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Effect of sodium acetate on butanol production by Clostridium saccharoperbutylacetonicum via ABE fermentation

Brigetta Super
University of Arkansas, Fayetteville

Effect of sodium acetate on butanol production by *Clostridium saccharoperbutylacetonicum* via ABE fermentation

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Ralph E. Martin Department of Chemical Engineering

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Brigetta Super

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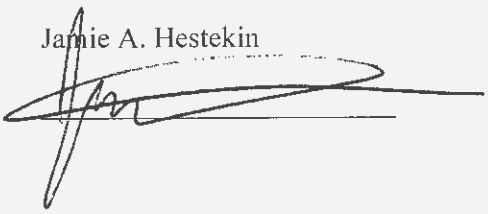
Thesis Advisor:

Jamie A. Hestekin



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Abstract

Clostridium saccharoperbutylacetonicum is used in ABE fermentation which historically produces a solvent mixture of 3:6:1 parts acetone, butanol, and ethanol respectively. The fermentation process undergoes two phases, an acidogenesis phase which produces butyric acid, and a solventogenic phase which reutilizes the butyric acid, making butanol. For ABE fermentation to compete economically with butanol production from petroleum, the concentration of butanol in the solvent product must be increased dramatically. Unpublished research by Carver student Kayla Kimbrough in the summer of 2012 showed that the addition of salts (sodium acetate) to PYG fermentation media increased butanol production from *Clostridium saccharoperbutylacetonicum* significantly. When compared to the control in this experiment, a 4 mM concentration of acetate in 20 g l^{-1} PYG media yielded a 400% increase in butanol concentration. This aim of this research was to confirm Kimbrough's results and perform a mass balance to account for the acetate after the fermentation process to ensure that this rise in production was not due to the salts being utilized as a food source. Although this experiment did not have the predicted high butanol yields, the post fermentation analysis accounted for >99% of the added acetate, which suggests that the acetate is acting as a stabilizer and not as a carbon source.

Introduction and Background

Butanol and other organic acids are naturally produced by *Clostridia*. *Clostridia* are rod-shaped, spore-forming, gram positive bacteria that are usually strict anaerobes. *Clostridia* are versatile in that they can utilize a variety of substrates including five- and six-carbon sugars and polysaccharides to produce solvents although an addition of yeast extract to the media broth is required for optimum bacteria growth and solvent production (Monot et al., 1982). The most widely used *clostridia* for solventogenic production are *C. acetobutylicum, C. beijerinckii, C. saccrobutyklicum,* and *C. saccroperbutylacetonicum* (Lee, 2008)

Production of butanol occurs in two phases: an acidogenic phase in which acetate, butyrate, hydrogen and carbon dioxide are produced and a solventogenic phase in which acids are reutilized to form acetone, butanol, and ethanol. The acidogenic phase occurs during the exponential growth phase. This switch from acidogenesis to solventogenesis is the result of a dramatic change in gene expression (Dürre et al, 1987).

The first record of producing butanol by ABE fermentation was in 1961 by Pasteur. By 1945 butanol was the second largest produced chemical after ethanol. In the 1960s the production of butanol by fermentation decreased substantially due to increased substrate costs and the rapid development of petrochemicals. Only Russia and South Africa continued to produce fermentative butanol up until the late 1980s and early 1990s (Lee et al, 2008).

The resurgence of interest in producing butanol from fermentation began with the oil embargo in 1973 when the U.S. had a vested interest in cutting dependency of foreign oil and began investigating alternative fuels. Much of the ABE research was focused on ethanol production usually using corn as the feedstock. Due to sustainability issues associated with producing ethanol from corn, academics are shifting their attention to butanol as a green fuel source. For efficiency, butanol is superior in that it has higher energy content per unit volume content and it absorbs less water than ethanol. The Energy

Independence and Security Act (EISA) was passed in 2007 included the Renewable Fuel Standard (RFS) which mandated that 35 billion gallons of ethanol-equivalent fuel and 1 billion gallons of biodiesel be produced by 2022 ("Renewable fuel," 2011). Butanol is widely thought to be the solution to fill the gap before this deadline. The concern for butanol production via ABE fermentation is the same for ethanol production in that the substrate costs are still high and that relative yields of solvents are still too low to be economically feasible.

