

Continuous In-Situ Removal of Butanol from *Clostridium acetobutylicum*

Fermentations via Expanded-bed Adsorption

by

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ABSTRACT

The use of petroleum for liquid-transportation fuels has strained the environment and caused the global crude oil reserves to diminish. Therefore, there exists a need to replace petroleum as the primary fuel derivative. Butanol is a four-carbon alcohol that can be used to effectively replace gasoline without changing the current automotive infrastructure. Additionally, butanol offers the same environmentally friendly effects as ethanol, but possess a 23% higher energy density. *Clostridium acetobutylicum* is an anaerobic bacterium that can ferment renewable biomass-derived sugars into butanol. However, this fermentation becomes limited by relatively low butanol concentrations (1.3% w/v), making this process uneconomical. To economically produce butanol, the in-situ product removal (ISPR) strategy is employed to the butanol fermentation. ISPR entails the removal of butanol as it is produced, effectively avoiding the toxicity limit and allowing for increased overall butanol production. This thesis explores the application of ISPR through integration of expanded-bed adsorption (EBA) with the *C. acetobutylicum* butanol fermentations. The goal is to enhance volumetric productivity and to develop a semi-continuous biofuel production process. The hydrophobic polymer resin adsorbent Dowex Optipore L-493 was characterized in cell-free studies to determine the impact of adsorbent mass and circulation rate on butanol loading capacity and removal rate. Additionally, the EBA column was optimized to use a superficial velocity of 9.5 cm/min and a resin fraction of 50 g/L. When EBA was applied to a fed-batch butanol fermentation performed under optimal operating conditions, a total of 25.5 g butanol was produced in 120 h, corresponding to an average yield on glucose of 18.6%. At this level, integration of EBA for in situ butanol recovered enabled the production of 33% more butanol than the control fermentation. These results are very promising for the production of butanol as a biofuel. Future work will entail the optimization of the fed-batch process for higher glucose utilization and development of a reliable butanol recovery system from the resin.

DEDICATION

Throughout my college career, I have had several long periods of time where communication with my family was scarce. Sometimes months would pass without a trip home. But the second I walked through that garage door, I felt loved and the chronic engineering-induced stress would melt away for the night. This is a special thank you to my family for always being there when I was down, and for enduring my long absences. I could not have achieved so much without my solid support system back home. To my extended family - thank you for the fun game nights and all of the care packages that provided a little taste of home during the long study-nights. Finally, I wanted to thank my best friend. You have been by my side to put a smile on my face through the worst of it (refer to Fall 2012). I could not have achieved so much without you, pulling me up when I fell. Taylor you are my rock and I cannot thank you enough for all that you have done. Looks like we made it.

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CHAPTER 1 INTRODUCTION

1.1. Petroleum Shortage

1.1.1. Current Energy Demand

Global populations continue to grow and resources are consumed at perpetually increasing rates (EIA, 2013). The global oil demand at the start of 2012 was 86.8 million barrels/day, and is projected to increase by 26.4% to 109.5 million barrels/day by 2030 (OPEC, 2012). Despite the proven crude oil reserve of 1.5 trillion barrels at the end of 2010 (OPEC, 2012), the world is consuming crude oil at a rate that far surpasses production (Kochaphum et al., 2012; Hui et al., 2012; Dijkstra and Langstein, 2012; Stambouli, 2011). Additionally, the constant consumption of fossil fuels generates carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O), all significant global warming contributors (Kochaphum et al., 2012; Antoni et al., 2007). Thus, there exists an environmental need to replace conventional, petroleum-derived liquid transportation fuels with a clean and sustainable alternative.

1.1.2. Purposed Biofuels

One of the emerging solutions to combat the dwindling oil reserves is biologically produced fuels (Dijkstra and Langstein, 2012; Antoni et al., 2007). Among the various naturally-occurring bioproducts suitable for biofuel applications, most notably including: alcohols, methylfurans (Wang et al., 2013), biodiesel (Amani et al., 2013; Yi et al., 2013; Strömberg et al., 2013), alcohols are among the most commonly pursued. This is due to their ideal anti-knock characteristics when used in internal combustion engines. Ethanol, a two-carbon alcohol, is at the forefront of biofuel production for its applicability in spark-ignition engines due to a high octane number (Wang et al., 2013). Additionally, when compared to gasoline, the combustion of alcohols results in a reduction in carbon monoxide and unburned hydrocarbons (Hsieh et al., 2002). Currently, gasoline is often mixed with ethanol to reduce particulate emissions by up to 40% compared to unmodified gasoline (Szybist et al., 2011). Therefore, the US Government mandated that 36 billion gallons of biofuel to be used as fuel as of 2022 (Berg, 2004; Schnepf and Yacobucci, 2012).

Conventional methods to produce ethanol begin with harvested lignocellulosic biomass (e.g. corn, switchgrass, biological waste, etc.) that is enzymatically digested to release simple sugars as substrates (Ben et al., 2013; Saxena and Tanner, 2012; Wirawan et al., 2012; Facts, 2007). The sugars can then be fermented by microorganisms which produce ethanol as a by-product. Common ethanologenic microorganisms include the yeast *Saccharomyces cerevisiae* (Aldiguier et al., 2004; Wirawan et al., 2012; Dodić et al., 2012; Lamsal and Johnson, 2012), and bacteria such as *Clostridium ragsdalei* (Saxena and Tanner, 2012), and *Zymomanas mobilis* (Wirawan et al., 2012; Letti et al., 2012).

While the bioproduction of ethanol offers a renewable solution to crude oil, the process is associated with several limiting drawbacks. The primary drawback is that the energy density of ethanol is 65-69% that of gasoline (Lynd, 1996). Additionally, it has been determined that gasoline blends containing 85% ethanol (E85) cause complications with the spray quality from a fuel injector in a direct-injection engine (Zhu et al., 2009). Therefore, a transition from petroleum to ethanol as a liquid-transportation fuel would require significant modifications to existing automobiles that would include a higher fuel pump to the engine and increased injector flow capacity (Zhu et al., 2009). Thus with current automobiles and gasoline infrastructure, the application of ethanol is limited to gasoline enhancement (MacLean et al., 2000).

1.1.3. *Butanol as a Gasoline Replacement*

Another potential biofuel that is rapidly gaining support is *n*-butanol (further referred to as butanol). Butanol is a four-carbon alcohol that is often used as a solvent in products such as cosmetics and paints. In 2002, global butanol usage reached 5.1 million metric tons (Dow, 2006). Current methods of butanol production entail the conversion of petroleum-generated propylene to butanol over a triphenylphosphine rhodium hydrocarbonyl catalyst (Dow, 2006).

Current fuel applications of butanol are very similar to ethanol and are likewise limited to gasoline and diesel fuel blends (Rakopoulos et al., 2011). When a 10% butanol/gasoline blend is used in a standard direct-injection, spark-ignition engine, the decrease in emissions and increase in fuel combustion was rival to a 10% ethanol/gasoline blend (Wallner et al., 2009). Thus, butanol

offers the environmentally-friendly advantages that are associated with ethanol. Unlike ethanol, butanol can be used directly in direct-injection engines without any modification (Cobalt, 2013). Furthermore, as seen in Table 1.1, the calorific value of butanol is over 23% higher than that of ethanol (Rakopoulos et al., 2011). While butanol does not have as high of an energy density as gasoline or diesel, it can serve as a suitable stand-alone transportation fuel replacement.

Table 1.1. Properties of liquid-fuel options (Jin et al., 2011).

Property	Gasoline	Diesel	Ethanol	Butanol
Molecular formula	C ₄ -C ₁₂	C ₁₂ -C ₂₅	C ₂ H ₅ OH	C ₄ H ₉ OH
Octane number	80-99	20-30	108	96
Lower heating value (MJ/kg)	42.7	42.5	26.8	33.1

1.2. Sustainable Approach for Butanol Production

1.2.1. Bioproduction of Butanol

Several organisms exist that have been engineered to ferment sugars to butanol under anaerobic conditions. Some of these organisms include: *Escherichia coli* (Atsumi et al., 2008), *Saccharomyces cerevisiae* (Steen et al., 2008), *Pseudomonas putida* (Nielsen et al., 2009), *Bacillus subtilis* (Nielsen et al., 2009), and *Lactobacillus brevis* (Berezina et al., 2010). However, renewable butanol production continues to center around the natural producer: *Clostridia*. *Clostridia* are strictly anaerobic bacteria, a select few of which contain the genes required for butanol fermentation. Where examples include the species *C. pasteurianum* (Malaviya et al., 2012), *C. saccharobutylicum* (Yang et al., 2011), *C. saccharoperbutylacetonicum* (Yang et al., 2011), *C. beijerinckii* (Qureshi and Blaschek, 2000), and *Clostridium acetobutylicum*, which remains the most commonly studied.

1.3. *Clostridium acetobutylicum* Butanol Fermentation

1.3.1. Acidogenic Phase

Traditional *C. acetobutylicum* fermentations undergo two stages, where the onset of butanol production is dependent of several precipitating factors including biomass density, pH, and organic acid concentration (Jones and Woods, 1986). The first stage of fermentation is

referred to as the acidogenic phase which is dominated by the production of acetate and butyrate with hydrogen and carbon dioxide as byproducts. Acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively (Figure 1.1) (Baba et al., 2012), with the aid of key enzyme pairs phosphate acetyltransferase and acetate kinase and phosphate butyryltransferase and butyrate kinase, respectively (Jones and Woods, 1986). Due to the release of acetate and butyrate, the pH of the media will fall roughly one point towards acidic conditions (Jones and Woods, 1986).

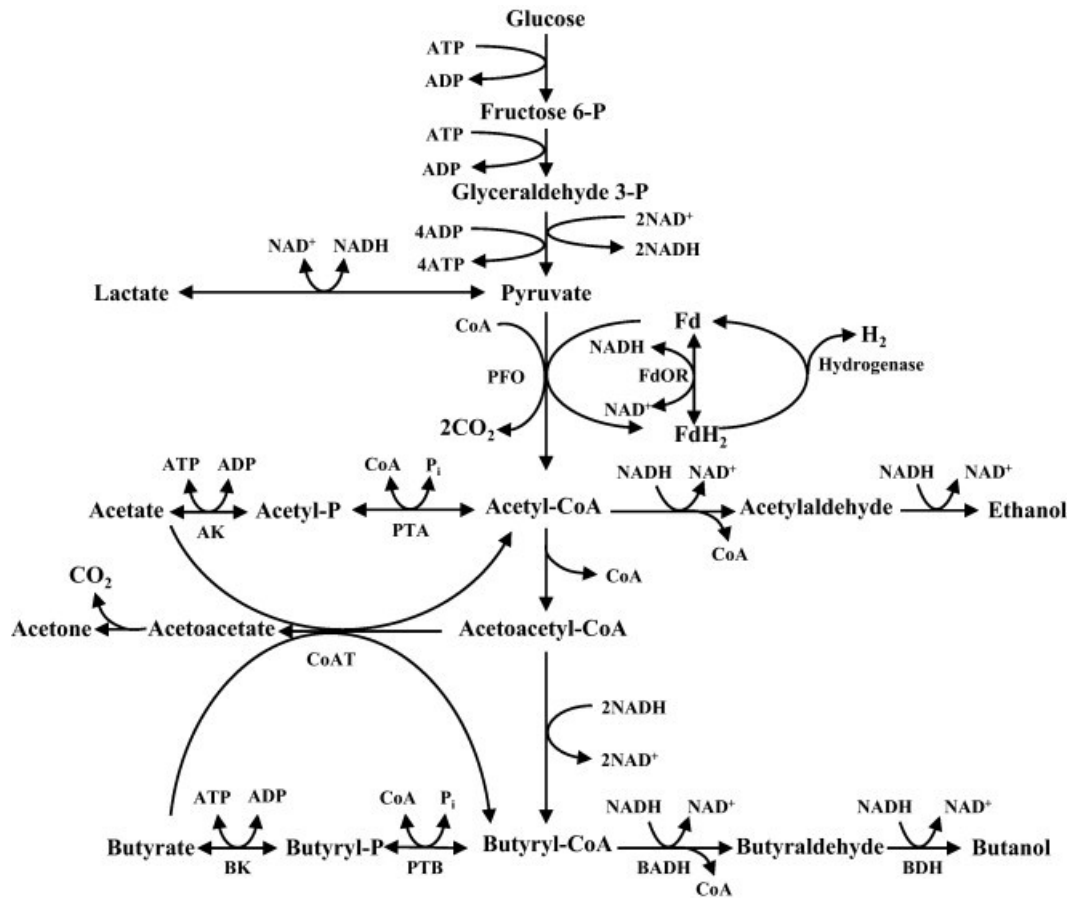


Figure 1.1. Butanol production pathway from glucose in *Clostridium acetobutylicum* ATCC 824 (Baba et al., 2012).

