 55g/L of glucose compared to other glucose concentrations but it was a marginal increase compared to the increase in succinic acid.



#### **4.4.3 Serum Bottle Preparation**

Sealed serum bottles were used to grow cells under anaerobic conditions. 50mL of the original growth medium was placed in a 125mL serum bottle. The headspace was sparged for 30 seconds with medical grade carbon dioxide and then the serum bottle was closed with a butyl rubber plug. A solution of 60g/L glucose was prepared separately and autoclaved with the serum bottles at 121°C for 15 minutes. Autoclaving the glucose separately ensured it did not caramelize in the presence of the growth medium, a phenomenon which prohibited growth with this microorganism. The serum bottles were allowed to cool to room temperature and then sealed with crimp caps. 10mL of the glucose solution was added to the serum bottles bringing the total volume to 60mL with 10g/L glucose in solution. The pH of each serum bottle was adjusted to 6.7 using autoclaved 5M H<sub>2</sub>SO<sub>4</sub>.

#### **4.4.4 Serum Bottle Preparation for Cell Exposure to a Low pH**

This experiment was designed to determine how bacteria responded to a low pH exposure and how long recovery took based on length of exposure. In this experiment, cell recovery was considered to be an increase in optical density. Nine serum bottles were prepared as described above, one of which was inoculated with 200µL of frozen cell stock and placed in an incubator at 37°C and 200rpm for 20 hours. A 3mL sample was drawn from this initial bottle and injected into one of the eight remaining abiotic or 'fresh' bottles. This serum bottle acted as the control for the study and was placed in the incubator. 5M H<sub>2</sub>SO<sub>4</sub> was added to the initial serum bottle to obtain a pH of 4.2. After 5, 10, 15, 30, 60, 120 and 240 minutes of low pH exposure, 3mL aliquots were removed from the low pH serum bottle and injected into fresh bottles. The optical density of the fresh serum bottles was monitored at intervals of 2, 4, 8, 12 and 24 hours after inoculation.

#### **4.4.5 Bioreactor Preparation**

A 5L BioFlo III (New Brunswick Scientific, Edison, NJ) with a 3L working volume was used for all bioreactor runs with control for pH and temperature. A 3x concentrated solution of the minimal growth medium was prepared in 1L of RO water and added to the bioreactor. An additional 1L of RO water was added to the bioreactor which was sealed and autoclaved at 121°C for 60 minutes. Separately, a 3x solution of 55g/L glucose was prepared in 1L of RO water and autoclaved, then added to the bioreactor aseptically. Cells were grown in serum bottles in an incubator at 37°C and 200rpm for 18 hours, then opened and added to the bioreactor to begin the biotransformation.

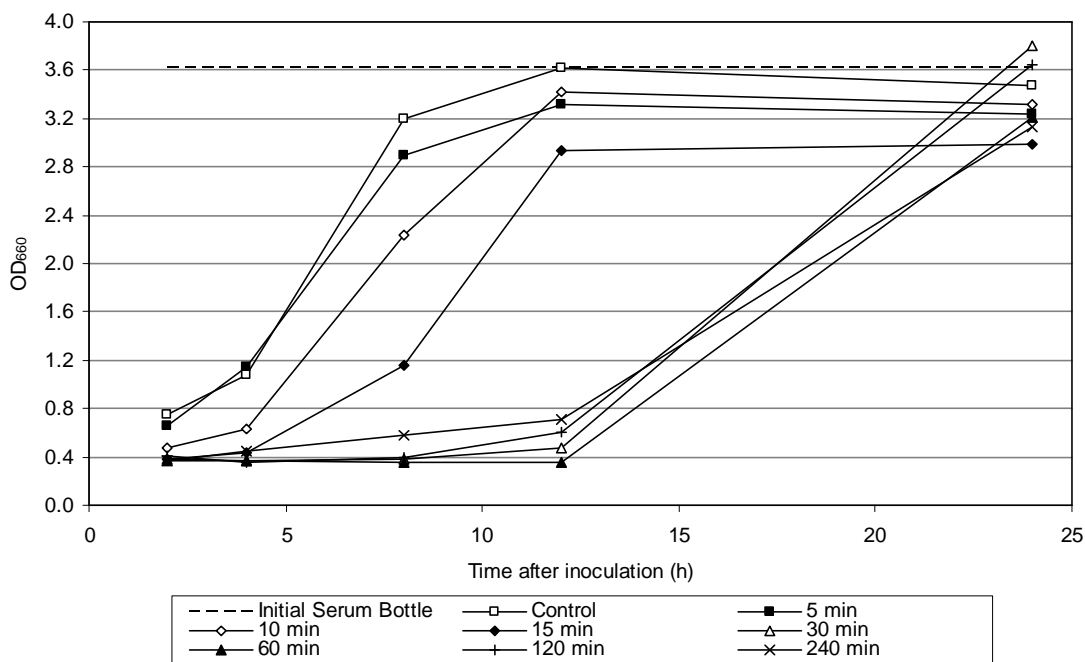
#### **4.4.6 Analytical Methods**

Cell concentration was measured using optical density in a spectrophotometer at 660nm ( $OD_{660}$ ) (Ultrospec 3000, Pharmacia Biotech, Sweden). Cell dry weight was calculated from a calibration curve where an absorbance of 1.0 equalled a cell dry weight of 401.2mg/L. Acid products were measured using High-Performance Liquid Chromatography (Varian Prostar, Mississauga, ON) using a Varian Hi-Plex H column (300 x 7.7 mm) with a 10mM H<sub>2</sub>SO<sub>4</sub> mobile phase at 0.7mL/min and 60°C. Glucose was measured using a DNS assay as described by Miller (1959) which contained the following components (in g/L): sodium potassium tartate, 200; NaOH, 10; dinitrosalicylic acid, 10; sodium sulphite, 4; phenol, 2. The absorbance of DNS assay samples were measured in a spectrophotometer at 540nm.

## **4.5 Results and Discussion**

### **4.5.1 Cell Exposure to a Low pH**

Figure 4-1 shows the optical density of the cells in serum bottles from inoculation after removal from low pH conditions. Each line represents a different exposure time to low pH conditions, ranging from 5 minutes to 4 hours as well as a control sample without any exposure. Also included in this figure is the optical density of the original serum bottle from which all samples were drawn. This optical density value acted as a standard to compare against all other samples in this test. The figure shows that the control test grew quickly and reached a maximum OD within 12 hours. 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 minutes shared the same trend and reached a maximum OD after 12 hours at normal pH conditions. Samples with exposure times of 30 minutes to 4 hours had a distinct lag phase which lasted for a minimum of 12 hours. At some point between 12 and 24 hours, the OD<sub>660</sub> of the serum bottles increased and rose to levels comparable with the control and samples showing no lag phase.



**Figure 4-1: Optical density over time after low pH exposure**

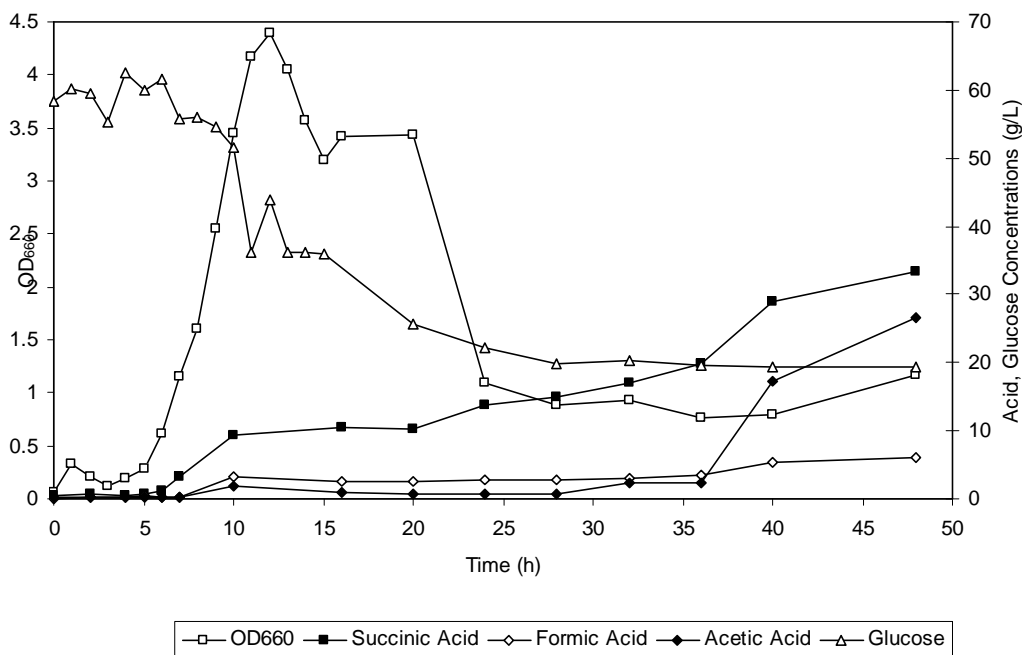
These results show that a longer cell exposure time led to a longer cell recovery time for the resumption of cell growth. However, the most important information from this test was that *Actinobacillus succinogenes* withstood up to four hours of low pH conditions and the biomass formation recovered to levels similar to those of 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 hindering cell growth. In this test, however, only the optical density of the cells was tracked, so effect on succinic acid production is unknown. Repeating the test and monitoring succinic acid production would determine whether there any negative effects on the cells and their production ability. Previous research into polymer uptake of target molecules indicated that times required to reach equilibrium were on the order of one hour, potentially, minimizing the negative effect of low pH exposure on cells [Daugulis et al., 2003, Prpich and Daugulis, 2004].

The above tests were conducted by inoculating the fresh serum bottles from the one containing the cells at pH 4.2. As such, the cells had to grow in the fresh serum bottles whereas in a bioreactor the cells would not have to regrow but only return to producing succinic acid, which would shorten lag times in the bioreactor compared to these serum bottle tests. Using a TPPB system, which does not require cell separation as a pre-treatment step, also eliminates the need to restart the bioreactor from frozen stock.

Previous experiments described in Chapter 3, Figure 3-13, indicated that lowering the pH of the system to 4.2 may not be effective based on succinic acid removal using Hytrel<sup>®</sup> 8206. Uptake at pH 3.8 was suggested in Chapter 3 to avoid low succinic acid uptake from fermentation broth. With a short exposure time to pH 3.8, it was expected that the cells would experience less lag before bioproduction continued than samples at pH 4.2 for longer periods.

#### **4.5.2 Initial Single-Phase Biotransformation**

The initial bioconversion of glucose to succinic acid was performed at 37°C and 200rpm. CO<sub>2</sub> gas was sparged into the system at 0.4vvm and the pH was maintained at 6.7 using 5M NaOH. The biomass, glucose and product concentrations over time are shown in Figure 4-2. The main point of interest in this figure was that the optical density reached over 4.0 after 12 hours, then dropped to 25% of its maximum value. This drop in optical density was due to cell flocculation which was confirmed visually by the presence of 1mm diameter particulates in bioreactor samples, similar to the results of Liu et al. (2008b). Overall, glucose was not completely consumed and the volumetric productivity of succinic acid was low, only reaching 0.688g/L·h after 48 hours. Previous research indicated that the same cell type in the same bioreactor operating conditions would completely consume an initial glucose concentration of 55g/L [Liu et al, 2008a].