In order for butanol production by fermentation to be commercially viable again, the final concentration of organic acids in the fermentation broth must be increased significantly to reduce recovery costs. The usual concentration of ABE solvents in the fermentation broth is typically 30 g l^{-1} with butanol accounting for 13 g l^{-1} (Qureshi and Blaschek, 2001). If the concentration of butanol could be increased from 10 g l^{-1} to 40 g l^{-1}, the ratio of fuel oil to 100% recovered butanol for distillation would be reduced from 1.5 t t^{-1} to 0.25 t t^{-1} (Phillips & Humphrey, 1983). In addition, ABE fermentation is product inhibited as butanol is highly toxic to *Clostridium acetobutylicum* cells. Therefore, batch reactors are limited to 20-60 g l^{-1} of sugar solutions (Ezeji & Qureshi, 2004).

It is also widely known that repeated subculturing of *clostridia* strains decreases the gene expression for butanol. A 20 mM addition of sodium acetate was shown to stabilize solvent production and maintain optical density of *C. beijerinckii* NCIMB. The control (no added acetate) showed a sharp decrease in solvent concentration and optical density. In this same study using a 60 g l^{-1} MP2 media solution, the production of butanol was 0.6, 5.3, and 13.9 g l^{-1} for added acetate concentrations of 0, 20, and 60 mM respectively (Chen & Blaschek, 1999).

Another study which investigated the effect of using different carbohydrate sources and media types for solvent production by *C. saccroperbutylacetonicum*. Al-Shorgani et al (2011) compared 30 g l^{-1} glucose, xylose, xylan, cellulose, and starch concentrations in typtone-yeast extract (TYA) or potato glucose media (PG). The highest yields were for glucose in PG media at 7.57 g l^{-1} and 5.36 g l^{-1} total ABE

concentration and butanol concentration, respectively. In the summer of 2012 University of Arkansas Carver student Kayla Kimbrough investigated the addition of acetate to *C. saccroperbutylacetonicum* with the aim of extending the bacteria's life as other studies have indicated it could (Chen & Blaschek, 1999). Using a procedure similar to Al-Shorgani, she inoculated bottles of 20 g l^{-1} glucose peptone-yeast-glucose (PYG) media with 0, 20, 40, 80, and 160 mM sodium acetate (Fig. 1). The experiment was repeated for 2, 5, and 10 mM acetate in 20 g l^{-1} PYG. The results of her experiments showed that moderate amounts of sodium acetate (4 g l^{-1}) increased the butanol concentration from 1.9 g l^{-1} (no added acetate) to 8.1 g l^{-1} (Fig 2).

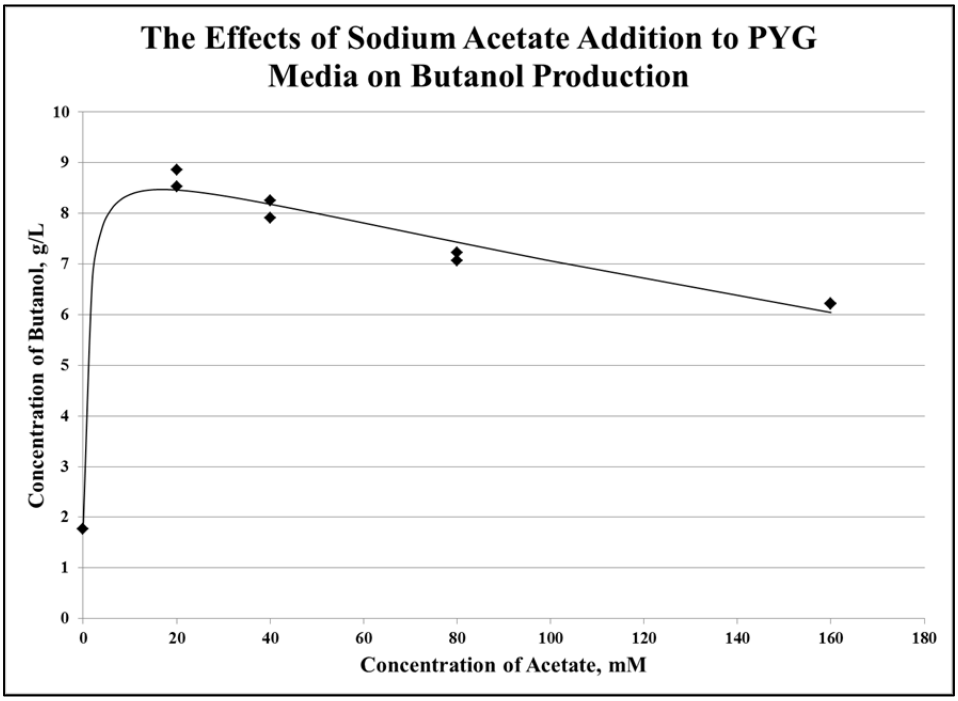

Figure 1: Kimbrough experiment trial 1

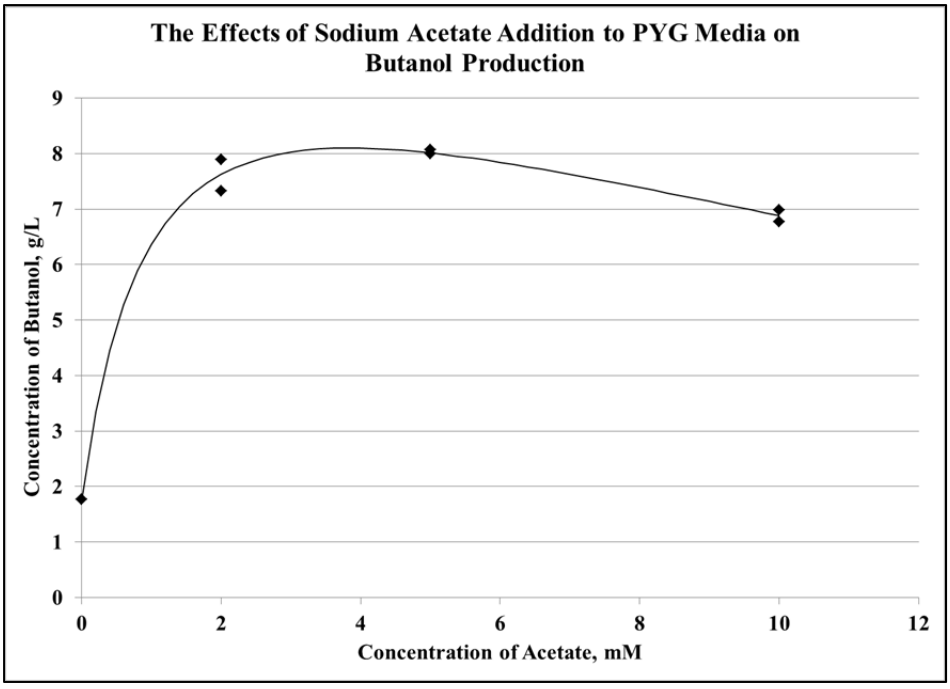

Figure 2: Kimbrough experiment trial 2

It was desired that this experiment be repeated and account for the acetate after the fermentation process was completed to ensure that this dramatic increase in butanol concentration was not due to the added acetate being utilized as a food source. This was the objective of my honors college thesis.

Methods and Materials

A stock of 20 g l^{-1} glucose PYG media (6.5 g l^{-1} peptone, 3.5 g l^{-1} yeast extract) was prepared with sodium acetate concentrations of 0, 2, 5, 10, and 20 mM acetate. All samples were done in duplicate. The media bottles were sterilized for 27 minutes at 121°C using a SANYO autoclave (MLS-3751L). The autoclaved bottles were inoculated with 10 mls of *C. saccroperbutylacetonicum* inoculum from culture bottles which contained no added acetate. The culture bottles were incubated at 32°C for 4 days then tested for acetone, butanol, ethanol, acetic acid, and butyric acid.

All the gas chromatography (GC) work was done on the Shimadzu 2014 gas chromatograph. This work was done with a Phenomenex ZB-FFAP capillary column with dimensions of 30 m x 0.32 mm x 0.25 um. Helium was used as the carrier gas at 30 cm/sec. The temperature profile was started at 40°C, raised to 60°C at 6 C/min, raised to 160 C at 20°C/min, raised to 230°C at 30°C/min, and held at 230°C for 4 min. Components were measured with the FID at 300°C, and calibrated to a 3 point series of each targeted component that had been done earlier. The calibration was checked by following the fermentation samples with a reference sample. The injection consisted of 2 microliters with a split of 10:1. The injector port was held at 250°C throughout.

Results and Discussion

Very little butanol was produced from this experiment as indicted in Figure 3. Instead large amounts of butyric acid and acetic acid were observed. It is evident that the culture did not proceed to the solventogenesis phase. A decrease or absence of solvent production can occur if the bacterium is exposed to oxygen or if the sample becomes contaminated (usually from *Streptococcus*). Since the problem occurred in all the sample bottles it is unlikely that oxygen was the culprit. Also, a visual analysis using a light microscope of the inoculum culture before inoculation and of the post-fermentation samples showed no signs of contamination.

The most reasonable explanation is that the culture had aged past the point of usefulness before inoculation. In general, *C. saccroperbutylacetonicum* will produce solvents up to its fourth split. The bacteria culture used had begun to lose its solvent producing function prior to inoculation. It is possible the acidic conditions proved to be too harsh to the weak bacteria which killed it before it could proceed to the solventogenic phase. However, the trend for butyric acid also shows a peak production near 10 mM acetate, which indicates that the salt addition would have increased the production of butanol, had the fermentation gone to completion. The butyric acid trend (Fig. 4) appears to be consistent with Kimbrough's results.

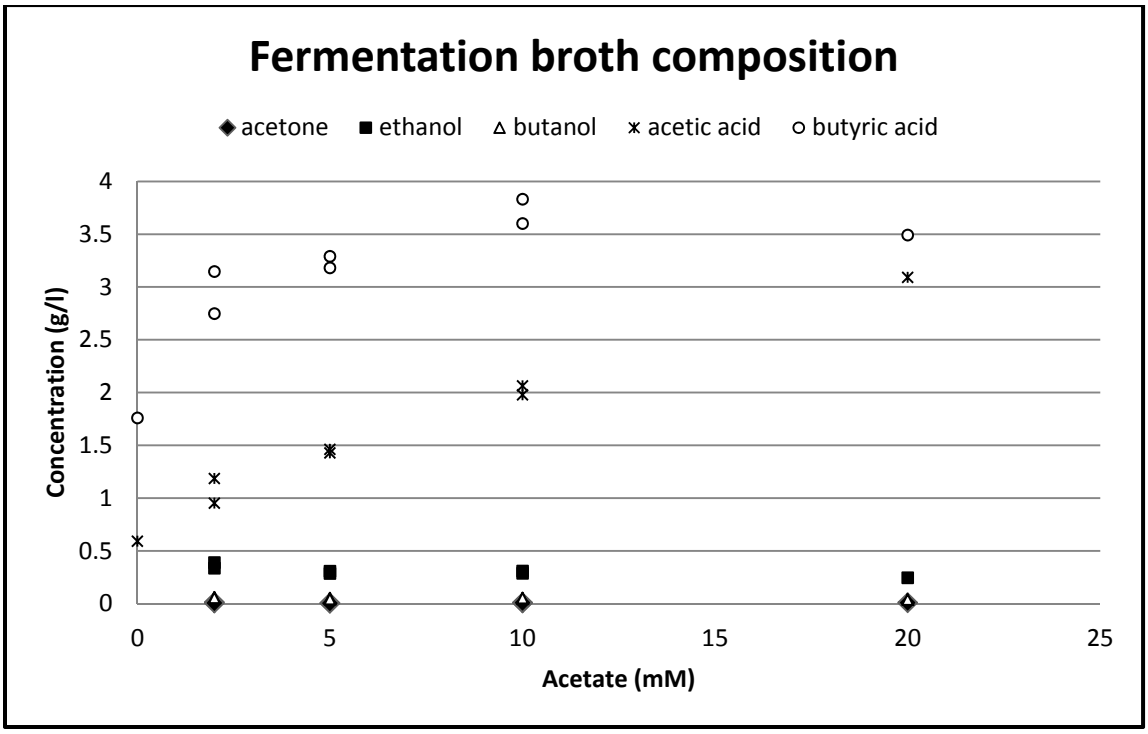

Figure 3. Final product concentrations with varying added acetate

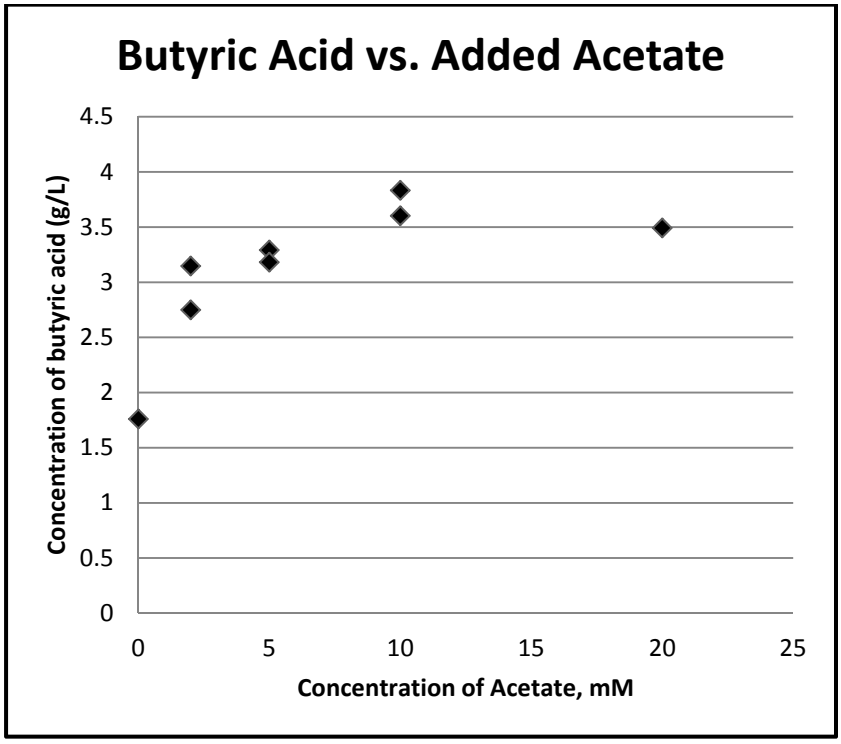

Figure 4. Butyric acid production with varying added acetate

The acidic conditions in the sample bottles provided a straight forward way to measure the acetate concentration in the post fermentation samples. The pH of the sample bottles ranged from 4.27-4.29, at which all the acetate was in the acid form (acetic acid) and not the salt form. Using the known concentration of acetate added to the samples, a simple mass balance was used account for the acetate at the end of the fermentation. It should be noted that a very small amount of acetic acid is naturally produced as a by-product. Using a linear fit of the initial acetate concentration versus the concentration of acetate in the fermentation samples (acetic acid), the amount of acetate before and after is nearly the same as an R^2 value of 0.98 indicates (Figure 5). This very conclusively proves that acetate is not being metabolized to butyrate in this case and the positive increase in butyric\butanol tolerance is a real effect.

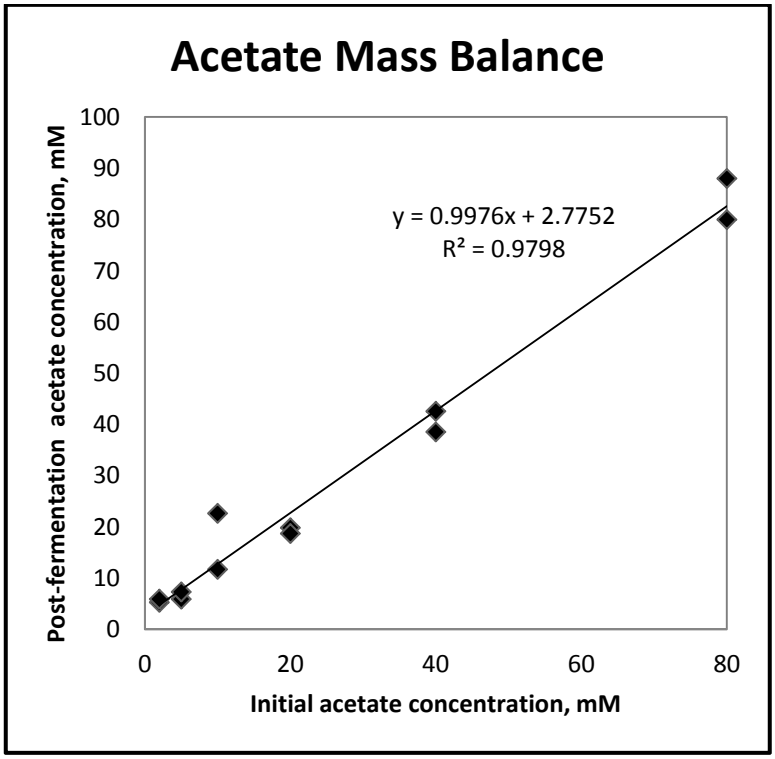

Figure 5. Acetate accountability

Conclusions

Although the results of the Kimbrough experiment were not entirely replicated, the results of this experiment corroborate her conclusions that additions of sodium acetate in dilute concentrations (< 10 mM) to PYG media will increase the overall production of butyric acid and therefore increase the production of butanol. Furthermore, it was concluded that acetate was not used as a carbon source for this fermentation process. It is believed that the acetate stabilizes the fermentation reactions, although the mechanism of how it does that is not well understood. This experiment was repeated with a fresh culture and the results will be summarized and hopefully published. Adding salts to fermentation media would be an inexpensive way to increase solvent production, and possibly prevent bacteria strain degradation; both results are a positive step to making ABE fermentation more economical for mass production.

Acknowledgements

I would like to acknowledge Dr. Jamie Hestekin for his encouragement and unfailing support for this project especially given the tight time constraints for this project. My interest in biofuels was largely due to his introductory Design class in which my group designed a production scale biobutanol plant.

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References

Al-Shorgani, N. K. N., Kalil, M. S., & Yusoff, W. M. W. (2011). The effect of different carbon sources on biobutanol production using clostridium saccharoperbutylacetonicum N1-4. *Biotechnology, 10*(3), 280-285.

Chen, C. K., & Blascheck, H. P. (1999a). Acetate enhances solvent production and prevents degeneration in clostridium beijerinchii BA101. *Applied Microbiology Biotechnology, 52*, 170-173.

Chen, C. K., & Blascheck, H. P. (1999b). Effect of acetate on molecular and physiological aspects of clostridium beijerinckii NCIMB 8052 solvent production and strain degredation. *Applied and Environmental Microbiology*, *65*(2), 499-505.

Dürre P. (1998). New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Applied Microbiology Biotechnology 49, 639-648.

Ezeji, T. C., Qureshi, N., & Blaschek, H. P. (2004). Acetone butanol ethanol (ABE) production from concentrated substrate: Reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. *Applied Microbiology Biotechnology*, *63*, 653-658.

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by clostridia. *Biotechnology and Bioengineering*, *101*(2), 209-228.

Monot F, Martin J-R, & Petitdemange H, Gay R. (1982). Acetone and butanol production by Closridium acetobutylicum in a synthetic medium, