INCREASING FERMENTATIVE BUTANOL PRODUCTION IN *CLOSTRIDIUM BEIJERINCKII* USING OXIDIZED EXTRACELLULAR ELECTRON SHUTTLING MOLECULES

BY

ANNE HALUSKA

THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering in Civil Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Adviser:

Professor Kevin T. Finneran
ABSTRACT

Continuing concerns over dependence on foreign oil and the environmental impacts of using oil as a major source of fuel have led to increased interest in more sustainable energy options. Bio-butanol, produced during fermentation of plant biomass, is one possible alternative. However, yields for butanol fermentation are low making recovery difficult. Higher yields are needed to make butanol a competitive option for an alternative fuel source.

This research focuses on using extracellular electron shuttles to alter the metabolic pathways in Clostridium beijerinckii NCIMB 8052 fermentation to increase butanol yield with glucose as a primary substrate. 1-5mM anthraquinone-2,6-disulfonate (AQDS), the oxidized form of the electron shuttle, were introduced into batch cultures of C. beijerinckii and increased butanol yields by 2.5 to 5 times relative to glucose alone fermentation batches. An electron mass balance of the system indicated that butyric acid equivalents decreased as butanol equivalents increased. Adding 20 mM Fe(III) (as ferrihydrite or ferric citrate), which also acts as an electron sink, increased butanol yield by 5 times relative to the glucose-only control. Adding both 1 mM AQDS and 20 mM Fe(III) increased the yield up to 5.5 times relative to the glucose-alone control, for a yield of 0.37 and final butanol concentration of 90 mM. The increase in butanol yield with Fe(III) is due to the fact that when AQDS is reduced, the hydroquinone immediately transfers electrons to the Fe(III) thus recycling the AQDS so that it can be reduced again. We anticipate that this strategy can be used to increase commercial bio-butanol yield, as well as provide insight into how fermentative metabolism can be modulated. Addition of 60mM supplementary acetate increased butanol yields 2.1 times compared to glucose-only control. 1mM AQDS and 60mM acetate increased butanol yields 2.27 times relative to the glucose controls. Adding 20mM ferric iron to cultures containing 60mM acetate decreased butanol yields compared to cultures amended with acetate but without ferric iron.
ACKNOWLEDGEMENTS

I would like to thank NSF for providing funding for this project. I am especially grateful to my advisor, Dr. Kevin Finneran for his advice and support and members of the Finneran lab for their help and suggestions.
# TABLE OF CONTENTS

- **INTRODUCTION** ................................................................. 1  
- **LITERATURE REVIEW** ...................................................... 3  
- **MATERIALS AND METHODS** ............................................ 17  
- **RESULTS** ............................................................................. 19  
- **DISCUSSION** ................................................................. 36  
- **CONCLUSIONS** ............................................................. 41  
- **REFERENCES** ..................................................................... 43
INTRODUCTION

Because of increasing concerns over climate change and dependence on foreign oil, the United States government has implemented energy initiatives with the goal of replacing 30% of transportation fuel in the U.S. with ethanol by the year 2030. However, current estimates that only 15% of the ethanol needed to achieve this goal can be produced using current processes without impacting global food and feed supply chains, indicate that other fuels and fuel production processes need to be explored. One solution is to use non-food, non-feed biomass as a substrate [1].

Currently, ethanol is produced by yeast which cannot metabolize many of the sugars found in alternative biomass sources. Bacteria from the genus Clostridium can metabolize a wide range of sugars to produce solvents like butanol. Butanol, like ethanol, can be used as a fuel or fuel extender but butanol holds several advantages over ethanol. Butanol has 30% greater energy content, lower vapor pressure making butanol easier to store, lower sensitivity to water allowing butanol to be distributed through current gasoline distribution pipelines while ethanol has to be hauled in trucks, lower flammability making butanol safer to handle and butanol can be used in current vehicles without any alterations to current engines [1].

Bacteria like Clostridium beijerinckii NCIMB 8052, the strain used in this study, have the potential to produce butanol for fuel on an industrial scale. C. beijerinckii NCIMB 8052 was chosen because it has been widely studied and its metabolic pathways are well characterized. It is a spore-forming, gram negative, anaerobic bacteria that can grow on a wide range of substrates including pentoses and hexoses. The ability to use a variety of substrates makes C. beijerinckii an attractive option for bio-fuel production from biomass not used for food or feed because non-food, non-feed biomass contains a wide range of sugars [2].

C. beijerinckii metabolism is characterized as biphasic. An acidogenic phase where microbes produce acetic and butyric acids corresponds to the exponential growth phase of the culture. When the culture enters stationary phase, the solventogenic phase of metabolism involving the production of acetone, ethanol and butanol begins. Because C. beijerinckii produces so many products, only a fraction of metabolized carbon and electrons are channeled towards the formation of butanol. Increasing the fraction of carbon and electrons directed to butanol production will result in higher butanol yields which are needed to make bio-butanol a viable fuel alternative [3].
One proposed method of altering carbon and electron flow in *C. beijerinckii* metabolism is through the use of external electron shuttling compounds which can influence the balance of redox reactions involved in metabolism. Extracellular electron shuttles are compounds that can be oxidized or reduced. They act as catalysts because they are not consumed in the reactions but allow redox reactions to occur without the reactants having to be in physical contact. Extracellular electron shuttles are found in all cells and are essential for proper cell function. One example is NAD\(^+\) which accepts electrons from the citric acid cycle and carries them to the cell membrane for cellular respiration. The use of electron shuttles to manipulate cellular metabolism is a well accepted concept. Electron shuttles are used in bioremediation, wastewater treatment and in microbial fuel cells [4].

The purpose of this research is to optimize butanol production in *Clostridium beijerinckii* NCIMB 8052 through the use of the oxidized extracellular electron shuttle anthraquinone-2,6-disulphonate (AQDS). This study aims to verify that AQDS can strip electrons out of the metabolic pathways and alter the flow of carbon and electrons. Successful manipulation of the metabolic pathways would result in greater product specificity. Butanol yields would increase at the expense of other, less desirable, final products. Increased butanol yields would make bio-butanol a more competitive energy option.
LITERATURE REVIEW

History of ABE Fermentation

Industrial ABE fermentation processes using Clostridial species date back to 1910 when Strange and Graham Ltd. began the manufacture rubber from butadiene, a compound derived from butanol [5, 6]. Strange and Graham hired Perkins and Weizmann (who later became the first president of Israel) from the University of Manchester and Fernbach and Schoen from the Institute Pasteur to develop solventogenic microbial cultures. In 1911, Fernbach isolated a strain designated FB that fermented potato mash with moderate butanol yields. Strange and Graham began production using the FB strain in 1913 [5].

At the outbreak of World War I in 1914, the British government commissioned Strange and Graham to produce acetone for use in munitions using the FB strain. But Weizmann, who had parted ways with Strange and Graham in 1911, convinced the British government that his BY strain which fermented maize mash was able to produce higher acetone yields than the FB strain and in 1916, Great Britain adopted the BY strain for their acetone plants in Great Britain, Canada and the United States [5].