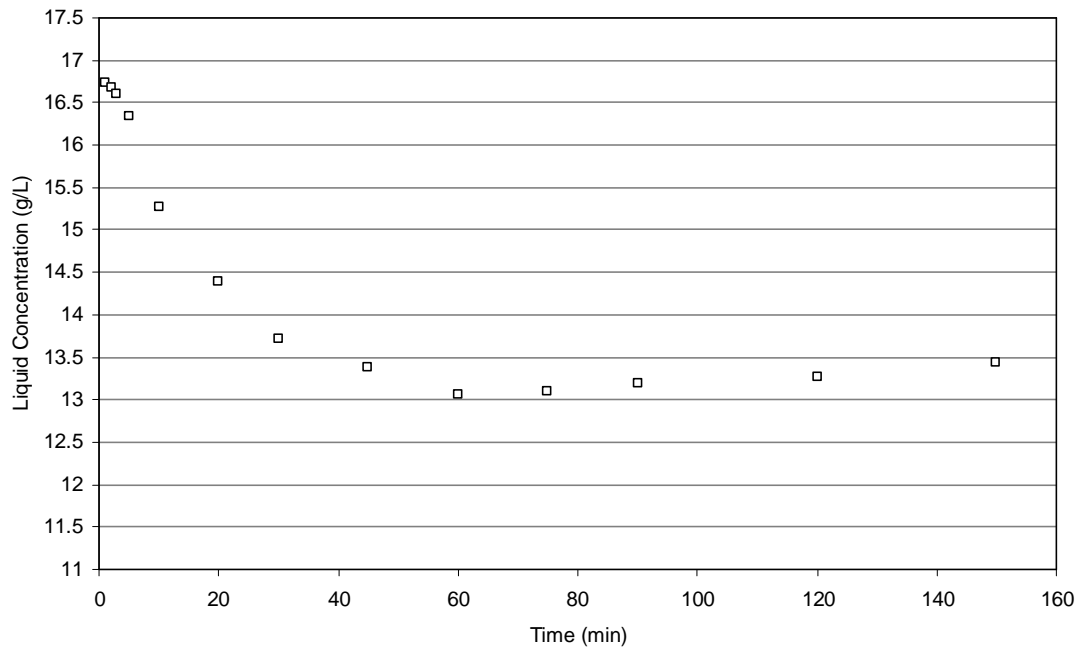
**Figure 4-2: Products, glucose and OD<sub>660</sub> for initial biotransformation**

The succinic acid concentration reached 33g/L after 48 hours while formic and acetic acid levels stayed close to 5g/L. After 36 hours, acetic acid apparently increased sharply, reaching 27g/L after 48 hours. This apparent increase was an artefact of overlap of fumarate and acetate elution in the HPLC and the accumulation of fumarate in the fermentation broth. Fumarate is an intermediate molecule on the anaerobic pathway to the production of succinic acid, causing it to increase as succinic acid production slowed. Overall, the results from this single-phase run indicated three areas for improvement with regard to enhancing cell growth and succinic acid production. The first recommendation was to replace NaOH with KOH for pH control. NaOH was shown by Liu et al (2008b) in previous research to cause cell flocculation, leading to a reduced succinic acid concentration. The second recommendation was to increase the agitation rate in the bioreactor from 200rpm to 500rpm. The increase in agitation would also reduce flocculation by increasing the shear forces on the flocs. The same study which showed the negative effects of NaOH for pH control determined that the use of magnesium carbonate

(MgCO<sub>3</sub>) for pH control and CO<sub>2</sub> addition gave the highest succinic acid concentration in their study, approximately 50g/L [Liu et al., 2008b]. While the third recommendation would be to use MgCO<sub>3</sub>, this strong buffer would greatly reduce the ability of carbon dioxide gas to lower the pH of water, as seen in Section 3.5.2. Its effect in the bioreactor was shown in Appendix A, but the conclusion was to add 5g/L MgSO<sub>4</sub> to the growth medium rather than MgCO<sub>3</sub>. The sulphate salt gave the benefit of the magnesium ion without adding a strong buffer to the system. A bioreactor run was prepared with KOH for pH control, an agitation rate of 500rpm and 5g/L MgSO<sub>4</sub> present. These changes led to an increase in glucose consumption and produced succinic acid to a concentration of 39g/L after 28 hours, a volumetric productivity of 1.39g/L·h (see Appendix A). Having improved the final succinic acid concentration and volumetric productivity, the focus of research became polymer uptake from fermentation broth.

#### **4.5.3 Polymer Uptake from Fermentation Broth**

To this point, polymer uptake was measured in abiotic solutions containing only succinic acid. New partition coefficient tests were undertaken in fermentation broth to determine the uptake results relative to the abiotic study. For this test of uptake over time, 1kg of Hytrel<sup>®</sup> 8206 was placed directly into a bioreactor containing 3.7L of fermentation broth at the end of a biotransformation. The polymer fraction in the bioreactor for this test was approximately 26% (w/v). The pH of the system was lowered to 3.8 and maintained using 5M H<sub>2</sub>SO<sub>4</sub>. The agitation rate was set to 500rpm and the liquid concentration was monitored over 3 hours. The results are shown in Figure 4-3.



**Figure 4-3: Polymer uptake of succinic acid from fermentation broth**

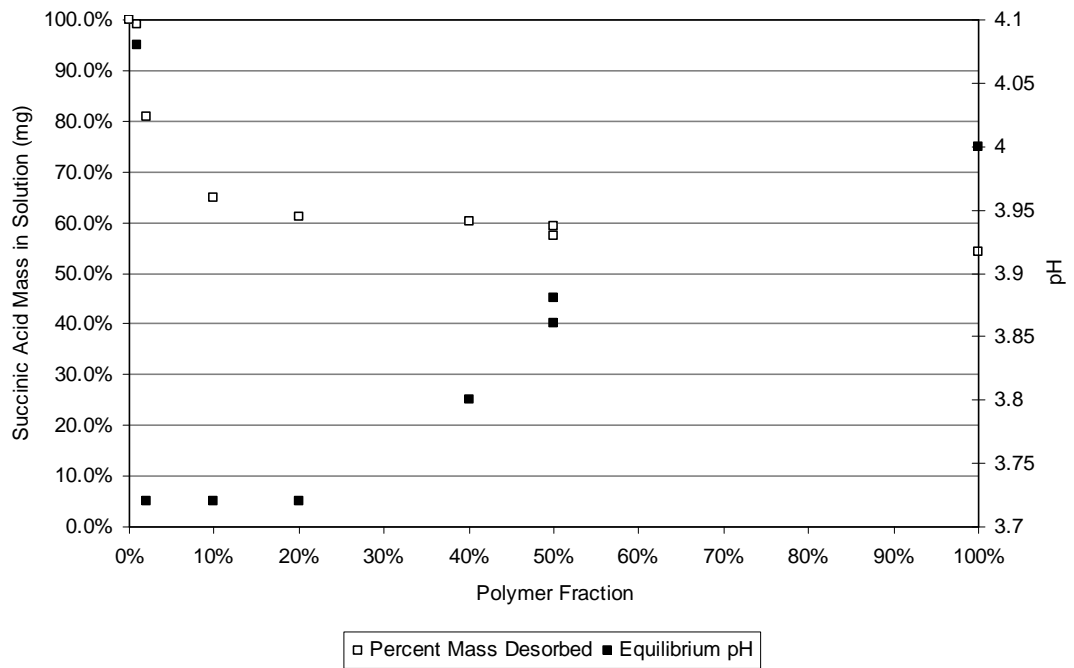
Figure 4-3 shows that the liquid concentration decreased quickly through the first hour, then levelled off for the remainder of the experiment. At the agitation rate used, the time required for succinic acid uptake into the polymer phase was 60 minutes. Based on the experiments subjecting cells to a low pH, one hour at pH 3.8 for succinic acid uptake was acceptable. The exposure time required for polymer uptake was 25% shorter than the maximum time tested in section 4.5.1 which showed that there would be no lasting negative effects on the cells. The succinic acid uptake from the fermentation broth gave a polymer capacity of 13.1mg/g, comparable to studies performed in Section 3.5.3 where the capacity was 12mg/g in abiotic capacity tests. The reason for this difference may have been due to pH maintenance at 3.8 rather than no pH control in abiotic tests in Section 3.5.3. pH increases during succinic acid uptake led to a decrease in the mass of succinic acid removed by changing the ratio of undissociated to dissociated molecules in the abiotic tests. Maintaining the pH at a fixed value ensured that the ratio of succinic acid to succinate remained unchanged.



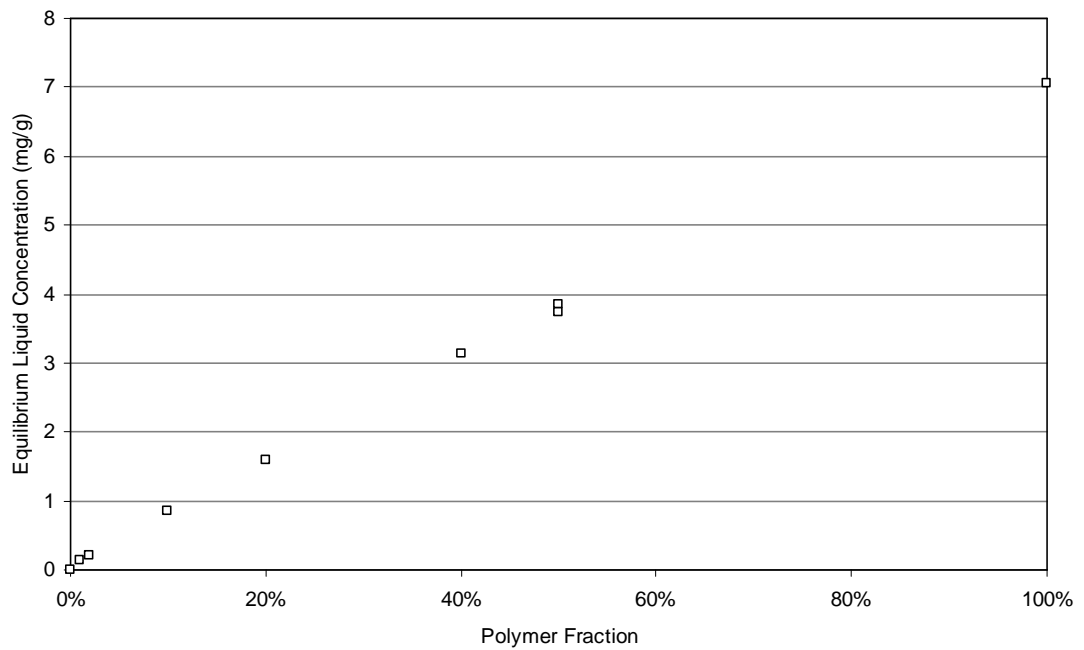
Using the equilibrium concentration in the liquid and polymer phases, a partition coefficient was calculated to be 1.00. This partition coefficient was lower than the previous values, but the difference may be due to the various salts in solution, possibly the other products and intermediates of the bioreaction; formate, malate and fumarate. The effects of succinic acid in its dissociated form were known to decrease the partition coefficient values in Section 3.5.4. The acids mentioned above have  $pK_A$  values below 3.8 and in order to maintain equilibrium they resisted the removal of protons from the solution through the protonation of succinate. In spite of this, succinic acid was successfully removed from the liquid phase using polymer materials, which were easily separated from the fermentation broth. The next research focus was desorbing succinic acid from the polymer beads.

#### **4.5.4 Desorption of Polymers Removed from Fermentation Broth**

Desorption was carried out using the polymers from the previous section with a succinic acid capacity of 13mg/g. Various polymer fractions were tested ranging from 1% to 100% (w/v) polymer to RO water. The percent of succinic acid desorbed from the polymer phase after one wash as well as the equilibrium pH values of solution are shown in Figure 4-4a. Figure 4-4b shows the equilibrium liquid phase concentrations of succinic acid as a function of polymer fraction. A polymer fraction of zero shows the control sample with a succinic acid mass of 130mg in 10g of polymers in Figure 4-4a and as a liquid equilibrium concentration of zero in Figure 4-4b.