The production of acetate and butyrate are necessary for cellular growth as the compounds partition in the cell membrane and allow protons to enter the cell from the medium, facilitating ATP generation (Foster and McLaughlin, 1974; Kell et al., 1981). However, excessive

production of acid to critical concentrations will result in the collapse of the pH gradient across the cellular membrane and cause total inhibition of metabolic function (Herrero, 1983; Herrero et al., 1985). The exact critical acid concentration is widely debated, but it is generally accepted that cellular growth is inhibited when the undissociated butyric acid concentration reaches 0.2 - 0.8 g/L, and solvent induction can occur from 0.5- 1.9 g/L (Monot et al., 1982; Martin et al., 1983). These conditions can occur when the pH of the media is high (above 5.5) for the duration of the fermentation (Jones and Woods, 1986). However, when the pH was allowed to decrease below 4.5 during the acidogenic phase, cellular growth and metabolism were inhibited and the cells only produced acids (Jones and Woods, 1986). Under certain conditions, the cellular metabolism shifts from the production of acids to the production of acetone, butanol, and ethanol (ABE), in what is called the solventogenic phase (Jones and Woods, 1986).

1.3.2. Solventogenic Phase

After *Clostridium acetobutylicum* undergoes a shift from acid production to solvent production, cellular growth slows to the stationary phase and hydrogen production decreases (Jones and Woods, 1986). This shift defines the onset of the second phase, solventogenesis, which is characterized by the production of acetone, ethanol, and butanol in a 3:1:6 molar ratio, respectively (Jones and Woods, 1986). When solvent production dominates the fermentation, all cell division has stopped. However, the optical density may continue to increase as the new cells that were formed prior to the induction of solventogenesis will continue to grow in size (Jones and Woods, 1986). During the solventogenic phase, the acids produced at the beginning of the fermentation will be consumed concurrently with glucose to produce the solvents (Davies, 1943), refer to Figure 1.1. Where butyric acid (butyrate) is consumed at a higher rate than acetic acid (acetate), accounting for the production of 6 mol of butanol per every 1 mol of ethanol (Ross, 1961). The uptake of the acids produced during the first stage is accordingly accompanied by an increase in pH (Jones and Woods, 1986).

The extracellular pH plays a very important role in solvent production. The generally accepted pH range for solvent production is between 4.5 and 5.0 (Jones and Woods, 1986; Li et

al., 2011). However, high solvent titers have been achieved using a pH as low as 4.0 (Napoli et al., 2010). One proven method of obtaining high butanol titers is through a 2-step pH approach. The pH is held at 5.5 until a dry cell weight of 0.5 g/L is achieved, then the pH is lowered to 4.9 (Guo et al., 2012).

While a low pH is necessary for solvent production, the actual trigger for solventogenesis is a combination of a low pH and the accumulation of acid end products (butyrate and acetate). The toxicity associated with the acid concentration is suspected to trigger the metabolic shift as the acids are consumed during solvent production (Hartmanis et al., 1984). In carbon-limited cultures, the critical acid concentration to cause the metabolic shift is not reached, and the cells only produce acids (Haggstorm, 1985; Bahl and Gottschalk, 1985). A similar effect is noted with nitrogen-limited media (Jones and Woods, 1986).

During solvent production, the intermediates used to make acids (acetyl-CoA and butyryl-CoA) are repurposed to make ethanol and butanol, refer to Figure 1.1 (Baba et al., 2012). To perform the reduction of butyryl-CoA to butanol, key enzymes are required: butyraldehyde dehydrogenase and butanol dehydrogenase (Andersch et al., 1983; Petitdemange et al., 1979). The maximum theoretical yield possible (assuming that the products of the fermentation were butanol and carbon dioxide) is 0.41 grams butanol per gram of glucose added (Kim and Zeikus, 1985). If one examines Figure 1.1, the production of acetone is coupled to the consumption of acid end products. Therefore, the production of acetone is a necessary step to achieving high butanol titers (Hartmanis et al., 1984).

1.3.3. Influence of Temperature on Fermentations

Operating temperatures for bacteria typically range between 30 to 37°C. When *Clostridium acetobutyicum* was grown at 30°C and 33°C, the solvent yield was relatively constant. However, when the temperature was increased to 37°C and 40°C, the solvent yield decreased (McCutchan and Hickey, 1954; McNeil and Kristiansen, 1985). Despite the decrease in total solvent yield (namely acetone), the butanol yield remained unaltered at the higher temperatures (Jones and Woods, 1986). Additionally, the fermentation time was decreased when

the cells were grown at the higher temperatures. Thus, the current growth standard for *Clostridium acetobutylicum* for butanol production is 37°C (Li et al., 2011; Qureshi and Blaschek, 2000; Guo et al., 2012).

1.3.4. Oxygen Toxicity

Clostridium acetobutylicum are strictly anaerobic bacteria, that is, oxygen is highly lethal to the cells. Therefore, before inoculation into the media, the environment must be thoroughly purged with an oxygen-free gas (e.g. Nitrogen). Typically, L-cystine hydrochloride is added to the growth media as the amino acid binds dissolved oxygen, effectively isolating it from the cells (Gorini, 1961). In small amounts, oxygen does not influence the cells; however, in excess, oxygen will inhibit glucose consumption and stop the formation of deoxyribonucleic acid, ribonucleic acid, and key proteins needed for growth (Jones and Woods, 1986). If oxygen is present in the system, the cells will still produce acetate, but butyrate production will not occur. Additionally, the cells will sporeulate to prevent the negative effects of oxygen. The negative effects of oxygen are reversible following a purge of the system with an oxygen-free gas. It is important to note that brief exposure (2-3 minutes every 1-2 hours) of oxygen can increase butanol production by 3.1 to 9.1% (Nakhmanovich and Kochkina, 1963).

1.3.5. Limitations of the Fermentation

The *Clostridium acetobutylicum* fermentation faces several limitations that make the process economically infeasible. While advancements have been made with the economics of the media components (e.g. replacing glucose with starch-based packing peanuts (Ezeji et al., 2003)), the resounding complication to fermentations is the low solvent titers attainable in wild-type fermentations. This is due to the toxicity of butanol to the cells and only allows for a final solvent concentration of about 20 g/L (Jones and Woods, 1986). When the butanol concentration reaches ~13 g/L, production tapers and the cells stop producing butanol (Costa and Moreira, 1983; Ryden, 1958; Monot et al., 1982). When the butanol concentration approaches the toxicity limit, the phospholipid component of the cellular membrane is disrupted (Bowles and Ellefson,

1985; Gottwald and Gottschalk, 1985). Disruption of the cell membrane results in the increased fluidity of the membrane causing vital nutrients to leak from the cells (Vollherbst-Schneck et al., 1984). Additionally, the ATPase activity in the membrane is abolished and the pH gradient within the cell that is necessary for survival collapses (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985).

As stated earlier, butanol is the key toxic species, not ethanol or acetone. For either of these species to become toxic, ethanol and acetone would need to accumulate to 50-60 g/L and ~70 g/L, respectively (Costa and Moreira, 1983; Leung and Wang, 1981). When the butanol concentration is under 4.0-4.8 g/L, cellular growth is unaffected. Whereas growth is completely inhibited between concentrations 12-16 g/L (Ounine et al., 1985). The effect of butanol toxicity is also dependent on the carbon source used. Cells grown using xylose sugars experienced a lower toxicity threshold compared to those grown using glucose (Ounine et al., 1985). Ultimately, for the *Clostridium acetobutylicum* fermentation to be an economical and feasible method for biofuel production, the butanol toxicity limit must be addressed.

1.4. Avoiding Butanol Toxicity

1.4.1. Cellular Manipulation

Avoiding the butanol toxicity limit in fermentation can be achieved by altering the cellular media and/or the genetics of the organism. One method of increasing butanol tolerance is to add saturated fatty acids to the media as the addition will increase the fatty acid content in the cellular membrane. Fatty acid enrichment has been shown to double the butanol tolerance in wild-type cells (Brosseau et al., 1986; Kim et al., 1984).

Advances in genetic engineering have lead to alterations of wild-type *Clostridia*. Mutagenesis and gene manipulations have produced strains that are more resistant to the toxic effects of butanol (Parekh et al., 1999; Annous and Blaschek, 1991). Engineering the bacteria for higher butanol tolerance and production is possible and has been achieved (Mermelstein et al., 1994). However, strains engineered for butanol tolerance often experience lower butanol production rates than their wild-type counterparts (Ezeji et al., 2004).

1.4.2. Immobilized Cells

Another method to avoid the restraints of low solvent titers is to immobilize the cells on a solid support and wash nutrient-rich media over said support. This method effectively allows the cells to produce solvents that are promptly washed away before an inhibitory concentration is reached. Typically, a low cost solid support is selected that can allow for the cells to be immobilized either by adsorption or size exclusion. Possible solid supports may include: brick (Yen and Li, 2011; Qureshi et al., 2000), bonechar (Qureshi and Maddox, 1995; Friedl et al., 1991); lignocellulosic materials (e.g. coconut and wood pulp fibers) (Survase et al., 2012), and corn stalk (Zhang et al., 2009). Cell immobilization has been shown to achieve high butanol production rates; however, if the dilution rate is too high, the cells will only produce acids (Yen and Li, 2011; Qureshi et al., 2000).

1.4.3. In-situ Butanol Removal

Due to the toxic nature of butanol, numerous different strategies have been developed to remove the butanol as it is produced, thereby precluding the accumulation to toxic levels. These can include, but are not limited to: gas stripping, liquid-liquid extraction, pervaporation, and adsorption. These processes all utilize physical and/or chemical differences between water and butanol as the driving force for removal (Table 1.2). These methods, commonly known as in-situ product removal (ISPR) strategies, can be applied to numerous toxicity limiting systems (Freeman et al., 1993), including the ABE fermentation.

Table 1.2. Physical properties of key fermentation components.

Chemical	Formula	Molar Mass (g/mol)	$\log K_{ow}$ ¹	Normal Boiling Point (°C) ²
Water	H ₂ O	18.02	--	119
Butanol	C ₄ H ₉ OH	74.12	0.88	100
Acetone	C ₃ H ₆ O	58.08	-0.24	56
Ethanol	C ₂ H ₅ OH	46.07	-0.31	78

¹ Spafiu et al., 2009

² Dow, 2006

1.5. In-situ Product Removal Strategy

1.5.1. Gas Stripping

Gas stripping is a strategy that exploits the volatility of the toxic species. Gas is constantly bubbled through the media, effectively vaporizing the toxic species that is then condensed outside of the system. This technique has been applied to the ABE fermentation using either pure nitrogen or fermentation gases (CO_2 and H_2) as the stripping gas (Ezeji et al., 2004).

Since the separation mechanism consists mainly of an inert gas being bubbled through a bioreactor, the cells are unharmed and the nutrients such as glucose are retained in the system (Qureshi and Blaschek, 2001). However, since water has a lower boiling point than butanol (refer to Table 1.2), a significant amount of water will be stripped from the media. When applied to the ABE fermentation, the condensate requires further processing as water, acetone, and ethanol will all also condense with the butanol. Despite the additional processing steps required, gas stripping has been shown to increase the total solvent yield by over 20% (Ezeji et al., 2004) and allow for over 500% extra sugar utilization (Qureshi and Blaschek, 2001).