After WWI, Commercial Solvents Corporation (CSC) bought the ABE production plant in the United States and began to explore commercial applications for fermentation products. Butanol and butyl acetate were in high demand as solvents for lacquers used in the booming automobile industry. CSC also continued to develop and isolate new bacterial strains that could grow on a wide variety of substrates to produce many different products. One of the new strains developed was Clostridium saccharo-butylicum-liquifaciens which fermented molasses to produce acetone during World War II when ABE fermentation was the second largest biotechnology process in the world [5, 7].

After WWII, when molasses prices increased and the petroleum industry was able to produce higher concentrations of solvents at lower prices, ABE fermentation was abandoned everywhere except Africa and China [5]. Since the 1970s oil crisis, concerns over fluctuating oil prices and dependence on foreign oil along with more recent initiatives to research clean energy alternatives to oil, have sparked renewed interest in ABE fermentation for the production of biofuels [8].
ABE Fermentation Limitations

There are currently three major hurdles to ABE fermentation competing with petroleum-based solvent production. They are (1) high cost of substrates, (2) low final product concentrations, and (3) high product recovery costs [7].

High Cost of Substrates

Up to 60% of the cost of fermentation processes can be attributed to the cost of substrate indicating that substrate cost is a major factor in the economic viability of ABE fermentation. Using lignocellulosic or starchy side streams from food crops or industrial processes would significantly lower the cost of the fermentation process. One advantage of Clostridial species over other fermentative bacteria or yeast is their ability to metabolize a wide variety of substrates. However, biological conversion of lignocelluloses is problematic due to the degradation-resistant crystalline structure which makes pretreatment a required processing step. Common pretreatment methods include steam expansion, acid hydrolysis and treatment with enzymes. These methods can produce by-products that inhibit fermentation. Contamination from heavy metals, xenobiotics or pathogens can also inhibit bacterial growth [7]. However, research indicates that pretreated wheat straw does not have an inhibitory effect but actually stimulates fermentation more than glucose alone [1]. Other methods to avoid inhibitory by-products of pretreatment include co-culturing. Co-culturing is the process where the fermentative bacteria are grown with another bacterial strain that can metabolize untreated cellulosic biomass. Solvent production from co-cultures has been low probably due to the kinetic limitations of degrading the biomass. Another method that has also met with limited success is adding cellulase from the fungus Trichoderma reesei to the medium which allows the fermentative bacteria to degrade the biomass [7].

Low Final Product Concentration

Low final product concentration stems from many factors including low product specificity, low bacterial tolerance to end products and strain degeneration. Because the metabolic pathways of ABE fermentation yield so many final products as shown in Figure 1,
concentrations of individual products remain low. Attempts to direct the metabolic pathways towards desired final products have been aimed at both the transcriptional level and the post-translational level [9]. At the transcriptional level, Tummala et al. used antisense RNA to inhibit transcription of the *ctfB* gene which codes for the CoA transferase enzyme and enhance expression of *aad*, the gene for aldehyde dehydrogenase to increase the butanol to acetone ratio. Nakayama downregulated transcription of the hydrogenase gene that encodes the enzyme responsible for producing hydrogen. Downregulation of the hydrogenase gene also increased butanol production [9]. Green et al. inactivated the *buk* gene, which encode the butyrate kinase enzyme, by inserting gene fragments into the gene. Butyrate kinase activity decreased when the *buk* gene was inactivated and butanol production increased [10].

Researchers have also attempted manipulation of metabolic pathways by regulation of enzyme activities. Lee at al. supplemented growth media of *C. beijerinckii* NCIMB 8052 with butyrate to increase solventogenic enzyme activity resulting in increased butanol production [11]. Others have inhibited hydrogenase, using CO or violegen dyes to guide carbon flow to butanol pathways [12, 13]. Blaschek has developed *C. beijerinckii* BA101, a hyper-butanol-producing mutant, that has increased solventogenic enzyme activity [14, 15].

A second problem is that bacteria are highly susceptible to butanol toxicity. High concentrations of butanol disrupt membrane permeability and solute transport, dissipate proton motive force and negatively affect the conformation and activity of membrane proteins [16]. The butanol-tolerant strain *C. beijerinckii* BA101 has an altered glucose uptake system which may indicate a change in membrane composition which allows higher butanol tolerance [9, 17]. Overexpression of the heat shock gene *groESL* in *C. acetobutylicum* allowed the culture to tolerate higher butanol concentrations. This could be due to the heat shock proteins expressed from *groESL* protecting the folding of membrane proteins under stressed conditions [9].

Finally, strain degeneration, when the culture loses the ability to produce solvents, also affects final product concentration. When the growth rate is uncontrolled, acetic and butyric acid concentrations build up to toxic levels before enzymes catalyzing solventogenesis can be activated to reassimilate the acids. To combat this problem, the cell growth can be slowed down by incubating the culture at a sub-optimal temperature or the media can be buffered which helps
to control the pH. Either of these methods allows time for induction of solventogenesis before toxic acid levels are achieved.

Strain degeneration can also be caused by mutations in the solvent genes. Mutants combining DNA from *C. beijerinckii* NCIMB 8052 and *Enterococcus faecalis* BM4110, a more genetically stable bacteria, have proven resistant to degeneration [7]. Chen and Blaschek also reported that cultures in the presence of excess acetate were more resistant to degeneration [18].

![Clostridium beijerinckii metabolic pathway](image)

**High Product Recovery Costs**

Recovery of low concentrations of butanol from liquid media is highly energy intensive and diminishes the economic viability of industrial fermentation processes. Recovery method research has focused on continuous product recovery which alleviates butanol inhibition of the bacteria [9]. Product recovery techniques include recovery using fermentation gases, extractive fermentation, perstraction, pervaporation and adsorption.

Several studies have reported successful recovery of products by continuously bubbling \( \text{N}_2 \) or gases produced in the fermentation (\( \text{H}_2 \) and \( \text{CO}_2 \)) through batch and continuous reactors. Volatile products like butanol, ethanol and acetone partition into the gas phase. The gas is captured and solvents can be condensed and collected. Continuous sparging of fermentation broth has allowed for increased butanol yield and productivity in both batch and continuous reactors [1, 8].

In extractive fermentation, an organic phase that butanol selectively concentrates in but is immiscible with the fermentation broth is added to the reactor. The organic phase can be easily separated from the media and butanol can be more easily extracted from the organic phase because of high butanol concentrations in the extractant. Evans reported a 72% increase in butanol production when using a mixture of decanol and oleyl alcohol as an extractant [16].

Some problems with extractive fermentation such as the extractant’s toxicity to cells, loss of solvent and the formation of organic emulsions in the liquid broth can be solved by perstraction. Perstraction operates on the same principles of extractive fermentation but the organic phase and the broth are separated by a selectively permeable membrane. By physically separating the extractant from the media, the possibility of forming emulsions is eliminated and the toxicity to the cells from the extractant is reduced. However, butanol diffusion through the membrane is slow and limits the rate at which butanol can be extracted [1].