(a)



(b)

**Figure 4-4: (a) Succinic acid mass desorbed as a percentage of total mass in the polymer phase and equilibrium pH after desorption and (b) equilibrium liquid concentration of succinic acid**

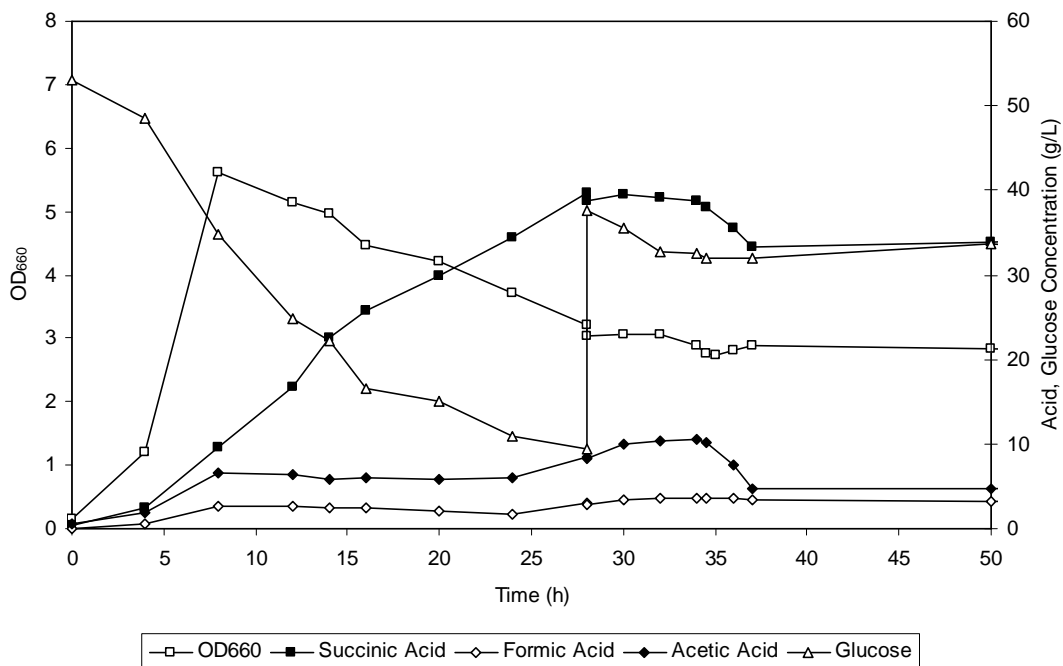
Acetic acid was present in all tests in the liquid phase at equilibrium, but its mass was less than 10% of the succinic acid mass in solution (data not shown). The 1% polymer fraction sample resulted in 99% desorption of succinic acid from the polymer phase. The high mass of succinic acid in the aqueous phase indicated almost complete desorption from the polymer with an equilibrium pH of 4.08. Doubling the polymer fraction led to a decrease in the percent of succinic acid desorbed from 99% to 81%. The next two samples also showed decreases of succinic acid in solution, but the interesting data were the equilibrium pH values at these polymer fractions. The samples with polymer fractions of 2%, 10% and 20% had an equilibrium pH of 3.72. This indicated that in these samples, desorption of succinic acid into the liquid phase was limited by low pH rather than solubility or partition coefficient. Similar to how a high pH impeded further uptake of succinic acid in Section 3.5.4, a low pH impeded further desorption of succinic acid into RO water. At pH 3.72, the succinic acid was more in the undissociated state rather than dissociated in solution. More desorption from the polymers would further decrease the pH, bringing it further away from the  $pK_{A2}$ . Instead, equilibrium was reached between the liquid phase and polymer phase where the pH limited further desorption from the polymers, even though the partition coefficient of these three samples was higher than values from the other desorption samples or those calculated in Section 3.5.5.

In desorption samples where the polymer fraction was 40% or higher, the partition coefficient was reached before decreasing pH hindered further desorption. As a consequence, the equilibrium pH was higher than the lower limit of 3.72. In all samples, over 50% of the total succinic acid was desorbed from the polymers and the lowest equilibrium liquid mass observed was 70.57mg. A second round of washing was prepared by replacing the water volume to maintain the same polymer fractions and allowing the samples to reach equilibrium again. The results from the second round of desorption indicated that polymer fractions of 50% or higher did not show at least 90% total desorption (data not shown). The conclusion from this experiment was that a polymer fraction of 40% and 2 washes with RO water gave the best desorption of

succinic acid. It used the least amount of water while removing over 90% of succinic acid from the polymer phase.

#### 4.5.5 Bioreactor pH Cycling to Remove Succinic Acid

The results from Section 4.5.1 on cell exposure to low pH and Section 4.5.3 on succinic acid uptake were used to perform a bioreactor cycle. The bioreactor was prepared as described previously, including 5g/L MgSO<sub>4</sub> and 1kg of Hytrel<sup>®</sup> 8206 added prior to autoclaving. Temperature and agitation were maintained at 37°C and 500rpm, respectively. pH was controlled at 6.7 using 5M KOH. The results of the bioproduction and cycling process are shown in Figure 4-5.



**Figure 4-5: Products, glucose and OD<sub>660</sub> for final biotransformation including pH cycling and polymer uptake**

Figure 4-5 shows that the optical density increased to a higher level than the initial single-phase run and remained at a high value for longer due to the addition of MgSO<sub>4</sub> to the growth

medium. At 28 hours into the biotransformation, a 300mL autoclaved bolus 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. 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 increase back to 55g/L or higher. Prior to bolus addition, the concentration of succinic acid was at its highest, reaching 40g/L at 28 hours, giving a volumetric productivity of 1.42g/L·h. The succinic acid concentration achieved was comparable to the final single-phase run from Appendix A.

At 34 hours, the pH of the system was lowered to 3.8 using 5M H<sub>2</sub>SO<sub>4</sub>, taking 30 minutes. 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 left for 60 minutes. The decrease in the succinic acid concentration from 34.5 hours to 35.5 hours was due to polymer uptake from the fermentation broth. The decrease in succinic acid over this time resulted in a polymer concentration of approximately 10g/L, a value close to those found in Section 4.5.3. After one hour had passed, the separation of polymer beads and fermentation broth was achieved by pumping the liquid from the first bioreactor into a second, autoclaved bioreactor. The pH of the fermentation broth was then raised to 6.7 using 5M KOH, which required 30 minutes. The bioreactor then continued to run after 36 hours but cell growth did not resume, glucose was not consumed and no additional products were formed. This lack of cell growth seemed to contradict the results of Section 4.5.1, but the difference can be explained by the presence of salts, both from pH adjustment and nutrient addition. In pH exposure tests using serum bottles, cells were transferred to fresh bottles which did not already contain products of the bioreaction. The increased presence of salts in the bioreactor, especially from the use of H<sub>2</sub>SO<sub>4</sub> and KOH to adjust the pH, hindered further cell growth and succinic acid production. In spite of this lack of cell growth, the two-phase run did prove that succinic acid can easily be removed

from solution using polymer beads with minimal downstream processing required and no fermentation broth pre-treatment.

## **4.6 Conclusion**

Cell exposure to pH 4.2 for up to four hours did not permanently affect the ability of cells to grow. A distinct difference in recovery time was noticed in tests with low pH exposure for 30 minutes or longer. A lag phase lasted for at least 12 hours but after 24 hours at operational pH conditions, all serum bottles showed the same optical density as the control test. The first attempt at a single-phase run showed incomplete consumption of glucose and a lower final succinic acid concentration than other values reported in the literature. After 48 hours, the succinic acid concentration was 33g/L, giving a volumetric productivity of 0.688g/L·h. The low productivity was remedied by using KOH for pH control, adding MgSO<sub>4</sub> to the growth medium and increasing the agitation rate of the impellers. The optimized single-phase run gave a final succinic acid concentration of 39g/L after 28 hours.

Succinic acid uptake into the polymer phase was tested using fermentation broth containing all products and intermediates. The results were comparable to those obtained during physical testing of Hytrel® 8206 in abiotic solutions. One hour was required for the polymer phase to reach equilibrium in removing succinic acid. Because of this short exposure time, there was to be no adverse effects on the cells in terms of exposure length to low pH conditions during polymer uptake. Desorption of succinic acid from the polymer phase showed that, at equilibrium, the majority of samples desorbed to match the partition coefficient calculated in Chapter 3. The suggested strategy was to use two RO water washes with a polymer fraction of 40% to desorb over 90% of the succinic acid present in the polymer phase.

Combining the information on low pH exposure, the single-phase run and the polymer uptake data, a two-phase run was performed in which the pH was 'cycled' to remove succinic

acid using polymer beads. While succinic acid removal was successful, the cells did not recover, no further glucose was consumed and succinic acid production ceased. The reason cell growth ceased was due to the presence of salts from pH adjustment hindering continued succinic acid production. This bioreactor cycling system was proof of concept that succinic acid can be removed using polymer materials. Using polymer beads to remove succinic acid can take place without pre-treatment of the fermentation broth. Cells were retained in the fermentation broth and no complex downstream separation methods were required to desorb succinic acid from the polymer phase. Future work should include system improvements in terms of cell growth and succinic acid production as well as mass of succinic acid removed using polymer beads. These improvements will allow succinic acid bioproduction to become effective on an industrial scale. Developing a system which allows for continued succinic acid production after polymer uptake will increase volumetric productivity by eliminating the cell growth period required for restarting a bioreactor. Finding a polymer which can absorb more succinic acid from the fermentation broth will increase separation efficiency and decrease end product inhibition, allowing for greater succinic acid production after pH cycling and polymer uptake occurs.

## 4.7 References

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## 5.0 Conclusions and Recommendations for Future Work

### 5.1 Conclusions

In a bioreactor containing only RO water, carbon dioxide gas at atmospheric pressure was able to lower the pH of solution to 3.85 in under two minutes. However, the presence of growth medium components and products in the bioreactor hindered pH reduction. If the pressure of CO<sub>2</sub> can be increased, the solubility of carbon dioxide in water will increase and a lower pH can be reached in the presence of growth medium. The use of CO<sub>2</sub> gas at high pressures will eliminate the need for H<sub>2</sub>SO<sub>4</sub> to lower pH for succinic acid uptake as was required in this research.

Hytrel<sup>®</sup> 8206 was the only polymer that absorbed succinic acid to any significant extent. It had a partition coefficient of 1.3 when the pH of succinic acid in RO water was below the pK<sub>A2</sub>. pH levels at 4.1 and above decreased uptake into the polymer and did not yield a partition coefficient. A pH of 3.8 was used for polymer uptake after bioproduction ceased. The components of the minimal growth medium used in the bioreactor had no effect on polymer uptake or the partition coefficient at or below pH 3.8. Studies of polymer uptake in a bioreactor at pH 3.8 showed that one hour was the maximum time required for succinic acid to absorb into the polymer phase. Desorption studies showed that a 40% (w/v) polymer fraction with 2 washes in RO water was sufficient in desorbing 90% of succinic acid from the polymer phase.

Studying the effect of low pH on *A. succinogenes* showed that cells could grow after up to 4 hours of exposure at pH 4.2. Samples with up to 15 minutes of exposure showed no growth lag while samples with exposure times of 30 minutes to 4 hours showed at least a 12-hour lag period before cell growth began. 24 hours after low pH exposure ceased, all samples reached the same optical density as the control sample.

Succinic acid was removed from fermentation broth using polymer beads during one cycle where the pH was lowered to 3.8 for one hour, then raised to 6.7. The cells in the bioreactor could not continue succinic acid production because of the salts added for adjusting the pH with

H<sub>2</sub>SO<sub>4</sub> and KOH. Succinic acid was removed from the bioreactor in one hour while retaining the cells and without the need for pre-treatment, proving the concept that a TPPB can improve succinic acid production through polymer uptake, reducing end product inhibition.

## **5.2 Future Work**

While carbon dioxide could reduce the pH of RO water to 3.8 at atmospheric conditions, K<sub>2</sub>HPO<sub>4</sub> and yeast extract in the growth medium prevented pH reduction to this level. An external vessel which can withstand high pressures should be used to increase the solubility of carbon dioxide in fermentation broth. This will increase the concentration of carbonic acid formed to lower the pH of the solution for succinic acid uptake. At higher pressures, decreasing the temperature of the solution increases the solubility of carbon dioxide in H<sub>2</sub>O. As such, tests should be performed with high CO<sub>2</sub> pressures while decreasing the temperature below those values tested in Chapter 3. As the fermentation broth will contain many salts, the freezing point of the liquid will be reduced. Studying the impact of temperature on CO<sub>2</sub> solubility and pH should include temperatures down to the freezing point of fermentation broth as they will give the highest solubility of carbon dioxide gas before the liquid freezes. To this point no data have been found for CO<sub>2</sub> solubility below 0°C and the effect of low temperatures on cells must also be studied.