1.5.2. Liquid-Liquid Extraction

Liquid-liquid extraction entails the addition of an immiscible solvent to the culture media with the intention that the solvent will extract the toxic species. When applied to the ABE fermentation, the octanol-water partition coefficient ($\log K_{OW}$) of the toxic species (refer to Table 1.2) can be used to measure of the driving force for separation. Thus butanol will more readily enter an organic solvent compared to acetone and ethanol. Complications arise as the selected solvent must follow the following criteria: (i) immiscible with water, (ii) not toxic to the cells, (iii) toxic species has a high partitioning coefficient into the solvent, (iv) selectively absorbs the desired compound (Seader et al., 2011).

Numerous solvents have been tested for applicability in the ABE fermentation, and successful extractants include: corn oil (Wang et al., 1979), kerosene (Wang et al., 1979), dibutylphthalate (Wang et al., 1979), oleyl alcohol (Taya et al., 1985; Qureshi and Maddox, 1995;

Bankar et al., 2012), soybean-derived biodiesel (Adhami et al., 2009), and methylated crude palm oil (Ishizaki et al., 1999). However oleyl alcohol is considered to be the standard for ABE fermentations (Ishizaki et al., 1999; Jones and Woods, 1986; Qureshi and Maddox, 1995) as the addition allows for double butanol production in fed-batch fermentations (Taya et al., 1985). Unfortunately, due to the low partition coefficients associated with the ABE fermentation, large amounts of solvent are required to effectively avoid the toxicity (Park and Geng, 1992). Additionally, with the exception of biodiesel (Adhami et al., 2009), further processing of the extractant is required for liquid-fuel application, making this process economically infeasible (Park and Geng, 1992).

1.5.3. Pervaporation

Pervaporation is a method that consists of a membrane with a liquid phase on one side, and a gaseous phase on the other (Seader et al., 2011). The membrane is designed to be selective and only permeable to the toxic species. Typically, a vacuum is drawn on the gaseous side, effectively vaporizing the toxic species that diffuses through the membrane (Jones and Woods, 1986; Seader et al., 2011). Thus with pervaporation, a constantly renewing driving force is developed.

Pervaporation membranes have been applied to model ABE solutions with promising results (Marszalek and Kamiński, 2012). Furthermore, when this method was applied to the actual ABE fermentation the sugar was completely converted and total butanol production increased by a factor of four (Groot and Luyben, 1987; Groot et al., 1991). Unfortunately, these results were obtained with immobilized cell systems as this process is highly subject to biofouling with free cells. The free cells readily plug the pores of the pervaporation membrane, reducing efficiency and separation (Groot et al., 1992; Groot et al., 1991). Therefore, this method is unattractive for in-situ ABE separation without cellular immobilization due to the biofouling aspects (Groot et al., 1992).

1.5.4. Adsorption

Adsorption is a method of solid-phase extraction that occurs when the solute adsorbs onto the surface of a rigid, insoluble adsorbent particle (Green and Perry, 2008). Examples of adsorption include the removal of carbon tetrachloride from drinking water onto activated carbon (Weber, 1983) or the adsorption of succinic acid onto anion-exchange resins in *Actinobacillus succinogenes* cultures to avoid the toxicity limit (Li et al., 2011). Adsorbents utilize two different binding mechanisms to separate the solute from the solvent: chemisorption and physical adsorption. Chemisorption involves the formation of a solute monolayer on the adsorbent through chemical bonds between the adsorbate and the adsorbent (Seader et al., 2011). However chemisorption can be irreversible, leading to low desorption returns (Jarvis et al., 2011). Physical adsorption utilizes the van der Waals interactions between the adsorbent and solute particle allowing for rapid adsorption. This method of adsorption is the more common mode of non-catalytic adsorption as it is reversible depending on the layering of adsorbate particles. If the adsorbate forms one layer on the adsorbent, then the process is reversible (Seader et al., 2011). Adsorption applicability to the ABE fermentation has been widely studied, and it has been shown that biofouling has little to no effect on the loading capacity of the adsorption particles (Groot and Luyben, 1986). Thus, adsorption provides a promising outlook for avoiding the toxicity threshold of butanol in fermentations.

1.6. Adsorption in the ABE Fermentation

1.6.1. Adsorbent Selection

If an adsorbent is chosen for a chemical separation, the adsorbent must maintain a high loading capacity to minimize adsorbent mass and reactor size. In the case of ABE fermentations, a high selectivity for the desired solute is required, that is, the solvent or other solutes (particularly nutrients) should not adsorb to the adsorbent. Rapid sorption kinetics of the adsorbent are also highly desirable, and the ability to desorb the solute is necessary for biofuel application. Additionally, the adsorbent must be mechanically, chemically, and thermally stable when present

in the fermentation broth. To minimize biofuel production costs, the ideal adsorbent has a long lifetime and can be regenerated indefinitely.

1.6.2. Adsorbent Characterization

The amount of solute that can be loaded onto an adsorbent particle at equilibrium varies widely between the different applicable adsorbents. To quantify the adsorption strength of a given adsorbent at equilibrium, the specific loading capacity can be determined by performing a solute material balance:

$$q = \frac{(C_0 - C)V_{aq}}{m_a} \quad (1)$$

where q is the specific loading capacity, C_0 and C are the initial and final solute concentration in the aqueous solution, and m_a and V_{aq} are the adsorbent mass and the volume of the aqueous solution, respectively. Equation 1 assumes that solvent adsorption is non-existent and that there is a negligible change in the volume of the mixture following solute adsorption (Seader et al., 2011). Since the specific loading varies as a function of equilibrium solute concentration, the specific loading can be plotted against equilibrium solute concentration to form an adsorption isotherm (Seader et al., 2011).

Adsorption isotherms can take a variety of shapes, but the most common are depicted in Figure 1.2.

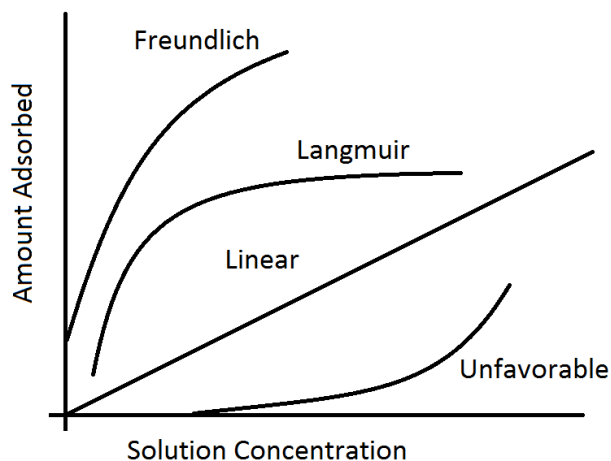


Figure 1.2. Adsorption isotherms in solid-liquid systems (Cussler, 2009).

One of the isotherms shown in Figure 1.2 is the Freundlich isotherm. Typically, adsorbents selected for ABE fermentation application follow the Freundlich isotherm and can be approximated by the following empirical relation:

$$q = kC^{1/n} \quad (2)$$

where k and n are system-specific, temperature-dependent constants. Another isotherm that is closely related to adsorption with the ISPR method is the Langmuir isotherm; however, this work focuses on adsorbents that follow the Freundlich model.

1.6.3. Screening of Adsorbents

An extensive amount of work has been performed on finding the ideal adsorbent for application in ABE fermentations. Suitable adsorbents for butanol adsorption can include: polymeric resins (Nielsen et al., 2010; Nielsen et al., 1988; Nielsen and Prather, 2008), zeolites (Oudshoorn et al., 2009; Oudshoorn et al., 2012), mesoporous carbon (Levario et al., 2011), activated carbon (Regdon et al., 1998; Groot and Luyben, 1986), starchy materials (Carmo et al., 2004), and silicate (Milestone and Bibby, 1981). All of these materials adsorb different amount of butanol per mass of adsorbent (Figure 1.3).

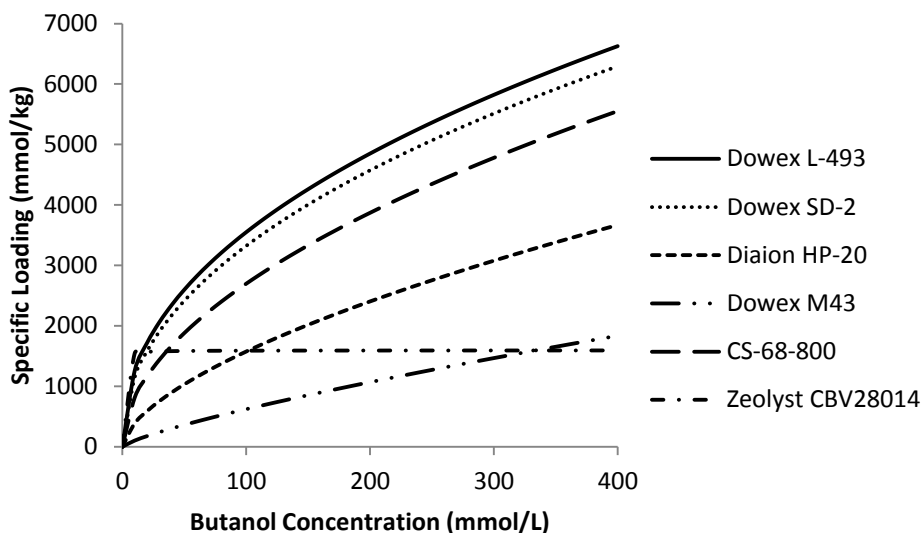


Figure 1.3. Selected adsorbents for butanol adsorption application.

The above figure shows isotherms for polymeric resins (DowexTM OptiporeTM L-493, DowexTM OptiporeTM SD-2, Diaion HP-20, DowexTM M43), mesoporous carbons (CS-68-800), and zeolites (Zeolyst CBV28014). CS-68-800 was developed at a bench-scale setting, therefore practical application of this particular adsorbent is limited. The polymeric resins, DowexTM OptiporeTM L-493 and DowexTM OptiporeTM SD-2, adsorb a significant amount of butanol at higher equilibrium concentrations compared to the other adsorbents, making these resins attractive for ABE fermentations. Unfortunately, DowexTM OptiporeTM SD-2 has been shown to adsorb vital nutrients needed by the cells (Nielsen and Prather, 2008). However, DowexTM OptiporeTM L-493 (hereon referred to as “L-493”) is more selective for butanol and highly biocompatible (Nielsen and Prather, 2008), making it an ideal adsorbent for ABE fermentation.

L-493 is a hydrophobic resin that is derived from poly(styrene-co-divinylbenene). The mechanism of adsorption is through van der Waals interactions with the $\pi - \pi$ bonds on the phenyl-side chain in the polymer matrix (Nielsen and Prather, 2008). Adsorbent properties are listed in Table 1.3.

Table 1.3. L-493 hydrophobic resin properties (Nielsen et al., 2010).

Specific surface area (m ² /g)	Pore size (nm)	Freundlich Parameters	
		<i>k</i> (mmol/kg)	<i>n</i>
1100	4.6	446 ± 115	2.22 ± 0.26

1.6.4. Adsorption Configurations

The use of adsorbents for removal of bioproducts has most commonly been applied via three configurations: (1) adding the adsorbent directly to the media, (2) containing the adsorbents in a column for packed-bed adsorption (PBA), and (3) using the adsorbents in an expanded-bed adsorption (EBA) column. Adding resin directly to the media for ISPR is the simplest of configurations as it requires no additional equipment. However, separation of the adsorbent from the media following fermentation is a challenge, and typically causes a disruption in the process. Regardless, adsorbent addition directly to culture media has been used as demonstration of concept in *Clostridium acetobutylicum* butanol fermentations (Nielsen et al., 2010; Nielsen and Prather, 2008), as well as to reduce feedback inhibition of enzymes such as α -glucosidase (Ahmed et al., 2001) and bacterial lipase (Millitzer et al., 2005).

PBA consists of the adsorbents tightly packed in (typically) a column geometry with minimal headspace. The media passes either upwards or downward through the bed which contains minimal void space between the adsorbent particles. This method is more advantageous than EBA from a separation view as the separation efficiency of the column is often greater for an equivalent adsorbent mass (Mankhaus and Glatz, 2005). However, PBA processes are highly limited when applied to biological systems as the presence of cells in the culture media cause fouling and blockage of the adsorbent bed. Thus to effectively apply PBA columns, the cells must be removed (typically by microfiltration) from the media prior to adsorption (Pierce et al., 1999). PBA has previously been applied to the *C. acetobutylicum* fermentation by filtering and recycling the cell prior to PBA, and reached an effective total solvent concentration of 47.2 g/L (Yang and Tsao, 1995).