Pervaporation uses a membrane that selectively allows the solvent to diffuse through the membrane as a vapor. The vapor can then be condensed and collected. Qureshi found that using a silicone membrane for pervaporation allowed for greater productivity to be achieved but required enough energy input for the heat of vaporization of the solvent [1, 14].
Adsorption is another removal strategy that is energy efficient. Butanol adsorbs to adsorbents in the media. After the fermentation is complete, the adsorbents are removed and heat treated to desorb the butanol. A common sorbent is silicate, a form of silica that is effective at adsorbing alcohols from dilute solutions [9]. Milestone and Bibby were able to concentrate a 0.5% (w/v) solution of butanol to a 98% (w/v) solution using silicate as an adsorbent and thermal desorption [19].

Many valuable products besides butanol can also be recovered from the fermentation and marketed to increase the value of Clostridial fermentation. Along with butanol, ethanol and hydrogen gas can be used as energy carriers. CO₂ can be captured and used to grow algal biomass for fuel and oils. Vitamin B12 can also be recovered from the media and used in vitamin supplements [1].

**The Future of Fermentation**

The US government has implemented energy initiatives with the goal of replacing 30% of transportation fuel with ethanol by 2030. According to current estimates, 65.3x10⁹ kg of ethanol can be produced annually from corn in the United States without impacting the food and feed supply. This accounts for only about 15% of the total ethanol needed to meet energy initiative goals. To produce more ethanol without endangering the global food market, fermentation processes need to use alternative substrates like cellulosic biomass. Clostridial species cannot compete with yeast on ethanol production but they can use a wider range of substrates and can produce butanol. Butanol holds several advantages other ethanol including 30% greater energy content, lower vapor pressure, lower sensitivity to water, lower volatility, lower flammability and higher miscibility with gasoline [1].

While butanol has a promising future in biotechnology applications, more research needs to be focused on developing organisms with higher butanol tolerance, better end product specificity, and more efficient cellulosic substrate pretreatments and product recovery methods.
**Electron Shuttles and Sinks**

Electron shuttles (ESs) or redox mediators are organic molecules that can be reversibly oxidized and reduced allowing them to carry electrons between different oxidation-reduction (redox) reactions. ESs are found inside all cells and play a crucial role in biological metabolism. Some examples include nicotinamide adenine dinucleotide (NAD) which carries electrons from glycolysis and the TCA cycle to the electron transport chain in respiring organisms and ubiquinone which transports electrons between protein complexes in the electron transport chain. External electron shuttling compounds like humics or the humic acid analogue, anthraquinone-2,6-disulphonate (AQDS), can also play a metabolic role [4].

Interest in the ability of extracellular electron shuttles (EESs) to stimulate or alter metabolic pathways has led to research in the application of EESs in bioremediation, wastewater treatment and microbial fuel cells. EESs have been shown effective in stimulating the biological reduction of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), an explosive, and methyl tert-butyl ether (MTBE), a fuel oxygenate. The EES cobalamin has been shown to allow non-halorespiring microbes to reduce chlorinated organics like trichloroethylene (TCE) [4].

Interactions between AQDS and ferric iron in bioremediation of MTBE and RDX have been extensively studied [4, 20, 21]. Ferric iron reduction is one of the major reactions occurring in anaerobic soils. Ferric iron reduction is stimulated in the presence of AQDS because the shuttling compound eliminates the need for physical contact between the microbe and the iron for electron transfer to occur. The presence of ferric iron in turn stimulates the microbial metabolism by rapidly recycling AQDS to accept more electrons. AQDS can accept electrons from microorganisms to produce AH$_2$QDS. AH$_2$QDS then abiotically reduces ferric iron to ferrous iron and regenerates AQDS which then restarts the cycle [20, 21].

EES applications to wastewater treatment have mainly been in the area of azo-dye decoloration. Azo-dye is a synthetic dye widely used in commercial applications. It changes the water color and is a suspected mutagen. The dye can be anaerobically reduced to aniline but the reduction rates are usually low. EESs can accelerate azo-dye reduction rates which allows the process to be housed in smaller, more economical reactors [4].
The third common biotechnology application is in microbial fuel cells (MFC). MFCs “...exploit the bacterial catabolic activities to generate electricity from organic matter” [4]. They are generally considered a sustainable energy source because they can use waste products as substrates. Dissimilatory Fe(III)-reducing bacteria (DFBR) like Geobacter and Shewanella species are the most commonly used microbes in MFCs. The microbes metabolize the substrate and in the process release electrons. When the electrons are transferred to the anode, electricity is generated. Electrons can be transferred to the anode either through direct physical contact between the cells and the electrode or indirectly through the use of electron shuttles. The electron shuttles increase the amount of electricity that can be generated from the process by eliminating the requirement for a cell to physically contact the electrode to donate electrons. Humics, AQDS, neutral red, thionine, resazurin and methylene blue are all EESs used in MFCs [4].

Background on Clostridia species

*Clostridium beijerinckii* NCIMB 8052 along with *C. acetobutylicum* ATCC 824 is one of the most commonly studied solventogenic Clostridia species [11]. The genus Clostridia is composed of a diverse group of species with industrial, agricultural and medical relevance [22]. They are characterized as spore-forming, mesophilic, Gram-positive staining anaerobes [2]. Unlike *C. acetobutylicum* ATCC 824, *C. beijerinckii* NCIMB 8052 contains a single circular chromosome [11]. Clostridia species have a biphasic metabolism composed of an acidogenic phase and a solventogenic phase. *C. beijerinckii* NCIMB 8052 produces acetic and butyric acid in the acidogenic phase which is concurrent with the exponential growth phase. As growth slows and enters the stationary phase, metabolism shifts to solventogenesis and ethanol, butanol and acetone are produced [3].
Enzymes Involved in Acid and Solvent Production

Enzymes Involved in Acetone Production

Acetoacetyl-CoA:acetate/butyrate CoA transferase, commonly shortened to CoA transferase catalyzes the transfer of coenzyme A to acetate or butyrate:

\[
\text{Acetoacetyl-CoA} + \text{acetate or butyrate} \leftrightarrow \text{acetoacetate} + \text{acetyl-CoA} \text{ or butyryl-CoA}
\]

This reversible reaction mainly acts to prevent over acidification of the media by removing the acetate and butyrate produced in the acidogenic phase of the fermentation [23]. Increased CoA transferase activity has been demonstrated to correspond with the onset of the solventogenic phase of metabolism [22, 24]. In vitro studies show that acetate is converted at a faster rate than butyrate but relative concentrations of acetate and butyrate in vivo may alter the selectivity of the CoA transferase enzyme [23].

CoA transferase enzyme was purified from \textit{C. acetobutylicum} ATCC 824. It is composed of two subunits with molecular weights of 23 and 25 kDa respectively and encoded by the genes \textit{ctfA} and \textit{ctfB} [23, 25]. \textit{ctfA} encodes the small subunit and \textit{ctfB} encodes the large subunit. The genes are adjacent to each other and form part of the \textit{sol} operon that also encodes an aldehyde/alcohol dehydrogenase (\textit{adhE} or \textit{aad}) [26].

Acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetate to form acetone [5]:

\[
\text{Acetoacetate} \leftrightarrow \text{acetone} + \text{CO}_2
\]

Acetoacetate decarboxylase works to pull the less favorable CoA transferase reaction towards uptake of acetic and butyric acids by keeping the acetoacetate concentration low [27]. Studies have reported that acetoacetate decarboxylase enzyme activity is higher in solvent-producing cells [24, 28].

Acetoacetate decarboxylase was purified from \textit{C. acetobutylicum} ATCC 824 and DSM 792 [25]. The enzyme is composed of 12 identical subunits, each with a molecular mass of 29 kDa [26]. The \textit{adc} gene which encodes the acetoacetate decarboxylase enzyme has been cloned.
It is located separately from the genes encoding CoA transferase and is transcribed with its own promoter [25].

**Enzymes Involved in Butanol Production**

Butyraldehyde dehydrogenase catalyzes the conversion of butyryl-CoA to butyraldehyde [5]:

\[
\text{Butyryl-CoA} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{butyraldehyde} + \text{CoASH} + \text{NAD(P)}^+ 
\]

Butyraldehyde dehydrogenase is induced during solvent production and serves to regenerate NAD(P)\(^+\) for glycolysis [5, 29]. No gene encoding butyraldehyde dehydrogenase has been cloned but the aldehyde/alcohol dehydrogenase gene \(adhE\) could play a role in butanol production. Studies of mutant complementation experiments where \(adhE\) encoded on a plasmid and inserted into \(adhE\) mutant \(C. acetobutylicum\) M5 restoring butanol production validate the possibility that enzymes encoded by \(adhE\) are involved in butanol production [5].

Butanol dehydrogenase catalyzes the formation of butanol from butyraldehyde [5]:

\[
\text{Butyraldehyde} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{butanol} + \text{NAD(P)}^+ 
\]

Several butanol dehydrogenase enzymes have been purified. Two isozymes, BdhI and BdhII can dehydrogenate both acetaldehyde and butyraldehyde but have higher activity with butyraldehyde. BdhI is twice as active at dehydrogenating butyraldehyde and BdhII is 46 times more active using butyraldehyde [5]. Studies have shown that BdhI is NADPH dependent in \(C. acetobutylicum\) P262 and in \(C. acetobutylicum\) DSM 1732 NADH and NADPH dependent butanol dehydrogenases could be separated by centrifugation [5, 29]. Both enzymes are most active at pH around 5.5 which closely corresponds to the reported internal pH of solventogenic cells. BdhI and BdhII are encoded by the genes \(bdhA\) and \(bdhB\) respectively. The genes are located next to each other but have separate promoters [26].
Enzymes Involved in Acetate Production

Acetate is produced through the PTA-ACK pathway. Phosphotransacetylase (PTA) catalyzes the phosphorylation reaction that produces acetyl-phosphate from acetyl-CoA [5]:

\[
\text{Acetyl-CoA} + \text{Pi} \leftrightarrow \text{acetyl-phosphate} + \text{CoASH}
\]

Acetate kinase catalyzes the dephosphorylation of acetyl-phosphate to form acetate and ATP:

\[
\text{Acetyl-phosphate} + \text{ADP} \leftrightarrow \text{acetate} + \text{ATP}
\]

Because this is an ATP producing pathway, low intracellular ATP concentrations lead to induction of acid forming metabolism [25]. After metabolism shifts to the solventogenic phase, PTA and AK activity decreases [24]. The genes encoding PTA (pta) and AK (ack) were identified in \textit{C. acetobutylicum} ATCC 824 and form the \textit{pta-ack} operon. Studies inactivating the \textit{pta} gene in \textit{C. acetobutylicum} ATCC 824 reported decreased PTA and AK activities and lower acetate production [10].

Enzymes Involved in Butyrate Production

The butyrate pathway is similar to the acetate pathway but the enzymes in each pathway are unique. Phosphotransbutyrylase (PTB) catalyzes the phosphorylation reaction that produces butyryl-phosphate from butyryl-CoA [5]:

\[
\text{Butyryl-CoA} + \text{Pi} \leftrightarrow \text{butyryl-phosphate} + \text{CoASH}
\]

Butyrate kinase catalyzes the dephosphorylation of butyryl-phosphate to form butyrate and ATP:

\[
\text{Butyryl-phosphate} + \text{ADP} \leftrightarrow \text{butyrate} + \text{ATP}
\]

As with the PTA-AK pathway, PTB and BK activities increase when ATP concentration in the cell is low and decrease with the onset of solventogenesis [24, 25]. The \textit{ptb} and \textit{buk} genes which encode phosphotransbutyrylase and butyrate kinase respectively were identified from \textit{C. acetobutylicum} ATCC 824 and form the \textit{ptb-buk} operon [3, 10]. Inactivating \textit{buk} in \textit{C. acetobutylicum}
acetobutylicum ATCC 824 decreased butyrate kinase activity, reduced butyrate production and increased butanol production [10].

**Regulatory Mechanisms for Solventogenesis**

*Spo0A*

One proposed regulatory mechanism for solventogenesis at the transcriptional level is the Spo0A protein, a regulatory protein in bacteria that controls responses to environmental stimuli. The transition from exponential growth to stationary phase is regulated by Spo0A in all endospore-forming bacteria [30]. Spo0A is activated through phosphorylation and enhances transcription of some genes and represses the transcription of others [18]. Spo0A increases or reduces the frequency of transcription initiation by binding to sequence motifs TGNCGAA called 0A boxes that are upstream of certain genes. Wilkinson found 0A boxes upstream of the *adc*, *ctfA*, *ctfB*, *adhE*, *bdhA*, *bdhB*, *ptb* and *buk* genes and also reported that mutants without Spo0A were asporegenic and unable to produce solvents [30].

*Acids and pH*

pH has long been considered a trigger for solventogenesis. High acid concentrations from the acetate and butyrate produced in acidogenesis dissipate the proton motive force and cause cell death. Consuming acids to form neutral solvents lowers the acid concentration and allows the cell to maintain a higher internal pH. In *C. acetobutylicum* DSM 1731, the difference in internal and external pH dropped from 0.7 to 0.14 units at the onset of solvent production and increased as solvent production continued [31].

However, low pH alone is not enough to induce solvent metabolism [32]. Holt et al. was able to induce solvent formation at neutral pH by supplementing the medium with acetic and butyric acid [33]. Grupe and Gottschalk found solventogenesis was only induced when the intracellular pH was low and there was an excess of butyric acid in the media [31]. Experiments supplementing growth medium for *C. beijerinckii* NCIMB 8052 with butyrate shortened the lag
phase for solvent production and increased butanol production. Butanol concentrations exceeded concentrations that could be accounted for from the added butyrate indicating that there may have been a shift in carbon and electron flow in the metabolic pathways [11].