Physical testing showed that only Hytrel<sup>®</sup> 8206 was effective at removing succinic acid from abiotic samples, although the partition coefficient was minimal compared to other compounds and polymers tested in the Daugulis group. Synthesizing polymers with greater succinic acid uptake must take place by systematically determining which polymer functional groups give the best succinic acid absorbance while maintaining a low crystallinity. pH 3.8 was recommended for succinic acid uptake in the bioreactor though no abiotic partition coefficient samples were tested at this initial pH level. Therefore, a test to determine the partition coefficient

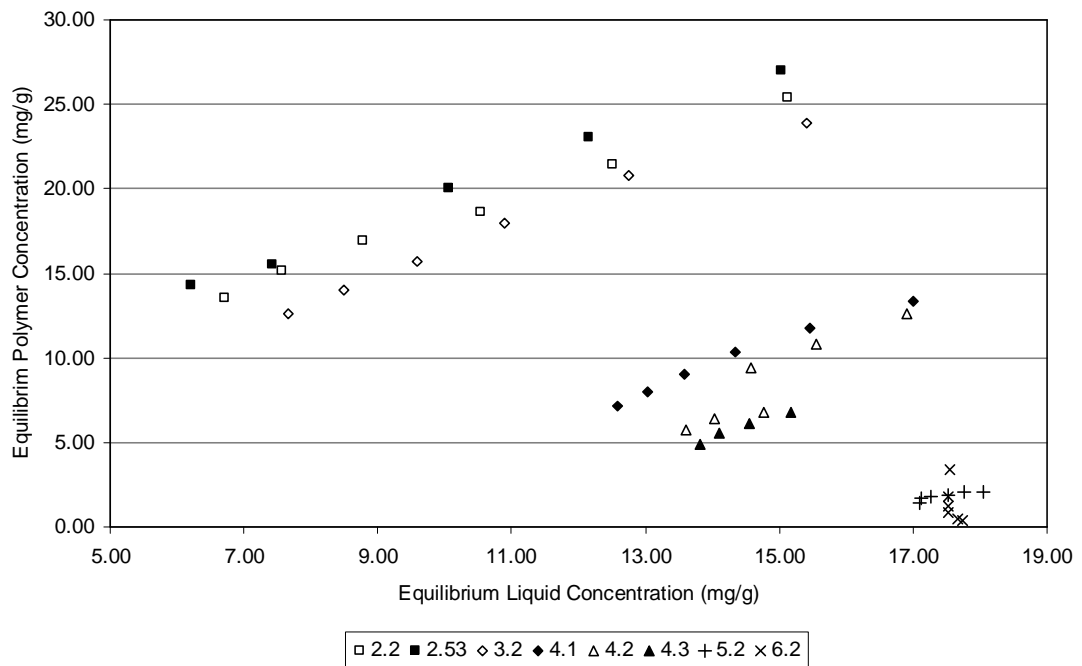
at pH 3.8 is required to confirm that succinic acid uptake remains high as the equilibrium pH approaches 4.2. Tests in Section 3.5.5 showed that succinic acid uptake was reduced as the pH of the solution approached 4.2. Testing the effect of pH on the partition coefficient must be repeated in the presence of pH control. These new tests with pH control will determine if succinic acid uptake is feasible near pH 4.2. Succinic acid uptake from a solution at 4.2 will reduce the CO<sub>2</sub> pressure and low temperature required to reach the target pH.

In terms of biological testing, another study is potentially required on cell exposure to a low pH, taking place at 3.8 rather than 4.2. A new pH exposure test will reflect the recommendation that succinic acid uptake occur at 3.8. As pH control reduced succinic acid production, new methods are required allowing cells to produce succinic acid to higher concentrations than with KOH. In Chapter 4, KOH in the presence of MgSO<sub>4</sub> gave a succinic acid concentration of 39g/L after 28 hours. However, 48g/L was reached after 28 hours when using MgCO<sub>3</sub> for CO<sub>2</sub> addition and pH control. Appendix A showed that a reduced concentration of MgCO<sub>3</sub> in the bioreactor did not hinder pH adjustment as it was entirely consumed. A hybrid pH control method is recommended where a low concentration of MgCO<sub>3</sub> is first used until pH 6.5 is reached, then KOH is used for the remainder of the biotransformation. While bolus addition to the bioreactor can replenish substrate and nutrients, decreased succinic acid production can occur with the addition of excess salts. In Section 4.5.5 the succinic concentration did not increase after bolus addition. Decreased succinic acid production from bolus addition cannot be confirmed, but the effect of the salt addition must be isolated and understood. A study is required to determine the mass of growth medium added before bioproduction and cell growth is halted due to excess salts in the system. The effect of salts hindering cell growth should include glucose additions as a large mass of glucose may reduce bioproduction as well. Literature shows data on maintaining glucose levels in the bioreactor yet no research exists on one-time glucose addition. The effect of one-time glucose addition on succinic acid production and cell growth is required.

## Appendix A: Water Uptake using Hytrel<sup>®</sup> 8206

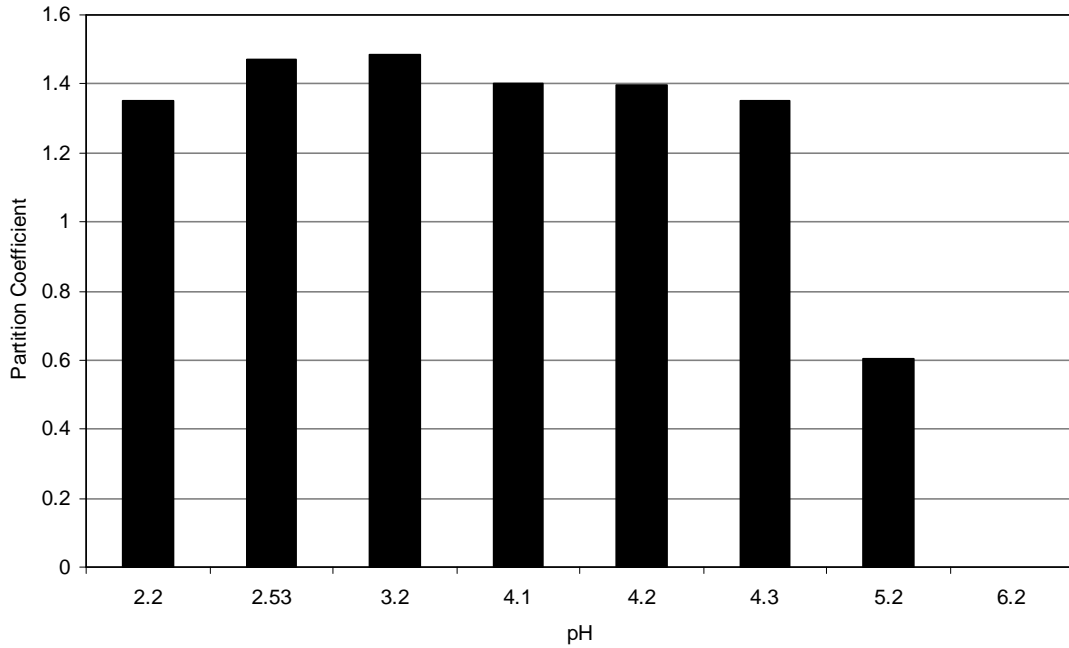
In Section 3.5.5, succinic acid uptake as a function of pH was studied with initial pH conditions in solution ranging from 2.2 to 6.2. The results indicated that when the pH was between 2.2 and 3.2, the equilibrium polymer concentrations changed similarly as polymer fractions increased. This led to similar partition coefficient values, as seen in Figure 3-13b. In tests where the initial pH ranged from 4.1 to 4.2, uptake was seen but as the polymer fraction increased, the linear trend seen in previous results broke down and at pH 4.3, all data points clustered in one location, yielding a partition coefficient of zero. Uptake at pH 5.2 and 6.2 showed an increase in the equilibrium liquid concentration of succinic acid, which led to consideration of water absorbance by the polymer. The only way that the liquid concentration of succinic acid could increase was through an addition of succinic acid to the liquid phase or a removal of water without succinic acid uptake. As the undissociated form of succinic acid is required for uptake, at pH 5.2 and 6.2, none of the target molecule was moving into the polymer phase, indicating that water was moving into the polymer phase, effectively concentrating the succinic acid in the liquid phase.

To determine a true estimate of succinic acid uptake into the polymer phase, the water absorbed by the polymer had to be factored into equilibrium liquid concentration measurements. Research from DuPont Canada indicated that the water absorbance of Hytrel<sup>®</sup> 8206 was 30%, leading to a water removal of 0.45 to 2.7 grams of water over the range of polymer fractions tested. Recalculating the equilibrium liquid concentrations with this water loss added led to the data seen in Figure A-1.



**Figure A-1: Equilibrium liquid and polymer concentrations as a function of initial pH, factoring in water absorbance by the polymer phase**

The data in the figure above closely resembled that in Figure 3-13a from pH 2.2 to 3.2. The main change in this new data was a decrease in the equilibrium liquid concentrations, leading to an increase in the equilibrium polymer concentrations. The change to the results caused a shift in the data up and to the left, though the trend in the data points remained the same. The most interesting data came from the results at pH 4.1 to 4.3 where the trends changed. In Figure A-1, the results at pH 4.1 to 4.3 showed very similar slopes to those at the lower pH values. It was not until pH 5.2 that the results formed a different curve. The equilibrium concentration of succinic acid in the polymer phase showed almost no change with increasing polymer fraction. At pH 6.2 the data showed no trend at all and were all located in one area with almost no uptake of succinic acid. Calculating the partition coefficient values from the figure above gave the results in Figure A-2.



**Figure A-2: Partition coefficient values as a function of initial pH**

Figure A-2 showed that the partition coefficient values are the same as in Section 3.5.5 when swelling was not factored into calculations for equilibrium liquid concentration. More importantly, however, was that through these new calculations, similar partition coefficient values were found up to pH 4.3. This indicated that while at higher pH values less succinic acid was removed from solution compared to the water absorbed, succinic acid was still removed to give a partition coefficient. At pH 5.2 where the percent of undissociated acid dropped to approximately 9%, succinic acid uptake was much lower and did not give a partition coefficient as high as at lower pH values. Again at pH 6.2 the partition coefficient was zero as there was no trend in the data in Figure A-1.

The conclusion from this set of calculations was that when the water absorbed from solution was factored into the equilibrium concentrations of succinic acid in the liquid phase, the results give a more expected trend. In spite of the water removed from solution, succinic acid was still being absorbed though the effect was masked by water absorption changing the succinic acid

concentration in solution. In practice, however, a higher mass of succinic acid was removed from the liquid phase at pH 3.2 and lower, so uptake at pH 4.1 to 4.3 was not practical. All following research in this project still continued to use pH 3.8 to ensure a high mass of succinic acid removed from solution.



## **Appendix B: Improved Organism Performance using MgCO<sub>3</sub>**

### ***B.1 Introduction***

The first biotransformation shown in Chapter 4 did not yield a high succinic acid concentration relative to other reports in the literature under the same conditions. In studies on the optimal glucose concentration for *A. succinogenes*, 55g/L in solution was shown to give the highest succinic acid concentration while showing no residual glucose in the bioreactor after 60 hours [Liu et al., 2008a]. This showed that the single-phase biotransformation in Chapter 4 was not as efficient as possible, which may have been due to cell flocculation as a stress response to non-ideal conditions in the system, particularly NaOH for pH control [Liu et al., 2008b]. Research by Liu et al. (2008b) showed similar results when NaOH was used for pH control in a bioreactor. Flocculation occurred after 16 hours, glucose was not completely consumed and succinic acid concentrations were low; below 30g/L after 40 hours [Liu et al., 2008b]. The explanation was that as the sodium ions accumulated in the fermentation broth, the osmolarity of the solution changed and the cells flocculated as a stress response. The study by Liu et al (2008b) was the only one to show the negative impacts of NaOH for pH control. Other research did not encounter flocculation using NaOH because the growth media contained buffering compounds in higher concentrations than the minimal growth medium used in this research.

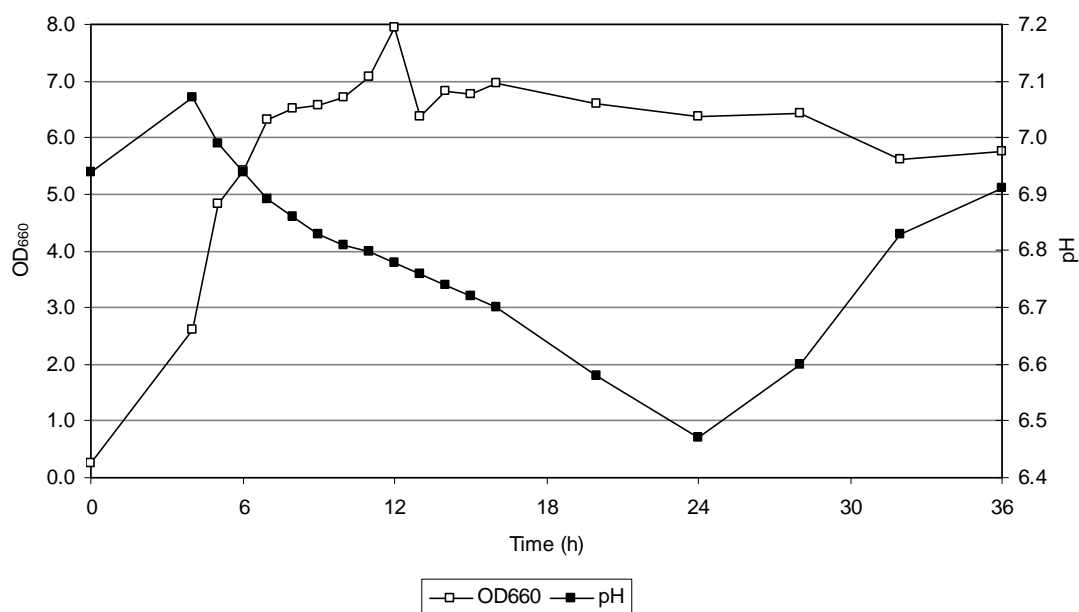
In an effort to improve the single-phase run and achieve a higher succinic acid concentration, recommendations were followed from Liu et al. (2008b) and magnesium carbonate was added to the bioreactor. The use of magnesium carbonate (MgCO<sub>3</sub>) for pH control was first used in the patent presented by Guettler et al. (1997). Solid magnesium carbonate was added to the bioreactor producing a slurry due to the low solubility of the salt. It dissociated into the magnesium cation and a carbonate anion, CO<sub>3</sub><sup>2-</sup>. The anion reacted with protons released during

bioproduction to form carbon dioxide and water. The use of magnesium carbonate acted as both a pH controller and a source of CO<sub>2</sub> for the tests in this study [Liu et al., 2008b].

Of five pH controlling agents tested by Liu et al. (2008b), MgCO<sub>3</sub> was the only one to effectively control the pH without causing cell flocculation. Tests using this buffer completely consumed 55g/L of glucose and produced the highest concentration of succinic acid in the study, reaching 40g/L after 32 hours. While the solid MgCO<sub>3</sub> did not maintain the pH at a specific value during fermentation, it was able to keep the pH within the optimal range for succinic acid production [Liu et al., 2008b]. Additionally, the impact of the magnesium ions on optical density and succinic acid production was tested using MgCl. The results showed that optical density remained high over the MgCl concentration tested; up to 0.3mol/L. NaCl and CaCl<sub>2</sub> were also tested and as the concentrations of these two salts reached 0.3mol/L, the optical density and succinic acid concentrations were drastically reduced.

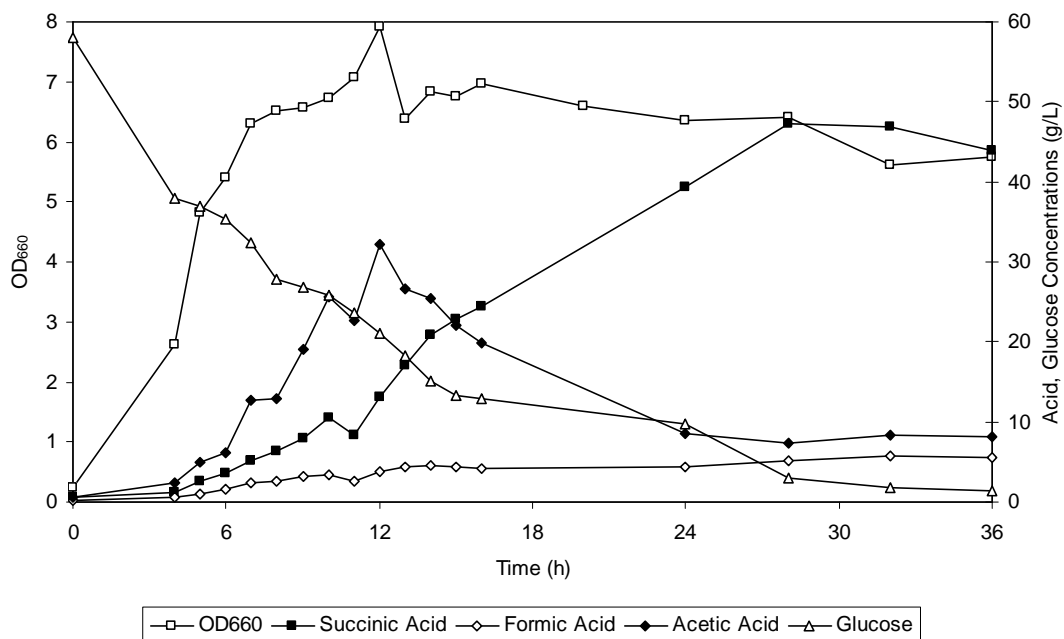
## ***B.2 Biotransformation with MgCO<sub>3</sub> for pH Control and CO<sub>2</sub> Addition***

This biotransformation was prepared as described in Chapter 4. It was conducted at 500rpm and 37°C with pH control from 55g/L MgCO<sub>3</sub>. Because CO<sub>2</sub> was generated through pH control, no gaseous CO<sub>2</sub> was added to the bioreactor. In preparation for this run, the 3x concentrated solutions of minimal growth medium, glucose and MgCO<sub>3</sub> in RO water were autoclaved separately to ensure there were no interactions between the growth medium, glucose and magnesium carbonate. After autoclaving, all three solutions were combined aseptically in the bioreactor. Prior to inoculation, the MgCO<sub>3</sub> caused the pH of the system to increase to over 8.0. Medical grade CO<sub>2</sub> gas was sparged into the bioreactor until the pH reached approximately 7.0. Figure B-1 shows the optical density and pH of the biotransformation.



**Figure B-1: OD<sub>660</sub> and pH versus time for the first MgCO<sub>3</sub> biotransformation**

Because MgCO<sub>3</sub> was used for pH control, no target pH could be set. Magnesium carbonate dissolved slowly in solution to keep the pH high, forming CO<sub>2</sub> and H<sub>2</sub>O. If the rate of proton release in the system was faster than MgCO<sub>3</sub> could dissolve, the pH decreased. As succinic acid production slowed, magnesium carbonate continued dissolving and the pH rose. Throughout Figure B-1 the pH remained above 6.4 and succinic acid production was not hindered, also evident as the optical density reached a high value of 7.0 and remained high. Measuring the optical density of the samples required dissolving solid MgCO<sub>3</sub> which was taken up with each bioreactor sample. Solid magnesium carbonate was dissolved by adding 5M H<sub>2</sub>SO<sub>4</sub> dropwise until no MgCO<sub>3</sub> was visible. Cells in solution without magnesium carbonate present were tested and the acid addition did not affect optical density results (data not shown). The product concentrations as well as glucose and optical density over time are presented in Figure B-2.



**Figure B-2: Products, glucose and OD<sub>660</sub> for the first MgCO<sub>3</sub> biotransformation**

The most important part of this biotransformation was the optical density which quickly increased and reached a level almost two fold higher than the first bioreactor run in Chapter 4. The level of optical density remained high throughout the experiment, only decreasing a small amount compared to the previous test where the OD<sub>660</sub> decreased to 25% of its peak value. Because the cells were not flocculating as a stress response to changes in osmolarity from NaOH addition, they maintained productivity, shown by the near complete consumption of glucose and high level of succinic acid produced. The glucose concentration was reduced to 3g/L after 28 hours whereas in Section 4.5.2 the glucose remained at 20g/L after 48 hours. The complete consumption of glucose in this biotransformation led to a succinic acid concentration of 48g/L, giving a volumetric productivity of 1.71g/L·h after 28 hours. While the volumetric productivity did not reach the target of 2.5 g/L·h set by the US Department of Energy, this result was comparable with other research into succinic acid production [Liu et al., 2008b, Du et al., 2008, Werpy and Petersen, 2004]. The peak in succinic acid concentration occurred at 28 hours then

began to slightly decrease as the glucose in the system approached zero. This decrease in succinic acid seemed to coincide with a slight increase in formic and acetic acid concentrations. In the production of succinic acid from *A. succinogenes*, two intermediates in anaerobic bioproduction can be converted to pyruvate; oxaloacetate and malate. This conversion is an irreversible process which removes CO<sub>2</sub> from the four-carbon molecules [McKinlay and Vieille, 2008]. This conversion to pyruvate is governed by enzymes which work to produce pyruvate as the carbon dioxide present in the system decreases. As the system's only supply of CO<sub>2</sub> came from MgCO<sub>3</sub> with its low solubility, the amount of carbon dioxide present in the system decreased as production progressed, leading to a shift towards formic and acetic acid production.

The growth medium used in this work had fewer components and used smaller concentrations of salts compared to other growth media used in the literature. This decrease in inputs will lower expenses for an industrial process, helping the bioproduction of succinic acid to better compete with chemical synthesis. Unfortunately, the MgCO<sub>3</sub> used in this biotransformation was not completely consumed and a large mass remained in the bioreactor after 36 hours. Given the previous study of individual growth medium components on pH adjustment, any magnesium carbonate present in the system would greatly hinder the ability of carbon dioxide gas to lower the pH. While this run was considered a success in terms of high optical density and succinic acid production, the excess magnesium carbonate presented a problem. Not only is MgCO<sub>3</sub> reduction required to allow for pH adjustment with CO<sub>2</sub>, but remaining tests in this research required acids and bases for pH adjustment. An excess of MgCO<sub>3</sub> would have required more acid to lower the pH to 3.8. Based on the excess magnesium carbonate in solution at the end of this biotransformation, a new concentration of 40g/L was calculated for the next bioreactor run.

### B.3 Biotransformation with Reduced $MgCO_3$ Concentration

The next biotransformation took place under the same conditions as above except the  $MgCO_3$  concentration was reduced from 55g/L to 40g/L. The results in Figure B-3 showed a high optical density for the length of the experiment, similar to the previous biotransformation.

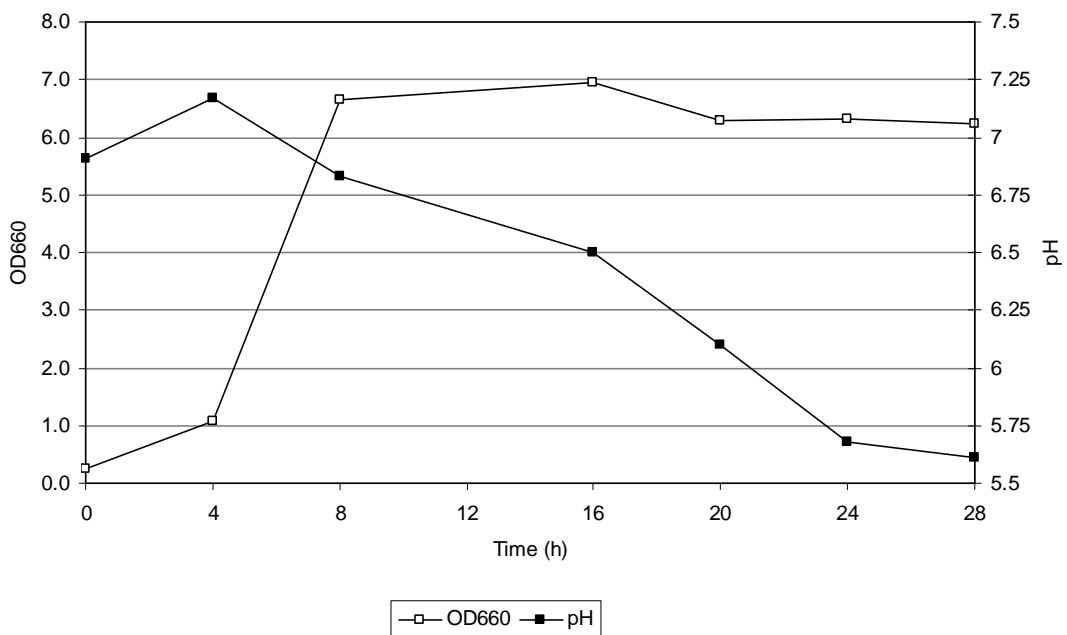
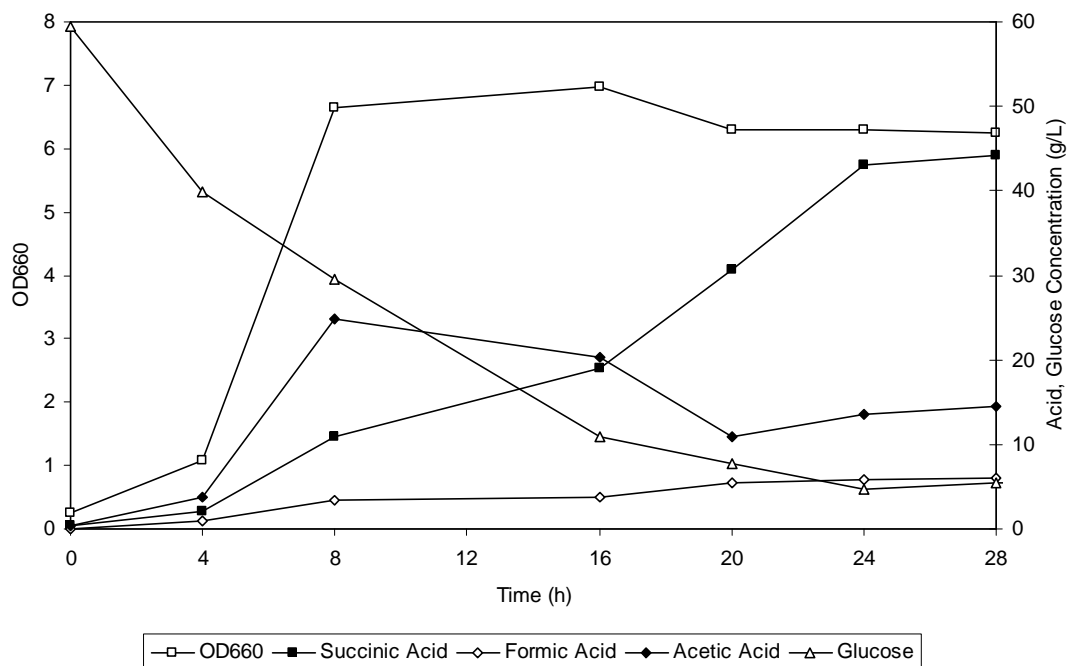


Figure B-3:  $OD_{660}$  and pH versus time for the reduced  $MgCO_3$  biotransformation

In measuring the optical density of the system, the magnesium carbonate was dissolved as described previously, but in this experiment, the magnesium carbonate had completely dissolved after 20 hours. As the pH continued to decrease after 20 hours, the  $MgCO_3$  dissolved in solution was completely consumed which caused different behaviour of the bioreactor pH. In the previous run, the pH reached its lowest point of 6.47 after 24 hours, then increased to 6.9. In this most recent test, pH 6.5 was passed just after 16 hours and continued to drop until the end of the experiment where the final measured pH was 5.61. Previous research showed that at this pH level, succinic acid production would not occur, which was visible in Figure B-4 [Liu et al., 2008b].



**Figure B-4: Products, glucose and OD<sub>660</sub> for third biotransformation**

In this biotransformation, the glucose was not completely consumed and appeared to level off near 24 hours, which occurred at the same time that the pH of the system fell below 6.0. Until this point, the consumption of glucose and production of succinic acid were similar to the previous run where MgCO<sub>3</sub> was in excess. Because of the incomplete glucose consumption, the final concentration of succinic acid was 44g/L, giving a volumetric productivity of 1.57g/L·h, lower than the previous run. This difference was small, but it reaffirmed that the pH must be maintained above 6.0 to ensure high succinic acid production. The succinic acid concentration in solution continued to rise after the pH fell below 6.0, but it was a small increase compared to the succinic acid production earlier in the biotransformation. This slight increase may have been due to the conversion of intermediates along the metabolic pathway to succinic acid, but no further glucose was consumed to produce succinic acid. The decreased pH also led to an increase in acetic acid production as the final concentration reached 14.5g/L, almost double the concentration

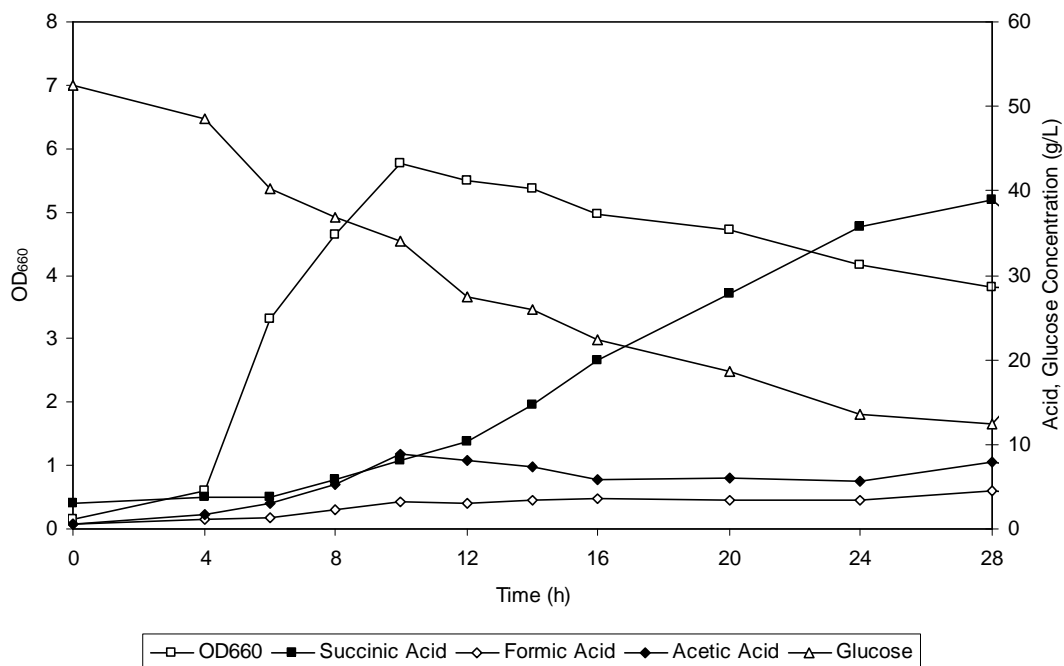
in the previous bioreactor run. This was, again, due to the lack of carbon dioxide present and the conversion of anaerobic intermediates to pyruvate, an intermediate itself for acetate and formate.

After 28 hours of running the bioreactor, CO<sub>2</sub> gas was sparged into the system to determine if the pH could be adjusted given the absence of magnesium carbonate. Unfortunately, the carbon dioxide had no effect on pH and it remained unchanged (data not shown). Even though the results indicated complete consumption of solid and dissolved magnesium carbonate, the pH could not be changed using carbon dioxide gas at atmospheric pressure. Because an excess of MgCO<sub>3</sub> would require additional acid to lower the pH to 3.8 and the minimized buffer amount led to an incomplete consumption of glucose, magnesium carbonate was ruled out as a candidate for pH control. Given the benefits of the magnesium ion using MgCO<sub>3</sub>, an alternate form of magnesium was supplied to the system to maintain a high optical density in future biotransformations.

#### ***B.4 Biotransformation using MgSO<sub>4</sub> for Flocculation Control***

For this biotransformation, pH was controlled at 6.7 using 5M KOH and 5g/L MgSO<sub>4</sub> was added to the growth medium. Temperature, agitation and CO<sub>2</sub> sparging rates remained the same from Chapter 4 experiments at 37°C, 500rpm and 0.4vvm, respectively. The results from this bioreactor run are presented below in Figure B-5.





**Figure B-5: Products, glucose and OD<sub>660</sub> for the biotransformation with MgSO<sub>4</sub> present**

The above figure indicated that the addition of MgSO<sub>4</sub> benefited the optical density. Flocculation still occurred though it was not as severe as the first biotransformation in Chapter 4 without magnesium sulphate. Additionally, the optical density did not reach the upper values shown in the two previous bioreactor runs. Because of flocculation, glucose was not completely consumed and was only reduced to 12g/L after 28 hours. The final succinic acid concentration was 39g/L, giving a volumetric productivity of 1.39g/L-h in 28 hours. The succinic acid concentration from this bioreactor run was lower than the previous work with MgCO<sub>3</sub> present in the system, but higher than the initial biotransformation with no magnesium present. Formic and acetic acid final concentrations were both below 10g/L.

An additional test was performed attempting to adjust the pH of the fermentation broth using CO<sub>2</sub> gas. The flowrate of the gas was increased to 1.6vvm but the pH showed no change as the system was already saturated with CO<sub>2</sub> (data not shown). When the temperature was decreased from 37°C to 12°C, the pH changed from 6.7 to 6.5 while maintaining CO<sub>2</sub> sparging at

1.6vvm (data not shown). While the pH change of 0.2 was insufficient for the purposes of this research, it was an indicator that the pH can be lowered with CO<sub>2</sub> provided the solubility of the gas in the liquid can be increased by reducing temperature and/or increasing the pressure in the system.

## ***B.5 Conclusion***

In an effort to increase succinic acid production in the bioreactor, MgCO<sub>3</sub> was added for CO<sub>2</sub> addition and pH and flocculation control. The final concentration of succinic acid was 48g/L after 28 hours with a much higher volumetric productivity compared to the initial bioreactor run in Chapter 4. Because MgCO<sub>3</sub> was in excess and hindered pH adjustment using CO<sub>2</sub>, a biotransformation took place with a reduced concentration of magnesium carbonate. The reduced MgCO<sub>3</sub> concentration could not keep the pH of the bioreactor above 6.0. As a consequence, the final succinic acid concentration in the bioreactor was 44g/L after 28 hours. The pH of the system could not be adjusted using CO<sub>2</sub> gas despite the complete consumption of MgCO<sub>3</sub>.

MgSO<sub>4</sub> was added to the minimal growth medium to gain the benefits of magnesium ions on flocculation without the buffering effects of carbonate. A final succinic acid concentration of 39g/L was reached after 28 hours; a volumetric productivity of 1.39g/L·h. Despite the lowered succinic acid production, the minimal growth medium with magnesium sulphate present became the formulation for all future work in this project. The use of carbon dioxide gas over MgCO<sub>3</sub> for CO<sub>2</sub> addition was also preferred as it enhanced the environmental benefits of succinic acid production. Both biomass growth and biosynthesis of succinic acid are CO<sub>2</sub>-fixing processes which can utilize waste carbon dioxide gas from other industrial processes. Having established the base-case single phase bioreactor run for succinic acid production, attention shifted to polymer uptake in fermentation broth and succinic acid desorption.