EBA entails pumping the media in and up-flow manner through an adsorbent-filled column, thereby fluidizing the adsorbent particles. The fluidization of the adsorbents generates larger void space between the particles. These larger spaces reduce the pressure drop and allow of easier navigation of cells through the column. For this reason, EBA is preferred to the packed-bed configuration in biological applications. A column that is external of the bioreactor can be easily changed to administer fresh adsorbent to the process. Additionally, the use of an adsorption column helps during product elution as the eluting solvent is easily passed through the column following fermentation. The use of EBA is highly versatile and has been applied to succinic acid production from *Actinobacillus* (Li et al., 2011) and to propionic acid production from *Propionibacterium* (Wang et al., 2012).

1.7. Research Objectives and Structure of Thesis

1.7.1. Thesis Objectives

The research conducted for this thesis is novel and addresses overcoming butanol toxicity as an important limiting factor in *Clostridium acetobutylicum* fermentations. The end-goal is to develop a cost-effective method of butanol production for biofuel applications. This will be accomplished by developing an integrated bioreactor process in which expanded-bed adsorption is used to remove the butanol as it is produced by *C. acetobutylicum*. Following a 'bottom up' design approach, the adsorption column was first studied under cell-free conditions to find the optimum operating parameters suited to ABE fermentation. After the system was well-defined, cells were grown with and without the aid of EBA to determine its effects.

1.7.2. Thesis Structure

This thesis is split into two main sections, characterizations performed in the absence of cells (Chapter 3) and those performed with active cultures in a bioreactor (Chapter 4). All experimental and analytical methods will be discussed in Chapter 2. This thesis will conclude with recommendations and final remarks in Chapter 5.

CHAPTER 2 MATERIALS AND METHODS

2.1. General Apparatus

2.1.1. Development of an Integrated Bioreactor Process

The fermentations and cell-free characterizations were carried out using a 2 L bioreactor (Biostat A Plus, Sartorius Stedium Biotech, Bohemia, NY), with attached agitator and control module (Figure 2.1). The broth was pumped from the bioreactor, through a glass column (450 mm length, 37 mm ID, Ace Glass, Vineland, NJ) with polypropylene end caps (#25 threads, 1/4" NPT inlet, Ace Glass, Vineland, NJ). The column was filled with Dowex™ Optipore™ L-493 hydrophobic resin (Sigma, St. Louis, MO). The media was pumped through the column using a peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL). As the media left the top of the column, it was recycled back into the bioreactor. The resin was thoroughly dried at 37°C for 72 hours before addition to the column to ensure accurate mass measurements.

Figure 2.1 shows the process flow diagram with the adsorption column (a) and the bioreactor (b). The bioreactor was maintained at a constant temperature of 37°C using the heating jacket (c). Ultra-high purity nitrogen was intermittently bubbled through the fermentation broth to maintain an oxygen free environment. A dissolved oxygen (DO) probe (Oxyferm FDA225, Hamilton, Reno, NV) was used to display the actual concentration of DO in the media. A pH probe (Easyferm Plus K8 200, Hamilton, Reno, NV) was used to monitor the pH of the solution. All relevant fermentation data (e.g. temperature, DO, pH, etc.) were displayed on the computer (Latitude E5410, Dell, Round Rock, TX) via the BioPAT MFCS/DA 3.0 software (Sartorius Stedium Biotech, Bohemia, NY). The computer software was used as a controller to maintain the pH with acid or base addition (f). To increase or decrease the pH of the solution, 5 M ammonium hydroxide and 1 M hydrochloric acid was used, respectively. The bioreactor was agitated at 300 rpm for all experiments (g).

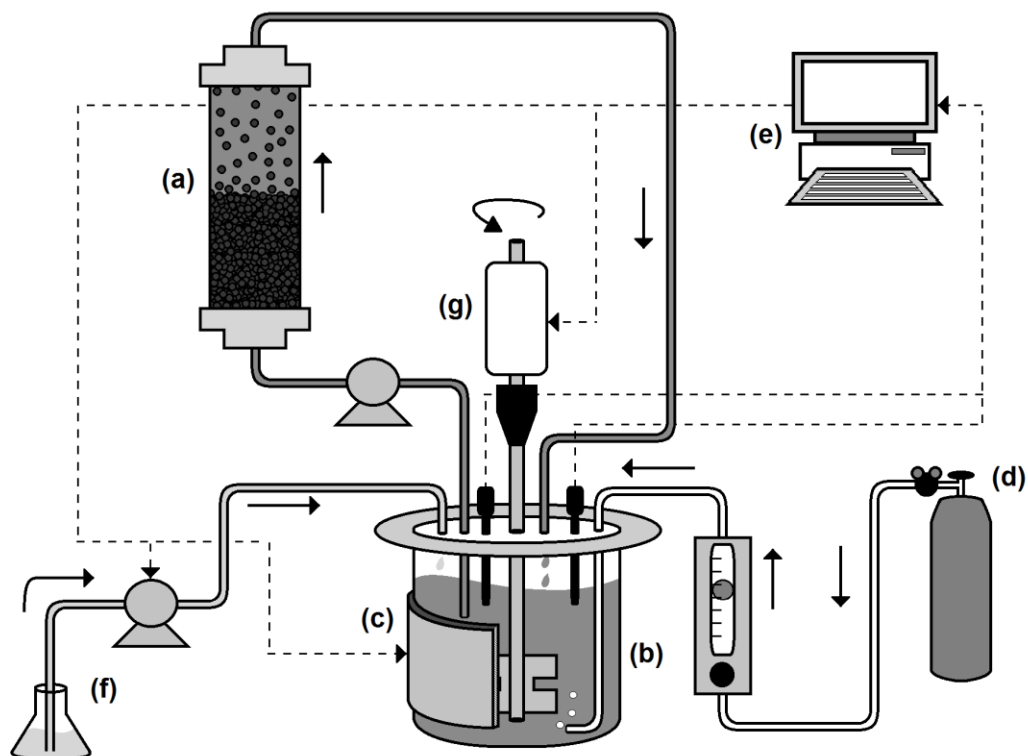


Figure 2.1. Fermentation process flow diagram with adsorption column.

2.2. Cell-free Characterizations

2.2.1. Adsorption Isotherms

To construct an adsorption isotherm, several model solutions were prepared with deionized water at varied initial solute concentrations. Solutes used to prepare isotherms included: butanol, acetone, ethanol, and glucose. A pre-weighed mass of dried L-493 resin (1-4 grams) was added to a 40 mL vial (VWR, Radnor, PA) with 25 mL of the prepared solution. The vial was tightly sealed and shaken on its side at 120 rpm and 37°C for 24 hours.

At the conclusion of the 24 hours (equilibrium was typically reached within 2 hours, data not shown), a sample of the equilibrated solution was taken and analyzed via high-performance liquid chromatography (HPLC; 1100 Series, Agilent, Santa Clara, CA), see section 2.4 for discussion on the HPLC. The concentration of the analyzed solutions were determined using external standards. The specific loading was determined using Equation 1.

$$q = \frac{(C_0 - C)V_{aq}}{m_a} \quad (1)$$

After repeating this process several times for varied initial concentrations, a curve was generated with specific loading vs. equilibrium concentration (refer to Figure 1.3). The same protocol was used for multi-component isotherms (e.g. solutions containing acetone, ethanol, and butanol). It is important to note that all isotherms included one triplicate trial for error analysis. Additionally, all isotherms were modeled using Equation 2.

$$q = kC^{1/n} \quad (2)$$

The model parameters were estimated using nonlinear least squares regression via the *nlinfit* function in MATLAB®.

2.2.2. Adsorption Kinetics in Column

To test the rate of butanol removal via adsorption with different circulation rates and adsorbent masses, the column was filled with dry L-493 and attached to the bioreactor. Added to the reactor was 1.5 L deionized water and butanol to make a solution with an initial concentration of ~14 g/L butanol. The liquid in the bioreactor was pumped at a flow rate between 10.7 and 95.3 mL/min through the column using a peristaltic pump (Cole Parmer, Vernon Hills, IL). A syringe (Integra 3 mL syringe, BD, Franklin Lakes, NJ) was used to take in-line samples from rubber tubing. The HPLC was used to analyze the concentration of butanol at different time points during the 100 minute experiment.

2.2.3. Simulated Fermentation

To test the viability of the designed adsorption column, a *C. acetobutylicum* fermentation was simulated. Water was added to the bioreactor, and butanol was added at the *C. acetobutylicum* wild-type production rate of 0.37 g/L·hr (Li et al., 2011). The butanol was added using a 60 mL Monoject syringe and an associated syringe pump (Auto Syringe AS40A, Baxter, Deerfield, IL). The solution in the bioreactor was circulated through the column at a superficial

velocity of 10.2 ± 0.3 cm/min. The average resin fraction during the experiment was 33.0 g/L. The butanol concentration in the bioreactor was sampled with time and analyzed via HPLC.

2.3. *Clostridium acetobutylicum* Fermentation

2.3.1. Media

The fermentation broth used was 2x Clostridial Reactor Media (CRM) and was prepared according to Mermelstein et al. (1994):

Table 2.1. 2x Clostridial Reactor Media components.

Compound	Mass added per liter
KH ₂ PO ₄	1.5 g
K ₂ HPO ₄	1.5 g
MgSO ₄	0.696 g
MnSO ₄ ·H ₂ O	20 mg
FeSO ₄ ·7H ₂ O	20 mg
p-Aminobenzoic acid	8 mg
NaCl	2 g
Asparagine	4 g
Yeast extract	10 g
(NH ₄) ₂ SO ₄	2 g

The fermentation broth was prepared and autoclaved in the bioreactor with the pH and oxygen probes 24 hours prior to inoculation. Glucose was autoclaved separately for 30 minutes at 121°C; when the glucose solution and the autoclaved broth were combined, the resulting media and glucose concentrations were 2x and 120 g/L, respectively. Antifoam 204 (Sigma, St. Louis, MO) was added in volumes of 100-1000 µL during fermentations to reduce foaming during ultra-high purity nitrogen purges.

2.3.2. Inoculum Preparation

To enrich the frozen or refrigerated stocks of *Clostridium acetobutylicum* ATCC 824 for inoculation into the bioreactor, the cells were added to oxygen-free, 15 mL anaerobic tubes filled with Reinforced Clostridial Medium (RCM; BD Difco, Franklin Lakes, NJ). The media was

prepared according to the provided instructions. To the media, 100 μL of 5 g/L resazurin was added. The resazurin was reduced by boiling the media for 10 minutes while stirring. Following the purple to pink color change of the media, pure nitrogen was bubbled into the media via a glass pipet until the media returned to its original color. At this point, 10 mL of the oxygen-free media was added into a nitrogen-flushed anaerobic tube. The tube was tightly capped and autoclaved. Following the autoclave, the tube was chilled to room temperature. At this point, 1 mL of frozen *C. acetobutylicum* stock was added to the tube. The newly inoculated tube was placed in the 37°C incubator overnight.

2.3.3. Bioreactor Start-up and Operation

After combining the glucose to the fermentation broth in the bioreactor, the media was sparged with nitrogen until the DO level was 0.0%. The pH of the media was adjusted to 5.5. Additionally, the temperature of the bioreactor was raised to 37°C. Following stabilization of the bioreactor, 15 mL of active cells enriched using RCM were added. When the dry weight of the cells was 0.5 g/L, the pH was changed to 4.9. To measure the dry weight of the cells, the optical density was used:

$$DW = 0.26 \cdot OD_{600} \quad (3)$$

where DW is the dry cell weight in g/L, and OD_{600} is the optical density analyzed at a wavelength of 600 nm (Guo et al., 2012). The optical density of the media was analyzed using a spectrophotometer (DU 800, Beckman Coulter, Brea, CA).

2.3.4. Adsorption Column Operation and Glucose Addition

As the culture entered solventogenesis (as marked by a subtle increase in the pH), media circulation through the adsorption column was initiated. As the glucose concentration approached 0 g/L (as anticipated from a control culture), 200 mL of 30 g/L glucose was added to the bioreactor. Following the addition of glucose, nitrogen sparging was used to re-establish and maintain an oxygen-free environment.

2.3.5. *Scanning Electron Microscopy*

Biofouling of the resin was studied using Scanning Electron Microscopy (SEM). Following extensive contact with the fermentation media and the cells, the resins were air-dried for 24 hours. The resin was fixed to carbon tape on pin stubs and then sputter coated with gold (Technics Sputter Coater). Following the gold coating, a Scanning Electron Microscope (JSM6300, JEOL, Tokyo, Japan) was used to image the resin.

2.4. **High-Performance Liquid Chromatography**

2.4.1. *Analysis of Model Solutions*

Several isotherms are constructed using single component solutes (e.g. butanol, acetone, ethanol, etc.). A HPLC (1100 Series, Agilent, Santa Clara, CA) was used to test the concentration. Double-deionized water was used as the mobile phase at a flow rate of 1 mL/min through a hydrophobic column (Hypersil GOLD aQ, Thermo Scientific, Waltham, MA). The volume of the sample injected into the HPLC was 5 μ L and the column was kept at 50°C. The concentration of the solute was determined using a refractive index detector (RID) and compared to external standards.

2.4.2. *Analysis of Fermentation Broth*

Due to the complex nature of fermentation broth, two columns were required in series to effectively separate the metabolites and nutrients. The sample (5 μ L) was carried in a 0.005 M H₂SO₄ mobile phase through the previously described hydrophobic column. The effluent of the hydrophobic column fed into an anionic column (Aminex HPX87H, Bio-Rad, Hercules, CA). The columns were maintained at 35°C. The samples were analyzed using a RID and external standards.

CHAPTER 3 CELL-FREE CHARACTERIZATIONS

3.1. Adsorption Capacity

3.1.1. *Multi-component Isotherms*

Adsorption isotherms were elucidated to determine the behavior of metabolite adsorption onto L-493 resin. It was postulated that the presence of acetone and ethanol would influence the adsorption of butanol, acting as direct competitors. Therefore, in addition to determining the adsorption behavior for individual component solutions (e.g. only butanol and water), it was determined for model mixtures of acetone, butanol, and ethanol (in a 3:6:1 molar ratio) in water (Figure 3.1).

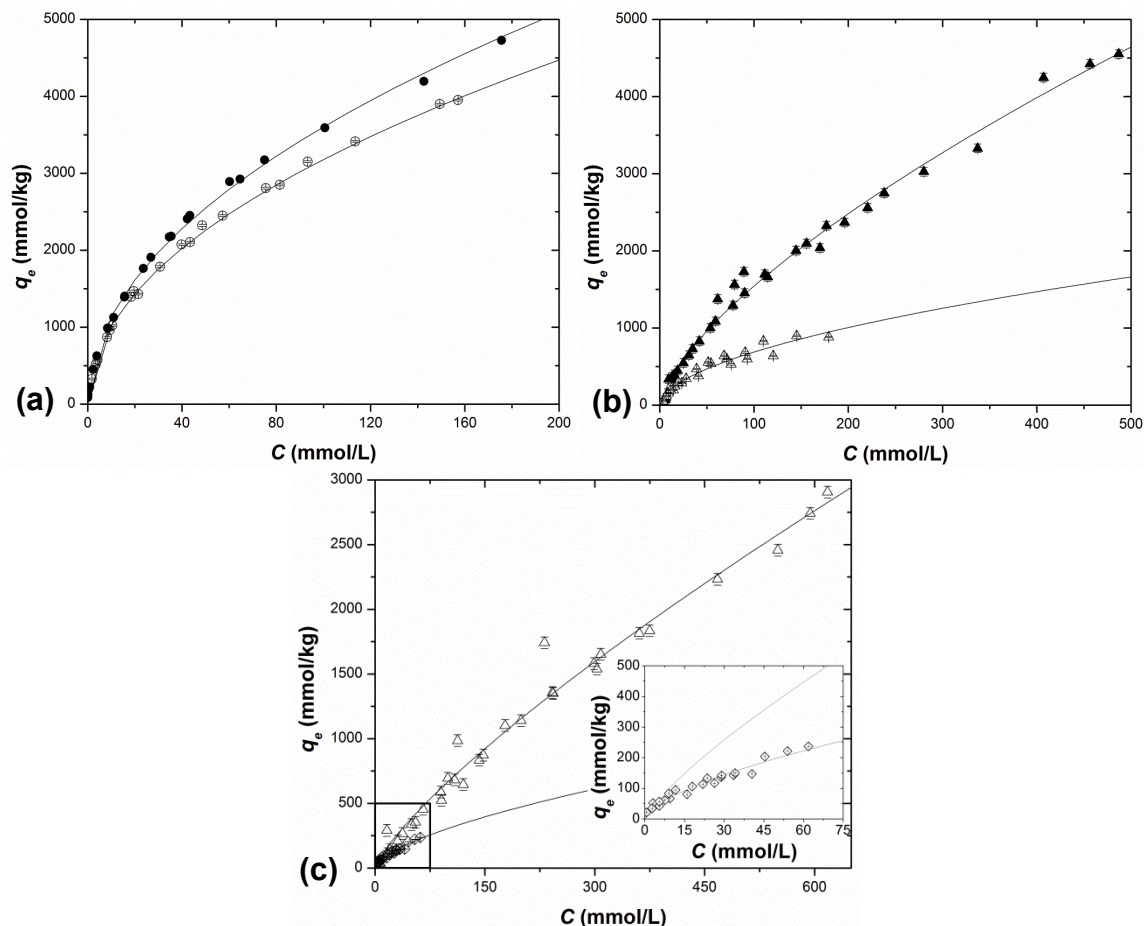


Figure 3.1. Single (closed) and multi-component (open) isotherms with model-fits: (a) acetone; (b) butanol; (c) ethanol, the box in magnified to the inset graph. All data include error bars that show standard deviation.

Figure 3.1 shows the single and multi-component isotherms for acetone, butanol, and ethanol, as well as the respective Freundlich model fits. Referring to Figure 3.1a, the specific loading of acetone significantly decrease when butanol and ethanol are present in the solution. At a concentration of 175 mM, roughly 2,260 mmol/kg was adsorbed when acetone was the only component present in the solution. However, with the addition of ethanol and butanol, 58.7% less acetone adsorbed onto the resin. The same was also true for ethanol (3.1c). In the case of ethanol at 175 mM, ~1,040 mmol/kg was adsorbed in a single component system. Similar to

acetone, addition of competitors (butanol and acetone in this case) decreased the amount of ethanol adsorbed by 58.2%.

However as seen in Figure 3.1b, the butanol specific loading was only slightly decreased in the presence of ethanol and acetone (i.e. in the multi-component model system). In the single component butanol system at 175 mM, about 4,760 mmol/kg was adsorbed, and the addition of competitors only decreased the butanol loading by 12.1%. Therefore, butanol adsorption is not nearly as affected by competitors (ethanol and acetone). It is important to note that 175 mM is ~13 g/L, the inhibitory butanol concentration in *C. acetobutylicum* fermentations.

The fact that the specific loading of butanol was not competitively inhibited by either acetone or ethanol, it was concluded that butanol adsorbs onto L-493 with the highest affinity. These results correlate with the functionality of the resin, L-493 is a hydrophobic resin. A compound with a higher $\log K_{ow}$ is more hydrophobic. Referring to Table 1.2, butanol has the highest $\log K_{ow}$ of 0.88. Acetone is second with -0.24, followed by ethanol with -0.31. Thus, while all three solutes adsorb, butanol dominates the majority of the adsorption sites on the resin surface due to its higher relative hydrophobicity. By inspecting the chemical structures of the three compounds (refer to Table 1.2), butanol has four carbons compared to the three in acetone, and two in ethanol. Carbon atoms in a molecule generally have a positive correlation with molecule hydrophobicity. Therefore the results shown above correlate well with theory.

In model solutions created to simulate fermentation broths, it has been shown that L-493 preferentially adsorbs butanol relative to the other potential metabolites (Eom et al., 2012). This phenomenon is further defined by examining the polymeric resin to aqueous solution partition coefficient (g solute loaded per kg resin per solute concentration in the solution) (Nielsen and Prather, 2008). For L-493 resin, the largest coefficient is butanol followed by acetone, butyrate, ethanol, acetate, and glucose with coefficients of 195.4, 16.4, 15.5, 9.9, 1.4, and 1.4, respectively (Nielsen and Prather, 2008). In the experimental results shown above, the resin selectively adsorbs butanol when present with acetone and ethanol. These results are consistent with Nielsen and Prather, as the resin to solution partition coefficient for L-493 favors butanol.

Despite the decrease in specific loading present in multi-component systems, the amount of butanol adsorbed per mass of resin still surpasses that of other adsorbents described in literature.

3.1.2. Effect of Glucose on Butanol Adsorption

Since the specific loading of butanol was influenced by the presence of other solutes in the media (acetone and ethanol), it is desired to know how the specific loading changes with the glucose concentration, as glucose will be in large excess during fermentations. The glucose concentrations tested with butanol in solution were 0%, 0.5%, 4%, 8%, and 12% (Figure 3.2).

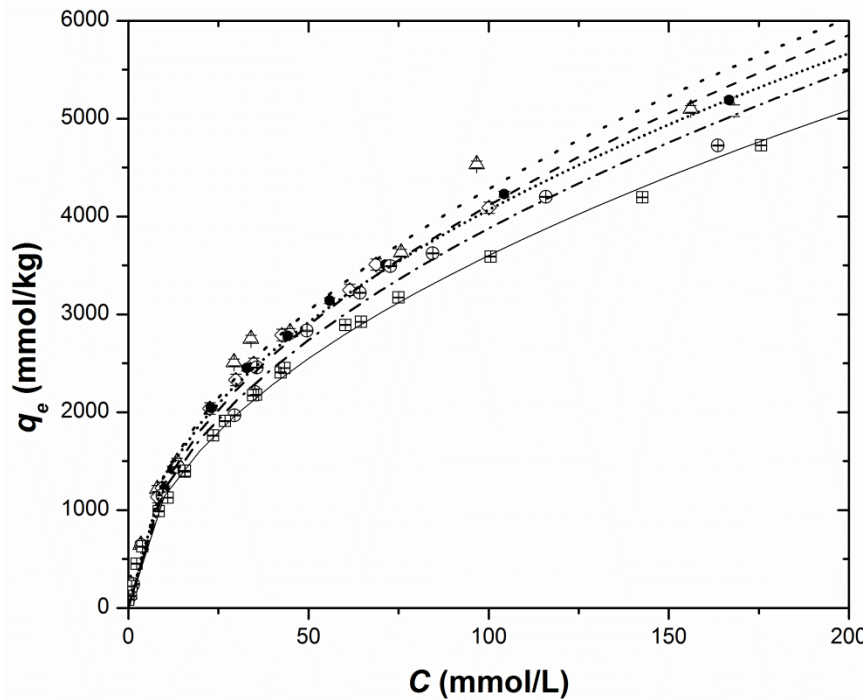


Figure 3.2. Specific loading of butanol onto L-493 resin glucose concentration of 0% (open squares), 0.5% (open circles), 4% (open triangles), 8% (open diamonds), and 12% (closed circles). All data sets are fit using the Freundlich model: 0% (solid line), 0.5% (dash-dot line), 4% (spaced dots), 8% (close dots), 12% (dashes). All data include error bars showing standard deviation.

Figure 3.2 compares the influence of glucose on the specific loading of butanol with the single-component butanol isotherm. While the glucose concentration did not have a concentration-dependent increase on the specific loading, all solutions containing glucose experienced increased butanol adsorption. Therefore it appears that the presence of glucose caused the hydrophobic effect and further drove butanol adsorption. The $\log K_{ow}$ of glucose was determined to be -2.82 (Mazzobre et al., 2005), showing that it strongly prefers to be in water, but has a weakly hydrophobic nature. Therefore it is speculated that the addition of glucose causes more butanol to adsorb onto the resin by lowering the butanol solubility in the solution. It was assumed that negligible glucose adsorption occurred during these experiments. Examination of the resin to solution partition coefficient for glucose and butanol (1.4:195.4) (Nielsen and Prather, 2008), butanol will be the dominant adsorbing species.

3.1.3. Isotherm Model Parameters

All isotherms were fit to the Freundlich isotherm model, to estimate value of k and n , which are compared in Table 3.1 below.

Table 3.1. Freundlich parameters for various isotherms. All estimates provided at one standard deviation.

Solute	Solution	k (mmol/kg)	n
Butanol	Single-component	362 ± 25	2.00 ± 0.06
	Multi-component	328 ± 21	2.03 ± 0.06
	0.5% Glucose	384 ± 74	1.99 ± 0.17
	4% Glucose	440 ± 100	2.02 ± 0.21
	8% Glucose	450 ± 62	2.09 ± 0.14
	12% Glucose	395 ± 69	1.96 ± 0.15
Acetone	Single-component	66 ± 12	1.46 ± 0.07
	Multi-component	55 ± 19	1.82 ± 0.26
Ethanol	Single-component	18 ± 6	1.26 ± 0.09
	Multi-component	17 ± 5	1.60 ± 0.20

Selected Freundlich fits were compared with previously published data for L-493 resin in Figure 3.3.

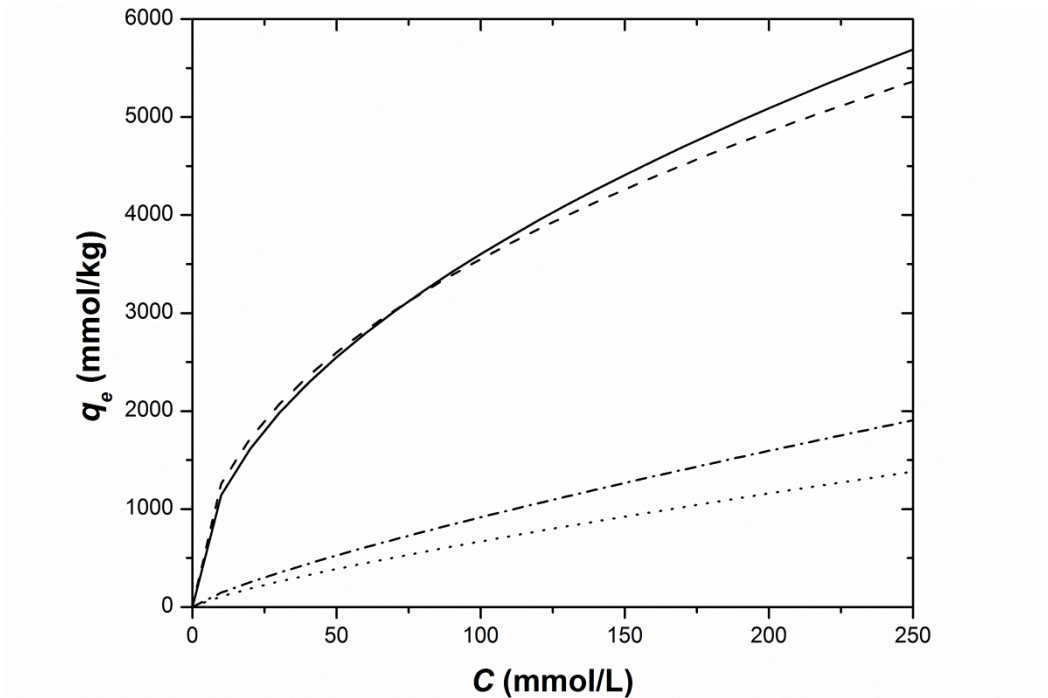


Figure 3.3. Experimental isotherm fits compared to published data: experimental butanol (solid line), experimental ethanol (dots), butanol (Nielsen and Prather, 2008) (dashes), ethanol (Nielsen and Prather, 2008) (dash-dot-dash).

As can be seen from Figure 3.3, the experimentally determined model fit for butanol adsorption onto L-493 very closely matches previously published data. The ethanol adsorption capacity does not align as well; however, this can be attributed to the error within the experiment. Thus, it can be concluded that the adsorption isotherms estimated here agree very well with published data.

3.1.4. Effect of Autoclaving on Butanol Adsorption

To maintain sterility in culture fermentations, all exposed surfaces and media components were subjected to steam autoclaving for 30 minutes. Therefore, for adsorbents to be a viable option for fermentations, the polymeric resins must be thermally and mechanically stable when subjected to the high (121°C) sterilization temperatures. The adsorbents were tested for butanol loading capacity before and after several cycles of autoclaving (Figure 3.4).

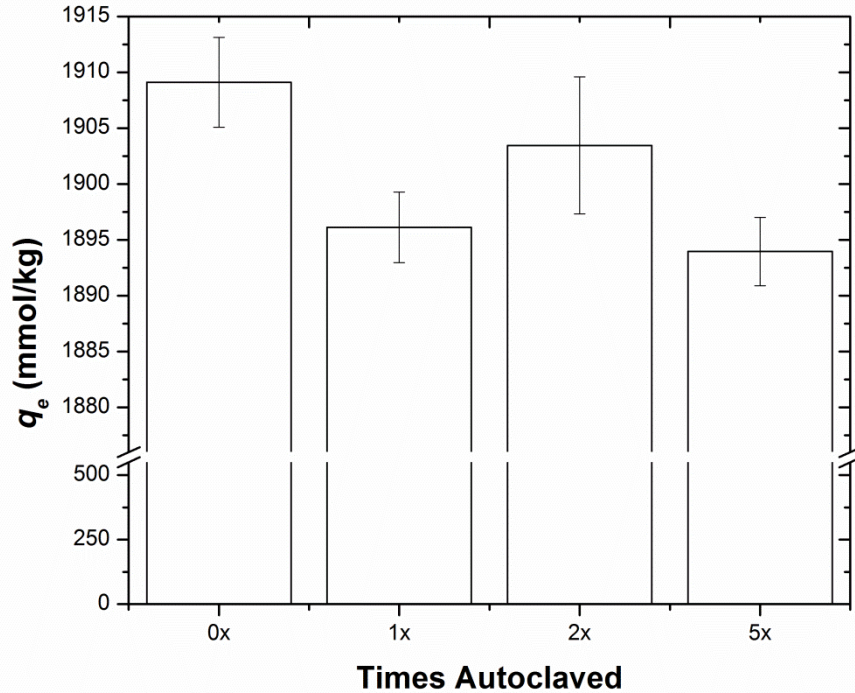


Figure 3.4. Butanol specific loading onto L-493 after multiple cycles of autoclaving autoclaving. Error bars are shown at one standard deviation.

Figure 3.4 shows the change in specific loading autoclaving the resin. This experiment was evaluated at a constant final concentration of 2.05 ± 0.05 g/L, a concentration that is often encountered during fermentations. Although the specific loading is highest when the resins are not autoclaved, but it appears that the high temperatures did not significantly influence the resin performance. For example, when autoclaved 5x, the specific loading decreased on average by only 0.79% compared to the unautoclaved resin. The variation of specific loading was within error of the experiment. It can be assumed that the unautoclaved resin will behave the same as autoclaved resin. Furthermore, this experiment shows that the resin can be autoclaved repeatedly, showing applicability to industrial fermentations.

3.2. Adsorption Kinetics in the Expanded Column

3.2.1. Effect of Adsorbent Mass

An important design variable for the adsorption column is the amount of adsorbent that it contains. To determine the influence of adsorbent mass on the butanol extraction rate, extent, and efficiency, several resin masses were tested in the adsorption column, each with constant superficial velocity (v) of about 9.6 ± 0.4 cm/min.

$$v = \frac{Q}{A_c} \quad (4)$$

Where Q is the volumetric flow rate, and A_c is the cross-sectional area of the column. The mass added to the column was normalized to the liquid volume in the bioreactor resulting in the resin fraction (X_r) defined as:

$$X_r = \frac{m_a}{V_{aq}} \quad (5)$$

The effect of three different X_r values was studied in the adsorption column: 10.5, 13.9, and 21.5 g/L, as seen in Figure 3.5. In all cases, the initial butanol concentration was ~ 14 g/L, which represents a titer well above the toxicity limit in *C. acetobutylicum* fermentations.

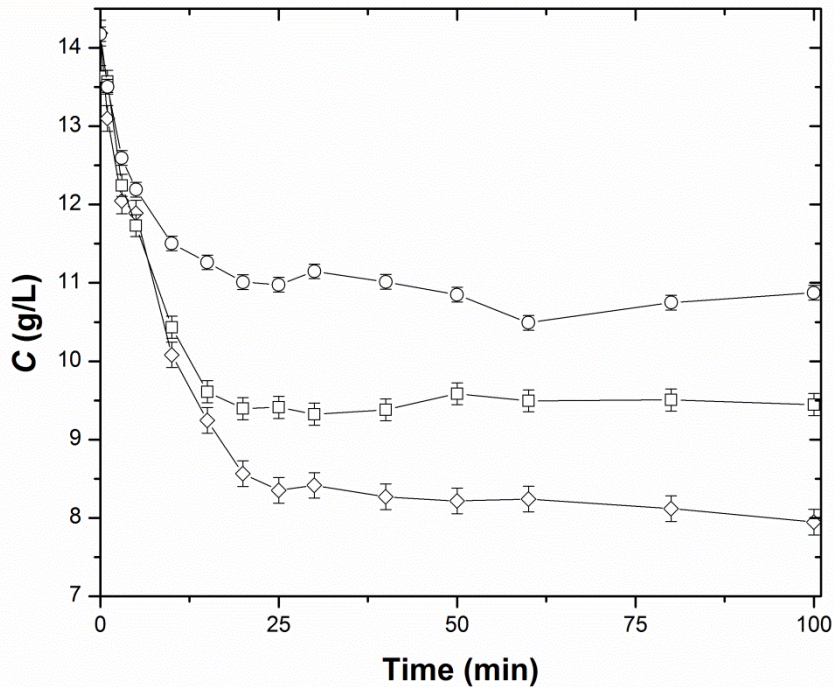


Figure 3.5. Decrease in butanol concentration with varied resin fraction: 10.5 g/L (diamonds), 13.9 g/L (squares), 21.5 g/L (circles).

In all cases, the butanol concentration was found to decrease sharply at first, before gradually approaching steady state by about 25 min. It is also notable that, independent of X_r , net initial rates of butanol removal were about equal. This shows that, under the conditions examined, X_r does not influence the rate of butanol adsorption, only the residual butanol concentration remaining in the bioreactor at steady state.

3.2.2. Effect of Circulation Rate

A second important design variable to be optimized in the expanded-bed design is the circulation rate of the butanol containing media through the column. To investigate the influence of superficial velocity on extraction rate, extent, and efficiency of butanol adsorption, four different superficial velocities were tested with a fixed X_r of 13.9 g/L. The initial concentration of butanol was again ~14 g/L in each case. The results are compared in Figure 3.6.

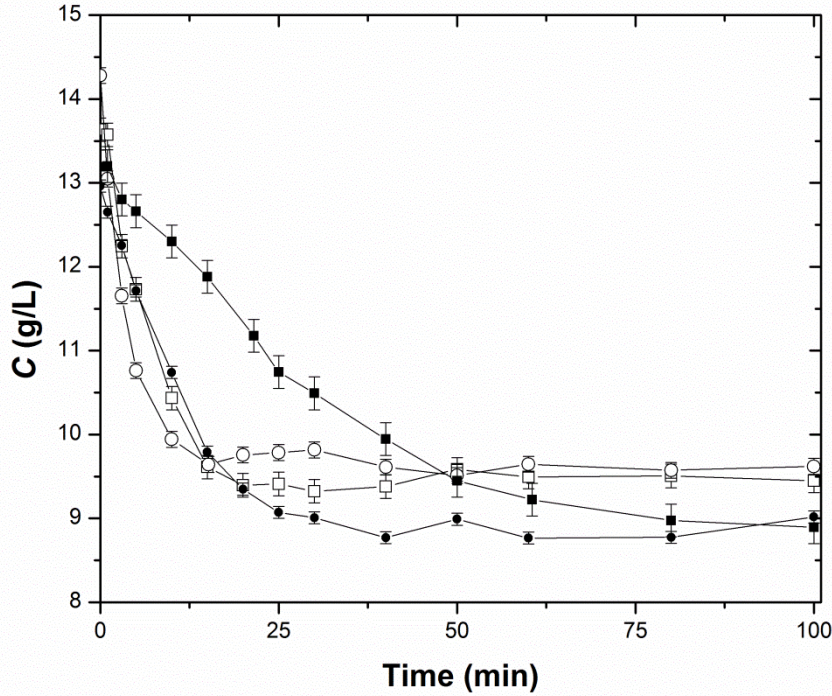


Figure 3.6. Decrease in butanol concentration with varied superficial velocity: 2.1 ± 0.1 cm/min (closed squares), 5.7 ± 0.2 cm/min (closed circles), 9.5 ± 0.2 cm/min (open squares), 18.5 ± 0.2 cm/min (open circles).

With a superficial velocity of 18.5 cm/min, the residual butanol concentration decreased rapidly, reaching equilibrium by ~15 minutes. However, as the superficial velocity was reduced, the system took proportionally longer to reach equilibrium. For superficial velocities of 9.5 and 5.7 cm/min, equilibrium was not reached until ~20 and ~30 min, respectively. Concurrently, when the lowest superficial velocity, 2.1 cm/min, was used, equilibrium was not reached until after 80 min. Clearly there exists a positive correlation between equilibration time or butanol removal rate and superficial velocity through the column. However, it can also be seen that increases in superficial velocity past 18.5 cm/min would likely result in diminishing returns which may not warrant the excess energy expenses associated with pumping solutions at faster rates. Additionally, high pumping and flow rates through the column could be a detriment to the cells as a result of increased shear and mechanical stresses. As at a superficial velocity of 9.5 cm/min

equilibrium could still be reached in ~20 min, this was selected as ideal for application with bioreactor cultures.

3.3. Simulated Butanol Fermentation

3.3.1. Butanol Recovery by Expanded-bed Adsorption in Simulation Cultures

To simulate butanol production in bioreactor cultures, pure butanol was slowly pumped into a bioreactor originally containing 1.5 L of pure water at a constant rate of 0.37 g/L·hr, representing typical, previously reported butanol production rates for wild-type *C. acetobutylicum* (Li et al., 2011). The adsorption column was operated with a resin fraction of 21.9 g/L and a superficial velocity of 10.2 ± 0.3 cm/min. The result is shown in Figure 3.7 where the data is compared to a control experiment in which no adsorption column was used.

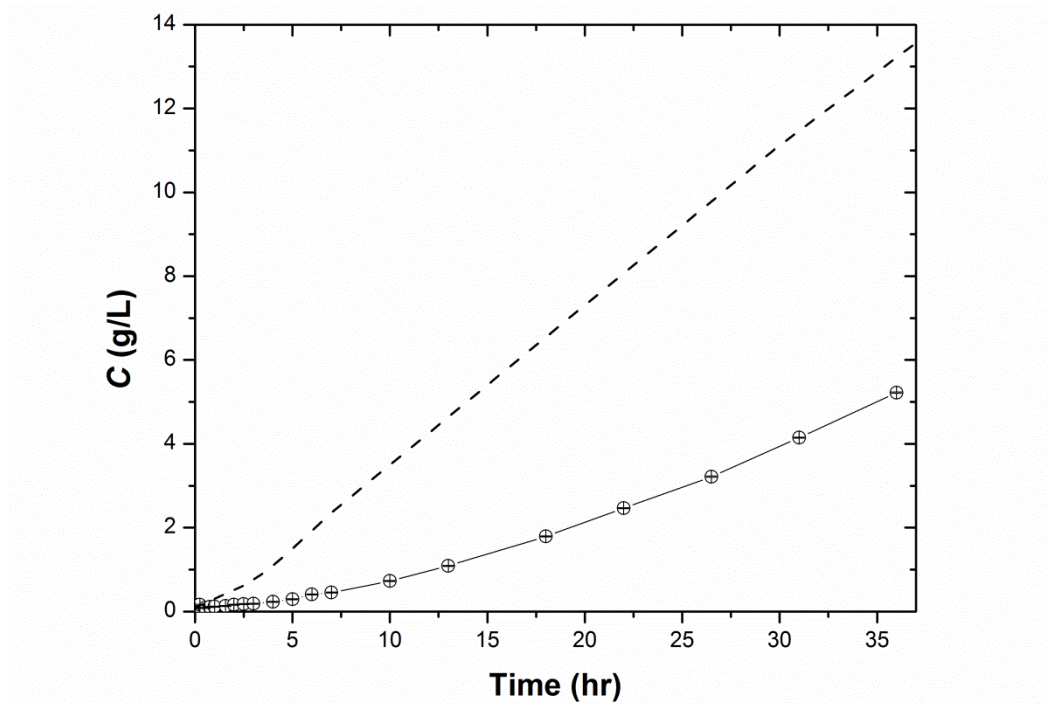


Figure 3.7. Butanol addition into a bioreactor without the addition of the adsorption column (dashed line) and with EBA (open circles).

In the absence of the adsorption column, it can be seen that the butanol concentration in the control (>13 g/L) exceeded the butanol inhibitory limit by the end of the experiment. In

contrast, when the media was continuously circulated through the adsorption column the butanol concentration in the bioreactor remained sub-inhibitory throughout. If the experiment were continued longer, the butanol concentration would eventually exceed the toxic limit even in the presence of the column; however, this event would occur at a later point in the fermentation and at a time by which the total butanol accumulated in the system would far surpass that of the control. By slowing the rate by which butanol accumulates in the bioreactor medium, the cells would theoretically consume more glucose and produce more butanol. By this experiment, it is shown that the expanded-bed adsorption system can avoid onset of inhibitory conditions in the bioreactor, enabling greater butanol accumulation and higher volumetric productivities.

CHAPTER 4 CLOSTRIDIUM ACETOBUTYLICUM FERMENTATIONS

4.1. Butanol Fermentation without Expanded-bed Adsorption

4.1.1. Conventional Batch Fermentation (without EBA)

To quantitatively assess the impact of in-situ butanol removal by expanded-bed adsorption, a control fermentation was first conducted without the use of EBA. The results are shown in Figure 4.1.

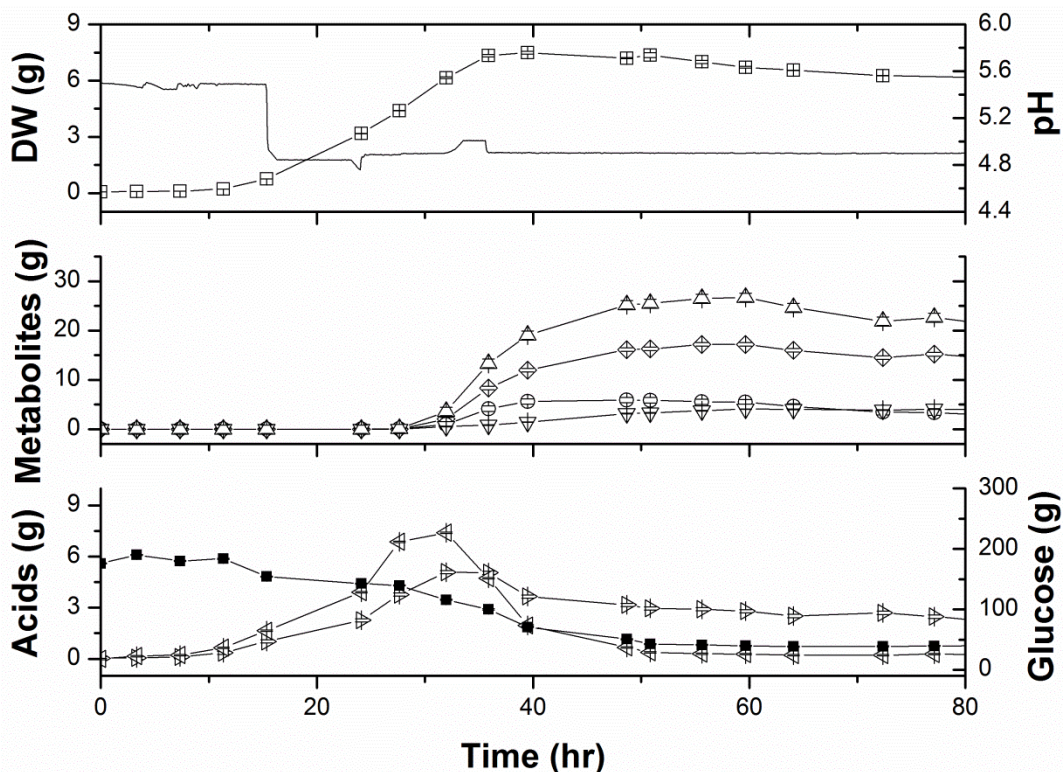


Figure 4.1. *C. Acetobutylicum* fermentation without EBA: pH (solid line), dry cell weight (open squares), solvent (triangles), butanol (diamonds), acetone (circles), ethanol (inverse triangles), glucose (closed squares), butyric acid (left triangle), acetic acid (right triangle).

As can be seen, following inoculation into the bioreactor, the cells underwent a slight lag phase. Following the lag phase, the cells entered the exponential growth phase, marked by a rapid increase in cell density and the increase of acid (butyric and acetic) production. The initial pH of the broth following inoculation was 5.5, but decreased slightly at around 5 hr, indicating that organic acid production by the cells had begun. After 16 hours, the optical density of the culture

reached ~1.95 and the pH was shifted to 4.9. At 32 hr, the pH began to increase, indicating a switch in the cell metabolism from acidogenesis to solventogenesis. Referring to Figure 1.1, acids are consumed as the solvents are produced. Consumption of acid in the fermentation broth causes a rise in pH (Jones and Woods, 1986).

Cells continued to produce solvents (butanol, acetone, and ethanol) until the inhibitory concentration of butanol was reached (~11.5 g/L for this experiment) at 55 hr. At this point, a total of 17.2 g butanol had been produced while ~40 g of unconsumed glucose still remained in the broth. Relevant results from this fermentation experiment are also summarized in Table 4.1.

4.2. Fermentation with Expanded-bed Adsorption

4.2.1. EBA Adsorption

A column containing 75 g fresh L-493 was integrated with a batch bioreactor that initially contained 1.5 L of CRM media with 120 g/L glucose. This represented an initial resin fraction of 50 g/L. At first, cells were cultured in conventional batch mode without circulation through the column to allow the cells to overcome the lag period and enter the solventogenic phase. Similar to the control fermentation, the pH was initially held at 5.5 and was switched to 4.9 when the dry cell weight reached ~0.5 g/L. Following inoculation, the cells underwent a lag phase followed by exponential growth to a maximum of 6.4 g cells. During this time, both acetic and butyric acid were produced; however, solvent production did not occur. As indicated in Figure 4.2, column circulation was initiated 42 h after starting the culture and continued through the remainder of the experiment. Following initiation of the EBA column, the cell density was not observed to decrease significantly, suggesting that the circulation through the EBA column was not harmful to the cells.

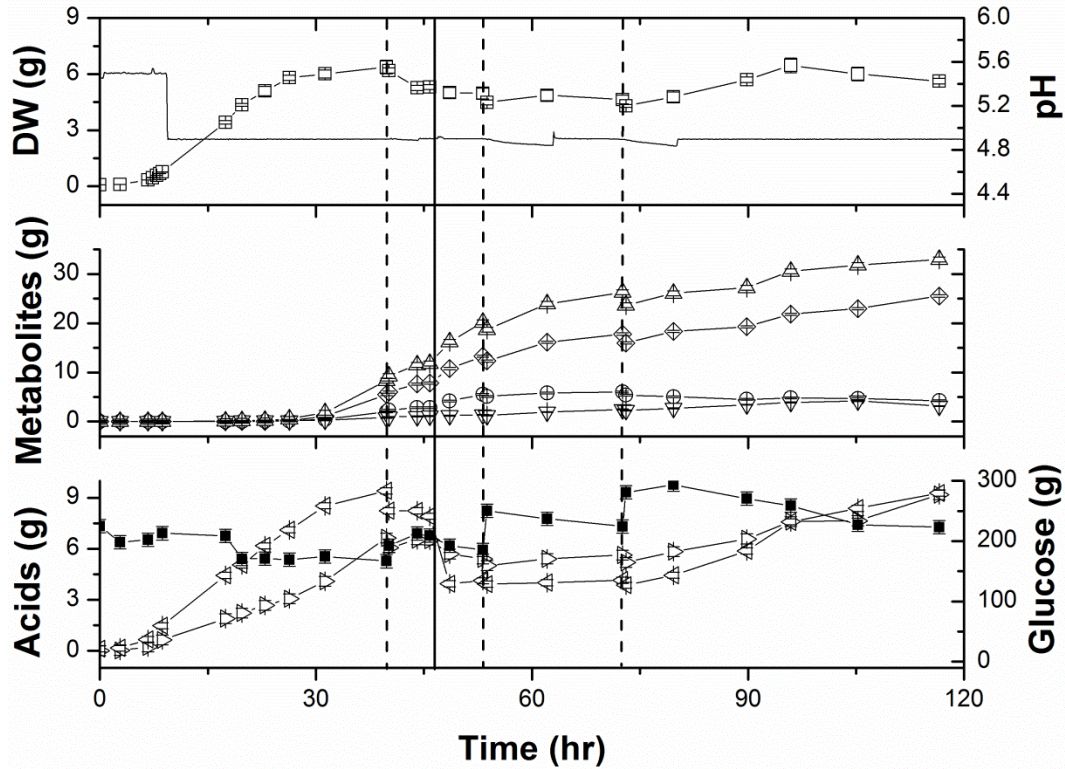


Figure 4.2. *C. acetobutylicum* fermentation with integrated EBA: pH (solid line), dry cell weight (open squares), solvent (triangles), butanol (diamonds), acetone (circles), ethanol (inverse triangles), glucose (closed squares), butyric acid (left triangle), acetic acid (right triangle), glucose additions (vertical dashed line), EBA induction (vertical solid line).

Figure 4.2 does not show the decrease from solvent adsorption as the data was corrected to show effective concentrations. To calculate the effective concentrations for acetone, butanol, and ethanol in the entire integrated process, the mass of each adsorbed species was predicted using model fits for the respective multi-component isotherms (Table 3.1), assuming that the adsorbent and aqueous phase were in equilibrium. Preliminary experiments indicated that, for the circulation rate used in this study, such an assumption could be considered as valid (data not shown).

Glucose was added in a fed-batch manner three times, at 40, 53, and 72 hr, during the fermentation to prevent nutrient limitation and to promote a continuous process. Following the first glucose addition at 40 hours, the cell density decreased. This was likely due to oxygen

present in the glucose added. The experiment ended with a large excess of glucose in the system, suggesting perhaps that another essential nutrient may in fact have been limiting to the process.

4.2.2. Comparison of Batch versus EBA

To determine the impact of EBA, the relevant fermentation data was summarized (Table 4.1).

Table 4.1. Comparison of fermentation results.

Parameter	Batch	EBA
Acetone (g)	5.48	4.28
Butanol (g)	17.20	25.53
Ethanol (g)	4.07	3.16
Total ABE (g)	26.75	32.97
Average Butanol Productivity (g/L·hr)	0.41	0.20
Maximum Butanol Productivity (g/ L·hr)	0.71	0.81
Average ABE Productivity (g/ L·hr)	0.63	0.31
Maximum ABE Productivity (g/ L·hr)	1.10	1.60
Butanol Yield (%)	12.3	18.6
ABE Yield (%)	19.1	24.1
Acetic Acid (g)	2.80	9.18
Butyric Acid (g)	0.27	9.28
Initial Glucose (g)	180	180
Total Glucose Added (g)	180	360
Final Glucose (g)	39.6	223.11
Glucose Availability (%)	78.0	38.0
Dry Cell Weight (g)	7.4	6.38
Dry Cell Yield (%)	5.3	4.7

While the batch fermentation produced slightly more acetone and ethanol, integration of EBA resulted in a 33% increase in net butanol production. The batch fermentation had an average butanol productivity that was over two times that of the EBA fermentation (0.41 v. 0.20 g/L·hr). However, the EBA fermentation had the larger maximum butanol production rate (0.81 v. 0.71 g/L·hr). The butanol productivity rates experienced are consistent with those previously reported, for example: 0.37 g/L·hr by Li et al. (2011), 0.32 g/L·hr by Lu et al. (2012), 0.48 g/L·hr

by Yen et al. (2011), and 0.71 g/L·hr by Yen et al. (2011). Similar to butanol productivity, the batch fermentation had a higher average ABE productivity than the EBA fermentation at 0.63 v. 0.31 g/L·hr. Meanwhile, the butanol and ABE yield per mass of glucose consumed was higher for the EBA fermentation (18.6% and 24.1%, respectively) compared to batch (12.3% and 19.1%).

The EBA fermentation had residual glucose remaining in the reactor, attributing to a low glucose availability of 38.0%. When no glucose was added, the batch fermentation, the glucose availability was 78.0%. The dry cell weight and yield were less for the EBA fermentation compared to the batch, likely do to minor inconsistencies between trials.

4.2.3. Assessing biofouling of the L-493 Adsorbent

To provide a preliminary assessment of the potential for adsorbent biofouling in the EBA column, a sample of L-493 was removed from the column following the experiment for imaging by scanning electron microscopy (SEM). The results compared with a sample of fresh L-493 as control, are shown in Figure 4.3.

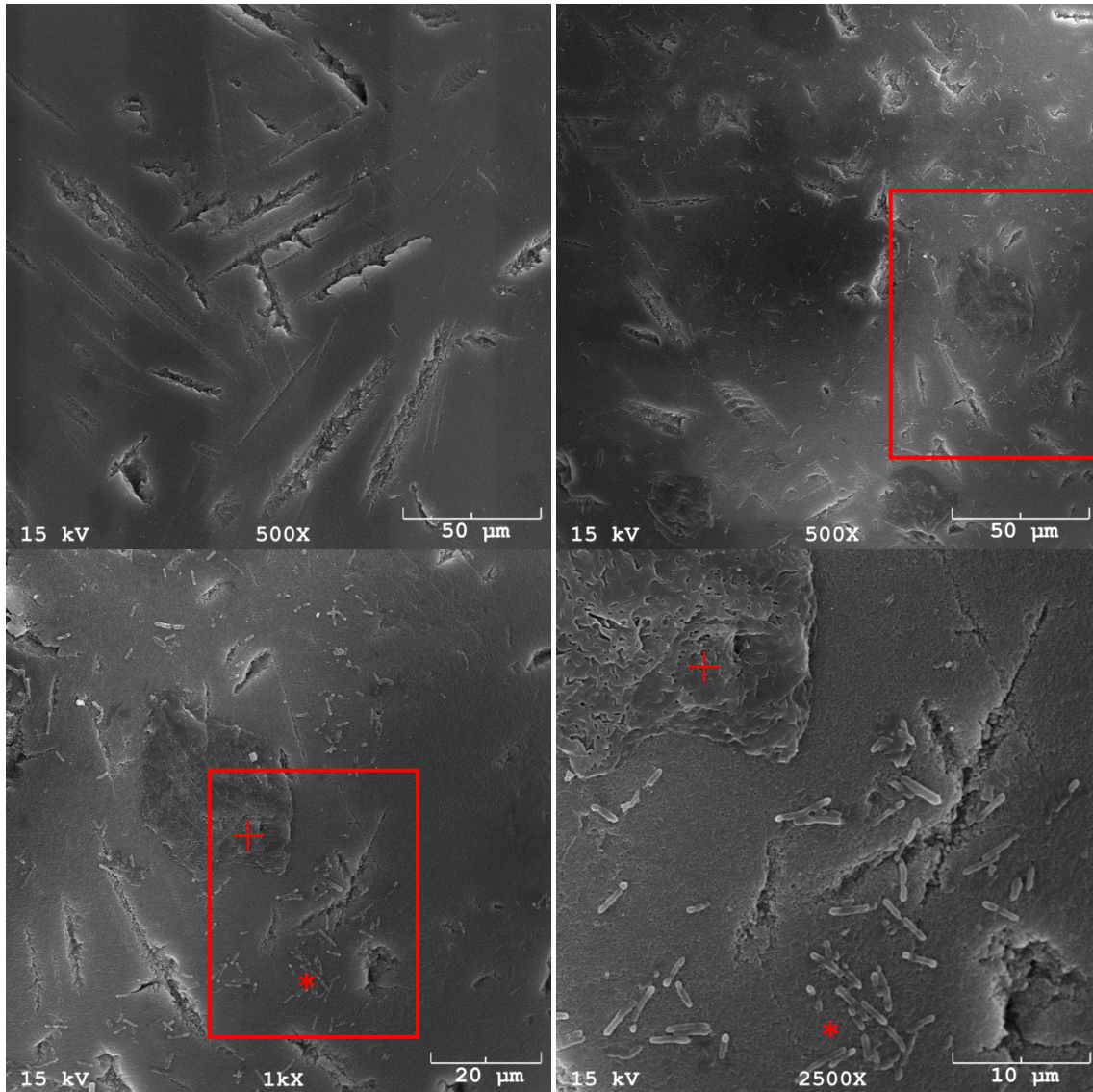


Figure 4.3. SEM images of L-493: fresh resin 500x (upper left), used resin 500x (upper right), used resin 1000x (lower left), used resin 2500x (lower right). The red boxes denote area of magnification. Examples of cells are indicated by an asterisk and extracellular polymeric substances with a cross.

Inspection of the fresh L-493 does not show any trace of cellular debris as expected. However, in the resin samples from the EBA column, cells can be observed in all images. In addition to whole cells, there also appears to be some evidence of extracellular polymeric substances (EPS) accumulation on the resin (Dohnalkova et al., 2011). Magnification to 2500x

clearly shows rod-shaped bacteria, characteristic of *Clostridia*. While *Clostridia* adsorbs onto the surface of the L-493, most of the adsorbent surface was not covered with an average cell count of $\sim 1.93 \cdot 10^6$ cells/cm². Similarly, Li et al. (2011) reported minimal biofouling of the ion-exchange adsorbents used with a coverage of $\sim 1.85 \cdot 10^6$ cells/cm². Furthermore, Groot and Luyben (1986) reported significant adsorbent biofouling, but concluded that the fouling had no measureable effect on the solute loading capacity. Thus it was concluded that the adsorbents used in these experiments were unaffected by fouling.

CHAPTER 5 CONCLUSIONS

5.1. Summary

The in-situ product removal strategy was applied to toxicity-limited butanol fermentations from *C. acetobutylicum* via adsorption. Cell-free characterizations have demonstrated that butanol was the highest affinity compound for adsorption when in solution with acetone and ethanol. It was determined that acetone and ethanol were the only compounds naturally found in fermentations that significantly decrease the butanol adsorption onto L-493 resin. Glucose had an inverse effect, causing more butanol to adsorb onto the hydrophobic resin.

The resin was contained in an expanded-bed adsorption column. The butanol extraction rate from the broth was found to be independent of resin mass, but highly dependent on the circulation rate. A circulation rate with a superficial velocity of 9.5 cm/min was determined to be ideal for application to butanol fermentations. Through a simulated fermentation, the EBA column was demonstrated to maintain the butanol concentration below the toxicity limit for an extended period of time. When applied to actual fermentations, 8.33 g more butanol was produced when the EBA column was used, netting a total of 25.53 g butanol (a 33% increase relative to the control). Additionally, the maximum butanol productivity was higher for the EBA fermentation (0.81 g/L·hr to 0.71 g/L·hr). EBA has been successfully applied to *C. acetobutylicum* fermentations to improve butanol production.

5.2. Recommendations for Future Work

Future work would include butanol recovery through, for example, solvent extraction or vaporization, following fermentation. The butanol adsorption was shown to be unchanged by autoclaving, but future work would include regeneration and re-use of resin. Additionally this process can be improved by scaling up the reactor size to produce larger quantities of butanol.

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