Addition of acetate to the media has produced conflicting results. Holt et al. reported that adding 20 mM of acetate to the medium with *C. beijerinckii* NCIMB 8052 decreased butanol production by half while Chen and Blaschek reported increased butanol production from *C. beijerinckii* NCIMB 8052 in media supplemented with acetate [18, 33]. George and Chen reported no change in solvent production with the addition of acetate or butyrate [34]. Acetate kinase and CoA transferase showed higher activity in acetate supplemented cultures. Chen and Blaschek proposed that increases in CoA transferase and acetate kinase activities increased the concentration of acetyl-phosphate, a compound that can donate a phosphate group to Spo0A. Increased concentrations of activated Spo0A could then enhance transcription of genes encoding solventogenic enzymes [18]. Alternatively, increasing acetate concentrations may competitively block CoA transferase from converting butyrate to butyryl-CoA which could account for a decrease in butanol yields. Gottschal and Morris found that the relative acetate and butyrate concentrations determined the final ratio of acetone to butanol [32].

**ATP and NADH**

ATP and NADH levels have also been proposed as mechanisms for inducing solvent production. The distribution of fermentation products determines the amount of ATP produced for each mole of substrate consumed. Acidogenesis produces more ATP than solventogenesis so a limited supply of ATP or a high demand (exponential growth), causes metabolism to switch to acidogenesis. Meyer and Papoutsakis found that acidogenic continuous cultures generated eight times more ATP than solventogenic continuous cultures [35]. High ATP concentrations may inhibit phosphotransbutyrylase, the enzyme catalyzing the formation of butyryl phosphate from butyryl CoA, effectively blocking the butyrate pathway and channeling the carbon flow into the butanol pathway [28].

NAD(P)H levels may also play a role in determining the ratio of end products. Butanol and ethanol pathways oxidize NAD(P)H to regenerate NAD(P)⁺ which can then be reduced
again in glycolysis. Grupe and Gottschalk found NAD(P)H concentrations increase up to the onset of solventogenesis. After inhibiting hydrogenase, the enzyme that catalyzes the formation of H₂, with CO or methyl violegen, they measured increased levels of NAD(P)H and increased butanol production with little acetone or ethanol production [31]. Rao and Murtharasan reported the same phenomenon using violegen dyes [12].
MATERIALS AND METHODS

Culture Growth and Maintenance

*Clostridium beijerinckii* NCIMB 8052 (ATCC #51743), a wild-type strain, was used in this study. The culture was stored frozen at -80 °C as spore suspensions in nanopure water and all experiments were started from spore stock. *C. beijerinckii* was grown in anaerobic pressure tubes sealed with butyl rubber stoppers and crimped aluminum caps. Spores were initially inoculated into TYG medium containing 3 g tryptone, 2 g glucose and 1 g yeast extract in one liter of distilled deionized water. The media and headspace was made anaerobic by flushing with nitrogen gas and sterilized by autoclaving at 121 °C for 20 minutes. After inoculation, the culture was incubated for 28 hours at 37 °C in a LabLine Imperial III incubator. 3% of the culture was transferred from TYG medium to P2 medium containing 6 g/L glucose after 28 hours. P2 medium is composed of 0.2 g MgSO₄·7H₂O, 0.01 g MnSO₄·H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g NaCl, 1 g P-aminobenzoic acid, 0.01 g biotin, 0.1 g thiamine, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄ and 2.0 g (NH₄)₂SO₄ in one liter of distilled deionized water. 5% of the culture is transferred from P2 medium to experimental tubes also containing P2 media after incubating at 37 °C for 28 hours. Glucose, AQDS, iron and acetate were amended from stock solutions that were sparged with nitrogen gas, sterilized in the autoclave at 121 °C for 20 minutes and added to experimental tubes right before inoculation of the culture.

Product Analysis

Solvents present in the cultures were analyzed on a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector and a capillary column (30 m x 0.250 mm) from Agilent. The samples were filtered through 0.2 μm sterile PTFE filters into HPLC autosampler vials to be used with an AOC-20i autoinjector and AOC-20s autosampler. The oven temperature was programmed to increase from 40 °C to 230 °C at the rate of 50 °C/minute. The injector and detector temperature were set at 220 °C. Helium was the carrier gas and was set at a flow rate of 28.5 mL/min.
Organic acids in the cultures were analyzed on a Dionex high performance liquid chromatograph equipped with a PDA-100 Photodiode Array Detector, ASI-100 Automated Sample Injector and P680 HPLC Pump. The column was a ICSep ICE-ION 310 column from Transgenomic and the eluent was Nanopure water with pH adjusted to 1.98 using \( \text{H}_2\text{SO}_4 \) and pumped through the column at 0.6 mL/min. The samples were filtered through 0.2 µm sterile PTFE filters into HPLC autosampler vials.

Biomass was measured using a Genesys 2 Spectrophotometer from ThermoSpectronic. Anaerobic pressure tubes were directly inserted in the spectrophotometer and optical density was measured at 600 nm wavelength.

Hydrogen concentrations were measured using an HP 5890A gas chromatograph equipped with a SRI110 thermal conductivity detector. The carrier gas was Nitrogen and the GC operated at room temperature. 0.5 mL samples from the headspace in the anaerobic pressure tubes were analyzed.

Glucose concentrations were determined using the phenol-sulfuric acid assay. 50µL of sample was combined with 150µL of sulfuric acid and 30µL of 5% phenol solution in a clear 96-well plate. Absorbance was measured in a Spectramax Plus plate reader from Molecular Devices after the samples were incubated in a Fisher Scientific ISOTEMP 2025 water bath at 80 °C for 30 minutes [36].

Ferrous iron concentrations were measured using the ferrozine assay. 100µL of sample was added to 4.9mL of 0.5N HCl. 100µL of sample in HCl is then transferred to 4.9mL of Ferrozine solution. Ferrozine solution contains 1 g/L Ferrozine and 11.62 g/L HEPES buffer. Absorbance of sample in Ferrozine solution is measured at 562nm in a Genesys 2 Spectrophotometer from ThermoSpectronic using disposable plastic cuvettes.

Poorly crystalline ferric oxide used in experiments was produced by dissolving 108 g FeCl\(_3\) in 1L of Nanopure water and adjusting the pH to 7 with 10N NaOH. Iron solution was centrifuged at 5K for 20 minutes. The supernatant was poured off and the iron was washed with Nanopure water. This process was repeated 9 times until the chloride concentration was less than 1mM.
RESULTS

Influence of Electron Shuttle Concentration

Addition of oxidized AQDS to *C. beijerinckii* cultures with 1.8 g/L glucose resulted in increased butanol concentrations between 2 and 5 times over unamended controls. AQDS concentrations were scaled from 0 mM to 5 mM. As quinone concentration increased, butanol concentration increased and butyric acid concentrations showed a corresponding decrease.

*Figure 2. Butanol and butyric acid concentrations with increasing AQDS concentration.*
All other fermentation by-product concentrations remained relatively constant with increasing AQDS concentration but as electron equivalents going to butyric acid decreases, electron equivalents to butanol show a corresponding increase.

![Graph showing electron distribution to final products as a function of AQDS concentration.](image)

**Figure 3.** Electron distribution to final products as a function of AQDS concentration.

**Influence of Substrate Concentration**

Batch cultures with 1 mM AQDS were compared to unamended cultures with 1.8 g/L, 18 g/L or 30 g/L glucose. Butanol concentrations increased with increasing substrate concentration. At each substrate concentration, butanol concentrations increased when AQDS was present in the culture.
Figure 4. Butanol concentrations in cultures with AQDS and unamended controls with 1.8 g/L glucose.

Figure 5. Butanol concentrations in cultures with AQDS and unamended controls with 18 g/L glucose.
Figure 6. Butanol concentrations in cultures with AQDS and unamended controls with 30 g/L glucose.

Glucose, acetone, ethanol and biomass concentrations were also monitored. Substrate utilization along with ethanol, acetone and biomass productions were not altered by the presence of AQDS. These results are in agreement with the AQDS scaling experiment where AQDS influenced butanol and butyric acid concentrations without impacting other by-product production. Glucose utilization was also unaffected by the presence of AQDS but was affected by starting substrate concentration. At low starting substrate concentration (1.8 g/L), 99% of the glucose was consumed but at higher substrate concentrations (18 g/L, 30 g/L) only 54% of substrate was consumed.
Figure 7. Optical density at 600 nm for C. beijerinckii cultures grown at different substrate concentrations with and without AQDS.

Figure 8. Acetone concentrations for C. beijerinckii cultures grown at different substrate concentrations with and without AQDS.
Figure 9. Ethanol concentrations for C. beijerinckii cultures grown at different substrate concentrations with and without AQDS.

Figure 10. Glucose concentrations for C. beijerinckii cultures grown at different substrate concentrations with and without AQDS.
Butanol yields were calculated as grams of butanol produced per gram of glucose consumed. Across substrate concentrations, the yields were similar with similar increases in yields with 1mM AQDS present. Yield increases between cultures with AQDS present and unamended controls ranged from 2 to 2.7 times greater.

![Figure 11. Butanol yields (grams of butanol produced per gram of substrate consumed) in cultures with 1mM AQDS compared to unamended controls.](image)

**Influence of Ferric Iron**

Ferric iron was added to cultures with and without AQDS in two forms, as insoluble poorly crystalline ferric oxide and as soluble ferric citrate. Adding 20mM of poorly crystalline ferric oxide increased butanol yield 3.6 times over the unamended control and adding both 1mM AQDS and 20mM poorly crystalline ferric oxide increased the butanol yield 5.1 times the unamended control for a final yield of 0.35 g butanol per g glucose consumed and a final butanol concentration of 89.6mM. 20mM of ferric citrate increased butanol yield 4.9 times the unamended yield and 20mM ferric citrate in the presence of 1 mM AQDS increased the butanol
yield further to 5.4 times the unamended control for a yield of 0.37 g/g and a final butanol concentration of 83.9 mM.

Figure 12. Butanol concentrations with 30 g/L glucose, 1 mM AQDS and 20 mM poorly crystalline ferric oxide (FeGel).
Figure 13. Butanol concentrations with 30 g/L glucose, 1mM AQDS and 20mM ferric citrate (FeCit).

Figure 14. Maximum butanol yields for 30 g/L glucose, 1mM AQDS and 20mM Fe(III) calculated as grams butanol produced per gram glucose consumed.
Ferric iron was reduced to ferrous iron in all iron containing cultures. Cultures with AQDS and ferric iron reduced more ferric iron than the cultures containing ferric iron and no AQDS. OD600 measurements increased as ferrous iron concentrations increased because ferrous iron is a growth factor for *C. beijerinckii*.

Figure 15. Ferrous iron concentrations in *C. beijerinckii* cultures.

Figure 16. Optical density at 600 nm in *C. beijerinckii* cultures.
In *C. beijerinckii* cultures with iron present, elevated acetone and ethanol concentrations were also detected as compared to cultures without iron present. AQDS without ferric iron did not influence acetone and ethanol concentrations.

*Figure 17. Ethanol concentrations in cultures containing poorly crystalline ferric oxide or ferric citrate.*

*Figure 18. Acetone concentrations in cultures containing poorly crystalline ferric oxide or ferric citrate.*
Influence of Supplementary Acetate

Previous studies have shown that supplementary acetate increases butanol production [18, 27, 32]. In *C. beijerinckii* cultures with 30 g/L glucose and 60mM acetate, butanol yields increased 2.10 times compared to cultures without supplementary acetate. 60mM of acetate with 1mM AQDS increased butanol yields 2.27 times compared to unamended controls. Cultures grown the presence of 60mM acetate and 20mM ferric iron (poorly crystalline ferric oxide or ferric citrate) or in the presence of 60mM acetate, 20mM ferric iron and 1mM AQDS had lower butanol yields compared to cultures grown with acetate or acetate and AQDS with butanol yields ranging from 1.62 to 1.76 times greater than glucose-only controls.

![Butanol Yield Chart](image)

*Figure 19. Maximum butanol yields for *C. beijerinckii* cultures with 30 g/L glucose and 60mM supplementary acetate compared to unamended controls.*
Figure 20. Butanol concentrations for *C. beijerinckii* with 30 g/L glucose and 60mM acetate.

While the yields vary for the different culture conditions, all cultures with 60mM acetate produced similar final butanol concentrations as shown in Figure 16. Acetone, ethanol and biomass concentrations also increased in the presence of acetate. Glucose consumption varied greatly between the different treatments which may account for decreased butanol yields in some cultures. Cultures grown in the presence of acetate used glucose more efficiently than cultures grown without supplementary acetate. Cultures grown in the presence of acetate or acetate and AQDS consumed twice as much glucose as cultures without acetate. Cultures with acetate and ferric iron consumed between 2.5 and 3 times more glucose compared to cultures without acetate.
Figure 21. Acetone concentrations in C. beijerinckii cultures with supplementary acetate.

Figure 22. Ethanol concentrations in C. beijerinckii cultures with supplementary acetate.
Figure 23. Cell growth of C. beijerinckii cultures with supplementary acetate.

Figure 24. Glucose concentration as a function of time for C. beijerinckii cultures with supplementary acetate.
Cultures grown with supplementary acetate had starting acetate concentrations between 60mM and 77mM. Acetate was consumed by *C. beijerinckii* to concentrations between 0mM and 16mM while cultures grown without supplementary acetate produced acetate concentrations between 2mM and 12mM. Butyric acid concentrations in cultures with acetate or acetate and AQDS were 2.5 greater than butyric acid concentrations in glucose-only controls. In all ferric iron containing cultures except cultures with acetate combined with AQDS and poorly crystalline ferric oxide, butyric acid concentrations reached a peak and then decreased during the solventogenic phase.

*Figure 25. Acetic acid concentrations in C. beijerinckii grown with supplementary acetate.*
Figure 26. Butyric acid concentrations for C. beijerinckii grown with supplementary acetate.
DISCUSSION

The goal of this study was to increase butanol yields in *C. beijerinckii* NCIMB 8052 cultures by manipulating redox reactions in the metabolic pathways through the use of oxidized extracellular electron shuttles. Increasing bio-butanol yields would make bio-butanol a viable alternative energy option. Since electron shuttles are not metabolized by the cell, they can be recovered and regenerated for use in new batch fermentations. Addition of AQDS, an oxidized electron shuttle, or ferric iron, an electron sink, or a combination of the two was shown to increase butanol yields in *C. beijerinckii* cultures.

Early experiments investigating the influence of electron shuttle concentration on butanol concentrations demonstrated that butanol concentration increases with increasing shuttle concentration while butyric acid concentration decreases with increasing shuttle concentration. These results confirm our conceptual model that the electron shuttle influences the flow of carbon and electrons through the metabolic pathways. AQDS shuts the “gate” on the butyric acid pathway and thus channels carbon and electrons into the butanol pathway.

Adding an electron acceptor to increase butanol yields may seem counterintuitive since butanol is a highly reduced end product which requires electrons donated from NAD(P)H. Addition of an oxidizing agent should keep NAD(P)^+ concentrations high which would be thermodynamically unfavorable for the production of butanol. Therefore, another mechanism must explain the increase in butanol production in the presence of oxidized extracellular electron shuttles.

One explanation for the shift from acidogenesis to solventogenesis in *Clostridial* species is that the microbes need to replenish the supply of NAD^+ to continue to run glycolysis. AQDS can oxidize NADH to replenish NAD^+ stores allowing the cells generate more ATP. Elevated ATP concentrations have been shown to inhibit the enzyme phosphotransbutyrylase which catalyzes the phosphorylation reaction that produces butyryl-phosphate from butyryl-CoA. Inhibiting phosphotransbutyrylase stops the production of butyric acid and all butyryl-CoA must then be channeled into the butanol pathway thus decreasing butyric acid concentrations and increasing butanol concentrations in the culture.
Increased ATP levels could allow for the phosphorylation of the Spo0A protein. Phosphorylated Spo0A is the active form of the protein. Activated Spo0A can then bind to 0A boxes and enhance the expression of genes for butanol producing enzymes or repress the expression of genes for butyric acid producing enzymes. Higher levels of butanol producing enzymes out-compete butyric acid producing enzymes for butyryl-CoA limiting the amount of butyric acid produced and stimulating production of butanol.

Substrate concentration had little effect on yield increases in cultures with AQDS compared to cultures without AQDS. All end-product concentrations increased when the starting substrate concentration was increased from 1.8 g/L up to 30 g/L but the amount of substrate consumed also increased. Increased butanol concentrations coupled with increased substrate utilization meant butanol yields were unchanged. Industrial fermentations operate at substrate concentrations ranging from 30 g/L to 60 g/L. Increasing butanol yields with 1mM AQDS at a substrate concentration of 30 g/L meant all subsequent experiments could run at an industrially relevant substrate concentration.

At low glucose concentration (1.8 g/L), the substrate utilization efficiency was 99% while at the higher glucose concentrations (18 g/L, 30 g/L), the substrate utilization efficiency was 54%. Increasing substrate utilization efficiencies at higher glucose concentrations could further increase final butanol concentrations if more substrate was channeled into the butanol pathway. End product inhibition at higher glucose concentrations may play a role in limiting the glucose utilization efficiency. Reactors that allow for continuous removal of fermentation by-products would remove the inhibitory compounds allowing for more complete substrate metabolism.

Oxidized electron shuttles used in in situ bioremediation have enhanced efficiency when ferric iron is present in the subsurface. The electron shuttle accepts electrons from the microbes and can then regenerate itself by donating electrons to ferric iron. In addition, ferric iron can directly accept electrons from the microbes further enhancing biological activity. This concept was applied to our batch reactors to further increase butanol yields. Adding 20mM of poorly crystalline ferric oxide increased butanol yield 3.6 times over the glucose-only control and adding both 1mM AQDS and 20mM poorly crystalline ferric oxide increased the butanol yield 5.1 times compared to the unamended control for a final yield of 0.35 g butanol per g glucose consumed and a final butanol concentration of 89.6mM. 20mM of ferric citrate increased
butanol yield 4.9 times the unamended yield and 20mM ferric citrate in the presence of 1 mM AQDS increased the butanol yield further to 5.4 times the unamended control for a yield of 0.37 g/g and a final butanol concentration of 83.9mM.

Butanol yields were higher in cultures containing soluble ferric iron (ferric citrate) compared to cultures containing insoluble poorly crystalline ferric oxide. This could be due to diffusion limitations in the iron. Electrons are transferred to the insoluble iron more slowly than to iron in solution because there is less iron in contact with the microbes. The presence of AQDS increased butanol yields in cultures containing soluble and insoluble ferric iron. Ferrous iron concentrations were also significantly increased in the presence of AQDS. AQDS eliminates the need for electron donor and acceptor to physically contact each other. Regeneration of the AQDS by reducing ferric iron to ferrous iron allows for more electrons to be stripped from the metabolic pathways increasing the butanol yield.

Acetone and ethanol concentrations also increased in the presence of iron and iron and AQDS. These increases may be an artifact of increased cell biomass in the system increasing overall solvent production as opposed to changes in the redox equilibrium of the metabolic pathways. Because ferrous iron is a growth factor for Clostridium beijerinckii, cell densities increased as the ferric iron in the system was reduced to ferrous iron. Cultures containing AQDS and ferric iron reduced more ferric iron to ferrous iron than cultures with only ferric iron. Cultures with more ferrous iron produced more biomass than cultures with only ferric iron.

Substrate utilization efficiency was also increased in cultures containing iron. Cultures with 20mM ferric iron and 1mM AQDS used three times more glucose as compared to glucose-only controls while the other iron containing cultures consumed between 2 and 2.5 times more glucose. Increased glucose consumption could be attributed to increasing ferrous iron concentrations. Ferrous iron stimulated growth and the increased biomass metabolized more substrate.

While increases in acetone and ethanol concentrations can be attributed to increases in biomass concentration leading to overall increased solvent production, influence on redox equilibrium of the metabolic pathways played a role in the increase of butanol production. Some increase in butanol production may be due to increased biomass production but butyric acid
concentrations decreased in all cultures containing AQDS, ferric iron or a combination of ferric iron and AQDS. This observation is in agreement with previous results where butanol production was favored at the expense of butyric acid production indicating that oxidized electron shuttles and electron sinks accepting electrons from the Clostridium metabolic pathways alter the flow of carbon and electrons as discussed previously.