## **B.7 References**

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## Appendix C: Calibration Curves

### C.1 HPLC Calibration Curves

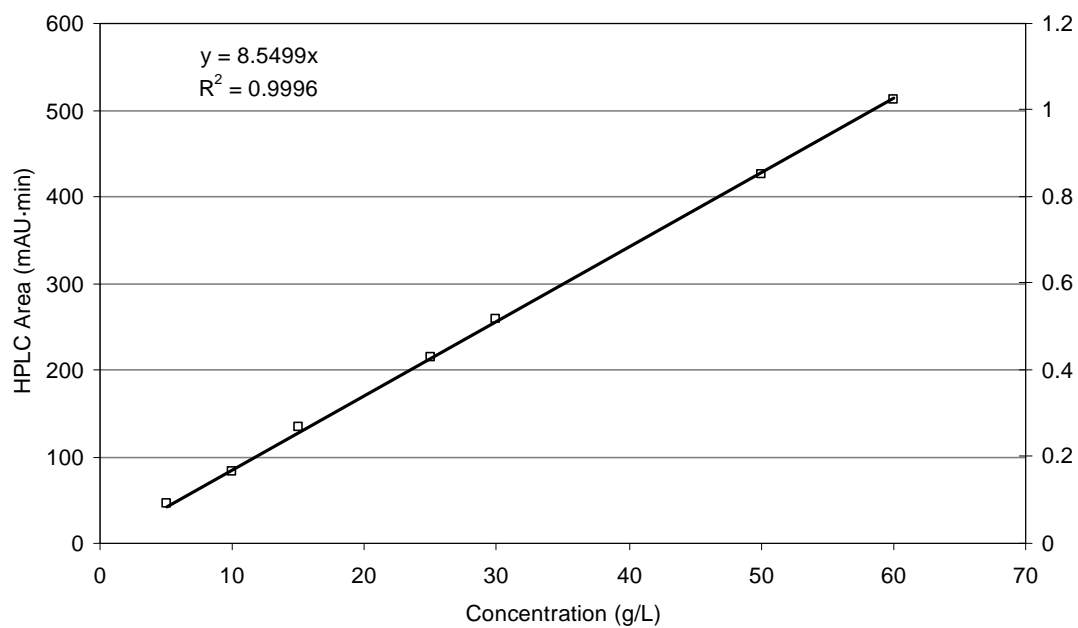
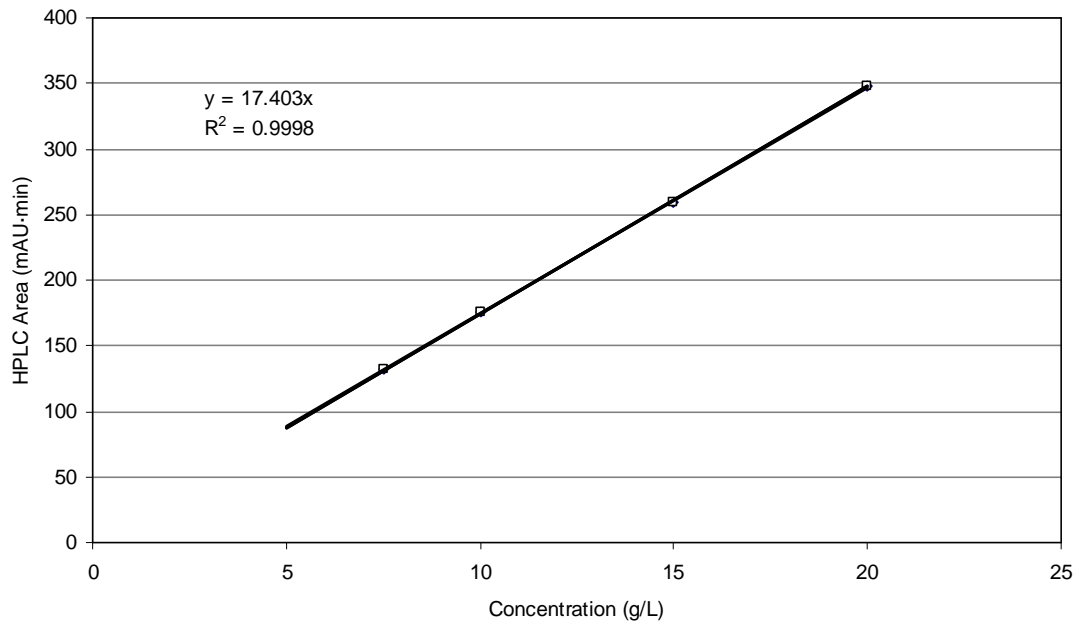
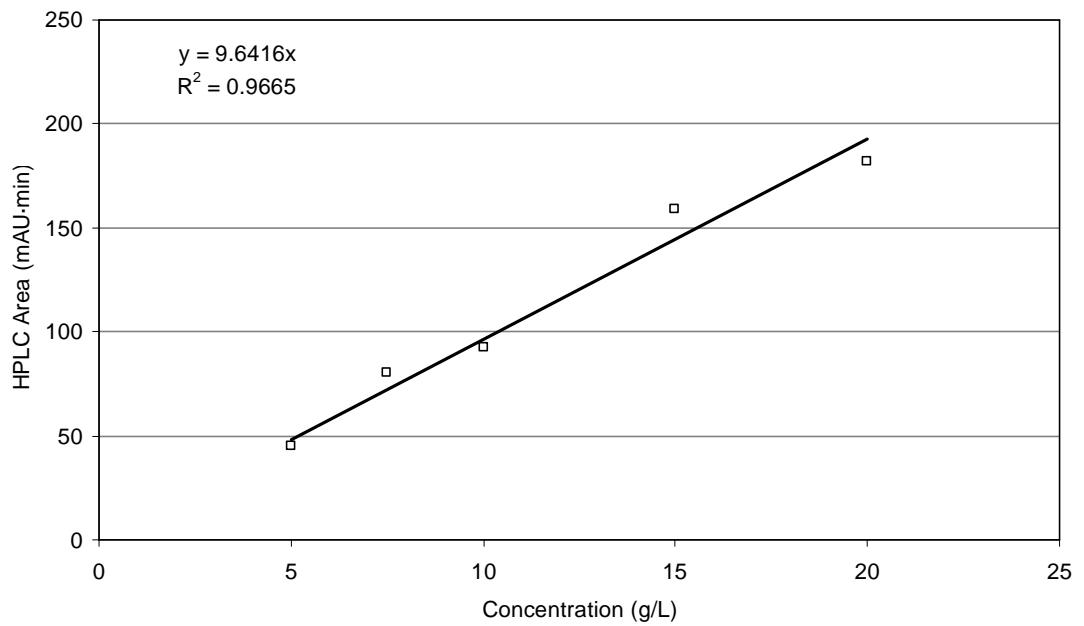


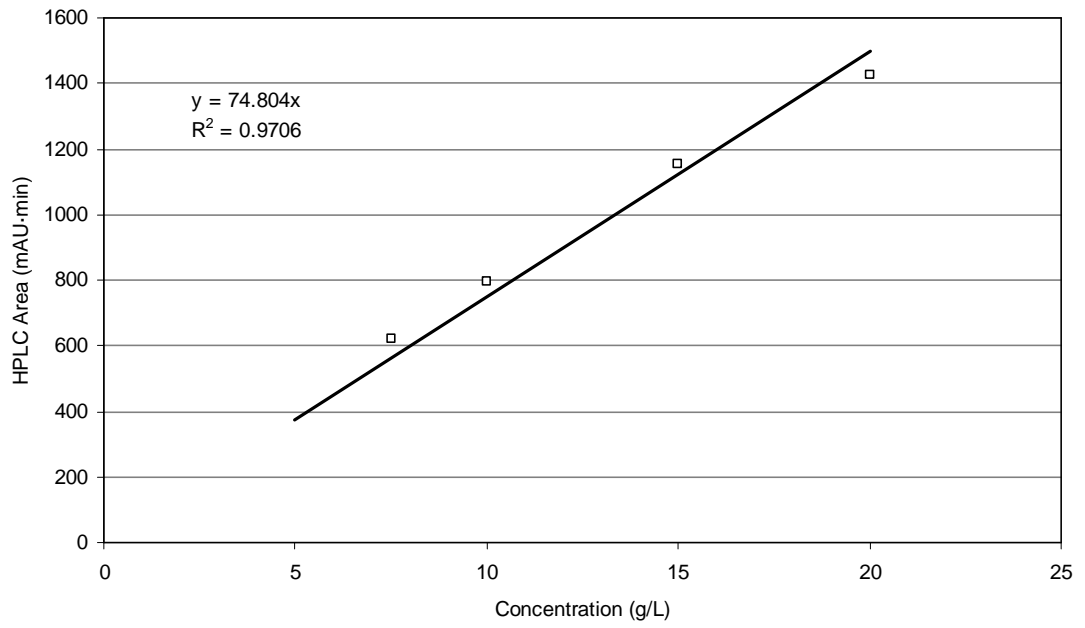
Figure C-1: UV-vis calibration curve for succinic acid, elution time 10.8 minutes



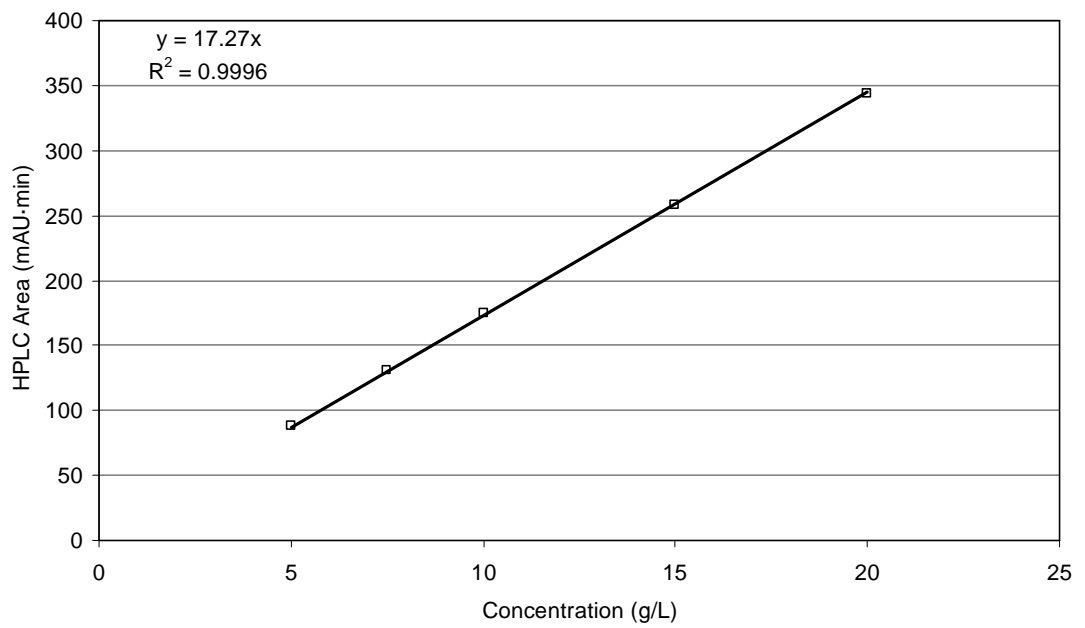
**Figure C-2: UV-vis calibration curve for formic acid, elution time 12.3 minutes**



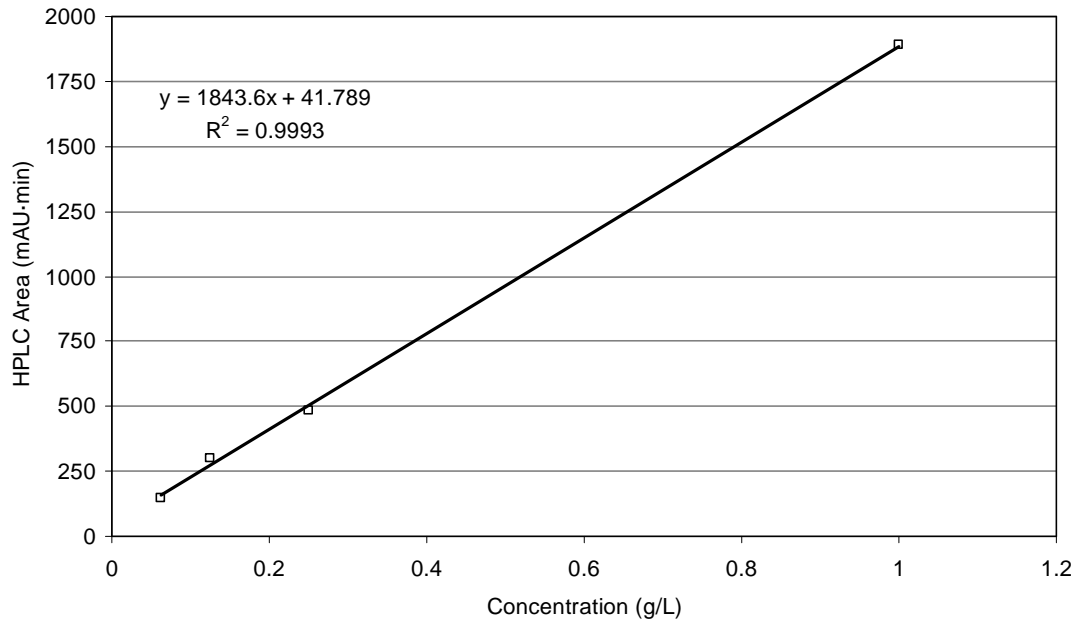
**Figure C-3: UV-vis calibration curve for acetic acid, elution time 13.2 minutes**



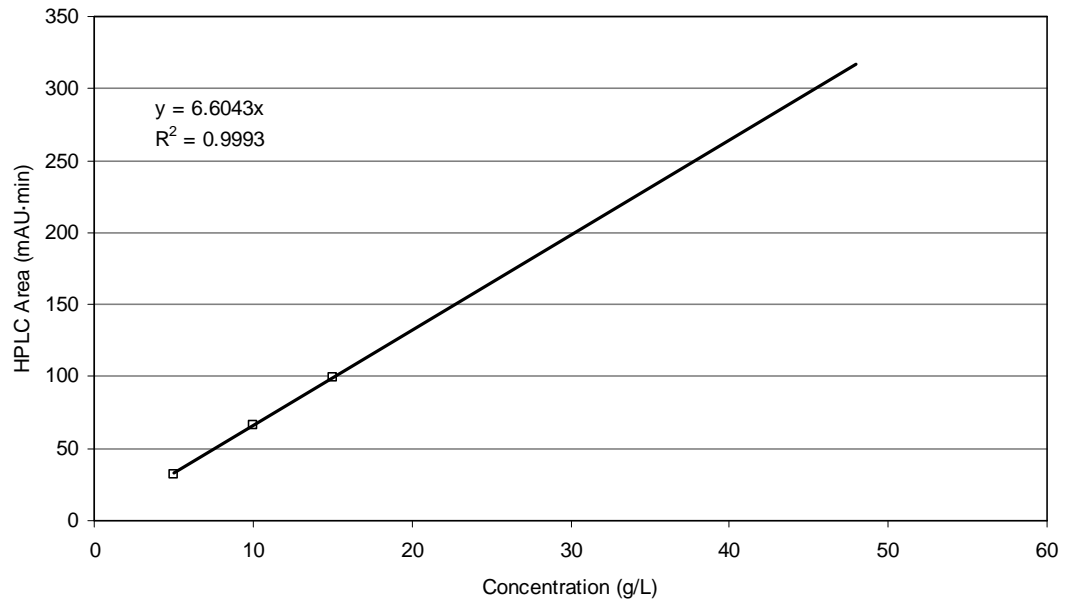
**Figure C-4: UV-vis calibration curve for oxaloacetic acid, elution time 9.75 minutes**



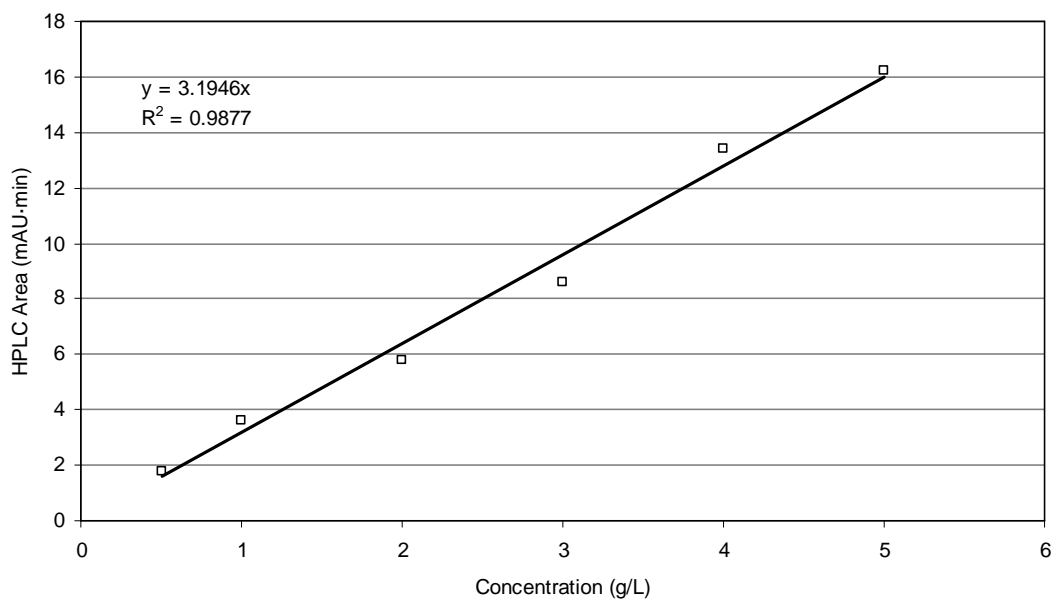
**Figure C-5: UV-vis calibration curve for malic acid, elution time 9.13 minutes**



**Figure C-6: UV-vis calibration curve for fumaric acid, elution time 13.2 minutes**

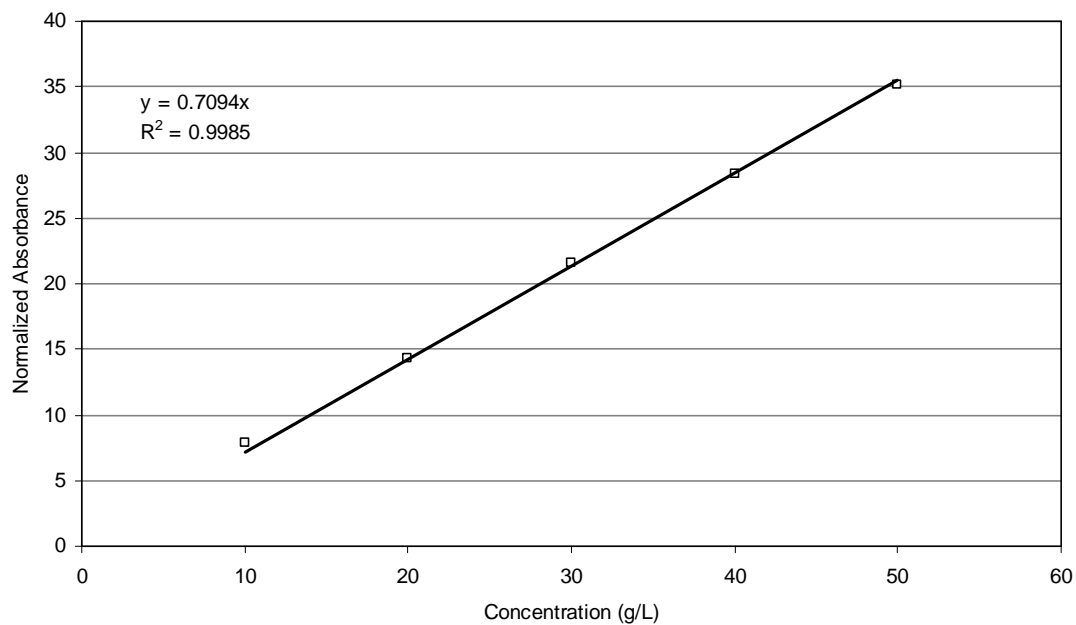


**Figure C-7: RI calibration curve for glucose, elution time 15.4 minutes**



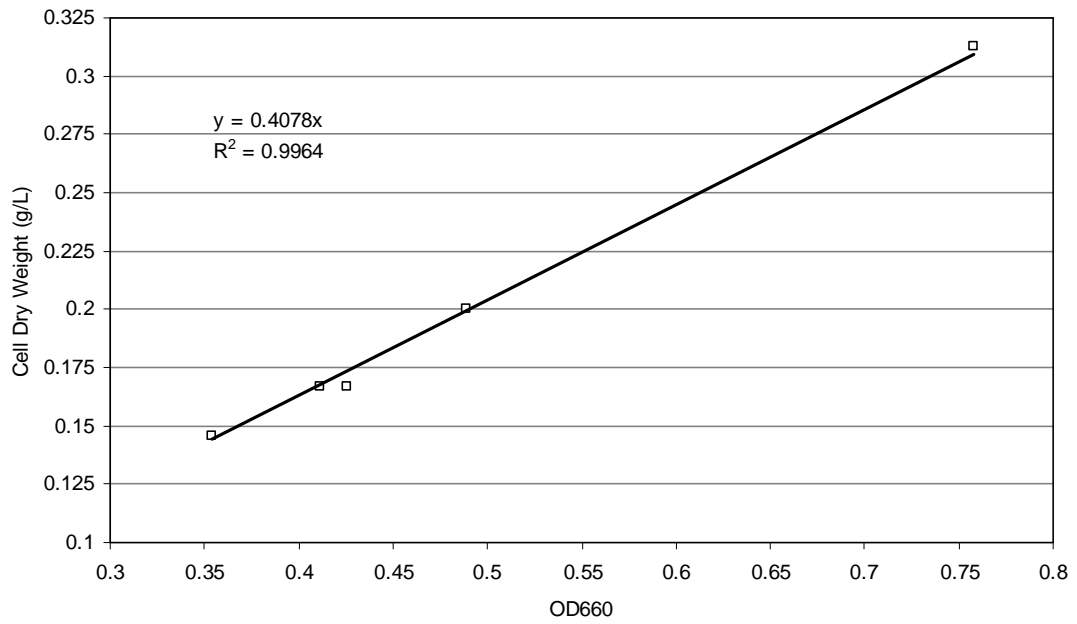
**Figure C-8: RI calibration curve for ethanol, elution time 33.1 minutes**

## ***C.2 UV Spectrophotometer Calibration Curves***

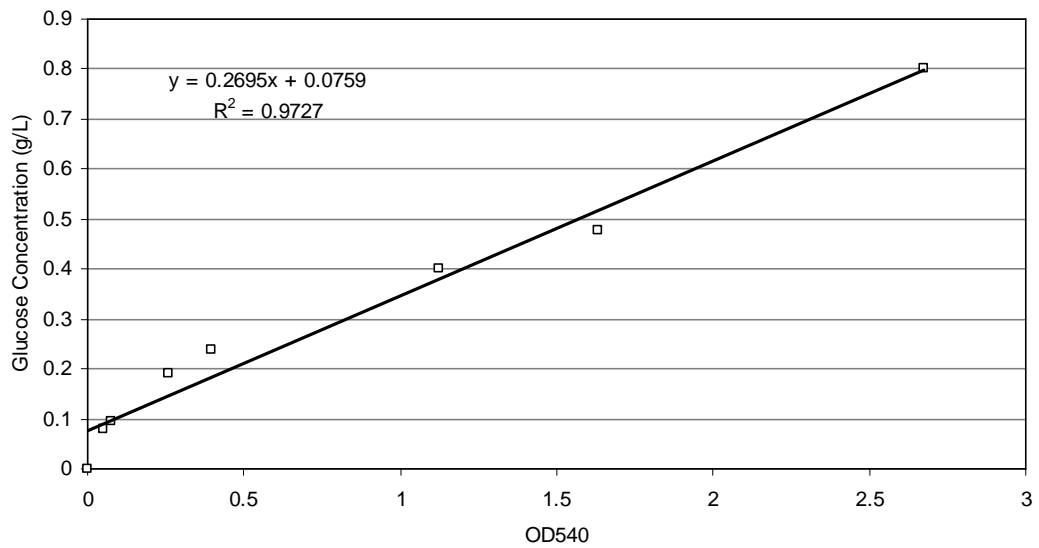


**Figure C-9: Spectrophotometer calibration curve for succinic acid, absorbance at 220nm**





**Figure C-10: Spectrophotometer calibration curve between optical density and cell dry weight, absorbance at 660nm**



**Figure C-11: Spectrophotometer calibration curve between optical density and glucose concentration using the DNS assay, absorbance at 540nm**