Supplementing media with acetate also resulted in increased butanol yields when compared to glucose-only controls. Addition of AQDS and acetate to culture media produced greater increases in butanol yields compared to cultures with only supplementary acetate. When ferric iron was added to cultures containing supplemental acetate or acetate and AQDS, butanol yield increases over unamended controls were lower than cultures containing acetate or acetate and AQDS without iron. The final butanol concentrations in all cultures containing supplementary acetate were similar but the amount of substrate consumed varied between cultures depending on the presence of iron. Cultures containing ferric iron consumed more substrate than cultures without ferric iron and cultures containing supplementary acetate used more substrate than cultures without supplementary acetate. So although the final butanol concentrations were similar in all cultures containing supplementary acetate, because some consumed more substrate, they produced less grams of butanol per gram of substrate consumed.

Additional carbon from increased substrate consumption in cultures with acetate and acetate and iron, was used to produce greater acetone, ethanol and biomass concentrations. As described in previous experiments, higher ferrous iron concentrations corresponded to higher biomass concentrations. Acetate may also stimulate cell growth since cultures containing supplementary acetate without iron also had greater biomass concentrations than cultures without supplementary acetate although not as great biomass concentrations as cultures also containing iron.

Acetone concentrations were highest in cultures containing iron although the addition of AQDS to the iron did not affect acetone concentrations. Cultures with acetate but no iron also produced elevated acetone concentrations although not as high as cultures with iron. The presence of AQDS did not affect acetone concentrations in cultures containing acetate. Acetone concentrations were elevated when acetate was added to the culture because acetate or butyrate is required to produce acetone. High acetate concentrations remove acetate limitations on acetone
production. AQDS may not affect the production of acetone in cultures containing supplementary acetate because the acetone pathway does not involve any redox reactions. The ability of AQDS to influence redox chemistry would have no impact on a pathway not associated with redox reactions. Ethanol concentrations were also increased in the presence of acetate and further increased in the presence of acetate and ferric iron. Acetate may stimulate cell growth and ferric iron further stimulates cell growth. Greater biomass can produce more solvents.

Cultures grown with acetate present may use acetate as an additional substrate. Acetic acid concentrations started around 66mM in cultures containing supplementary acetate and decreased over time. Cultures with poorly crystalline ferric oxide without AQDS or ferric citrate consumed the most acetate with final acetic acid concentrations lower than in glucose-only cultures. Increased carbon demands due to increased cell growth in cultures with iron may account for low final acetate concentrations.

Butyric acid concentrations increased in cultures containing acetate and acetate with AQDS. This finding corroborates the idea that acetate is not influencing the metabolic pathways but just increasing the total amount of end products produced. Cultures containing acetate, AQDS and poorly crystalline ferric oxide also produced more butyric acid. This set of amendments resulted in the highest ferric iron reduction and the highest biomass density. High biomass density could account for the high butyric acid production. All other cultures containing iron produced high levels of butyric acid initially but over time the butyric acid concentrations dropped to levels similar to cultures grown in the presence of AQDS. The drop in butyric acid concentrations may result from reassimilation of butyric acid into butanol during solventogenesis. However, final butanol concentrations in these cultures were equivalent to final butanol concentrations in the other cultures containing acetate. Butanol concentrations in cultures containing ferric iron and AQDS were slightly lower than the final butanol concentrations in corresponding iron-containing cultures without AQDS. Acetate is more reduced than glucose. The change in oxidation state of one substrate could play a role in influencing the redox pathways in Clostridium metabolism.
CONCLUSIONS

The addition of AQDS, an oxidized extracellular electron shuttle, to wild-type *Clostridium beijerinckii* NCIMB 8052 cultures produced 2.5 to 5 fold increases in butanol yields. Increasing butanol yields is essential to making bio-butanol an economically viable alternative energy source. AQDS can be recovered from culture media after the fermentation and reused because AQDS functions as a catalyst that it is not consumed in the metabolic pathways. Reusing AQDS will lower fermentation costs over other proposed methods for increasing butanol yields like adding supplementary acetate. Acetate is metabolized by the culture and cannot be reused.

By stripping electrons out of the metabolic pathways, the distribution of carbon and electrons to the final products is altered. Carbon and electron flow favors the butanol pathway at the expense of the butyric acid pathway. The mechanism triggering the formation of more butanol is unknown at this time. One possibility is that AQDS may more efficiently regenerate NAD⁺, by accepting electrons from NADH, for use in glycolysis allowing the culture to produce more ATP. High levels of ATP inhibit the phosphotransbutyrylase enzyme which catalyzes the transformation of butyryl-CoA to butyryl-phosphate, effectively blocking the butyric acid pathway. With the butyric acid pathway blocked, all butyryl-CoA will be forced into the butanol pathway which increases the butanol yield. More research into the actual mechanism behind the yield increases is needed. Measuring ATP, NAD(P)⁺, NAD(P)H and activity levels for enzymes involved in butanol and butyric acid production in the presence and absence of AQDS is essential for understanding the factors that trigger increased butanol production when AQDS is in the system.

Substrate concentration did not affect the ability of AQDS to alter carbon and electron flow to favor butanol production. While overall solvent and acid production increased with increasing substrate concentration, the yield increase in the presence of AQDS compared to cultures without AQDS at the same substrate concentration remained constant. Minimizing the amount of AQDS required to increase butanol yield is crucial to reducing the cost of industrial bio-butanol production. These results show that a relatively low AQDS to substrate ratio can still achieve large increases in butanol yield.
Adding ferric iron to cultures had a similar effect on butanol yields as adding AQDS. Ferric iron accepts electrons from the metabolic pathways and is reduced to ferrous iron. The electron removal shifts the carbon and electron flow away from the butyric acid pathway and channels the flow into the butanol pathway thus increasing butanol yields. Ferrous iron also stimulates culture growth resulting in more biomass. More biomass results in greater overall solvent and acid production. Adding ferric iron and AQDS together resulted in even greater increases in butanol yields. Iron can accept electrons directly from the microbes or from AQDS which regenerates the AQDS allowing it to strip more electrons from the culture. Like AQDS, ferrous iron can be recovered from the media, regenerated and reused in subsequent fermentations. Recycling the iron can reduce the costs of implementing this strategy in the fermentation process.

Adding supplementary acetate to cultures also increased butanol yields and adding supplementary acetate to cultures with AQDS further increased butanol yields. However, supplementary acetate and ferric iron resulted in similar final butanol concentrations but lower butanol yields compared to cultures with acetate or acetate and AQDS. This result is due to increased substrate utilization in cultures containing iron. Adding acetate to media is not an effective means of increasing butanol yields. Acetate is metabolized by the microbes to produce more solvents and acids so that acetate cannot be recovered and reused. Every fermentation batch would require more acetate. Using AQDS or AQDS and ferric iron is a more promising method for increasing butanol yields in C. beijerinckii cultures.
REFERENCES


31. Grupe, H. and G. Gottschalk, *Physiological Events in Clostridium acetobutylicum during the Shift from Acidogenesis to Solventogenesis in Continuous Culture and Presentation of*


35. Meyer, C.L. and E.T. Papoutsakis, Increased levels of ATP and NADH are associated with increased solvent production in continuous cultures of Clostridium acetobutylicum. Applied Microbiology and Biotechnology, 1989. 30: p. 450-459.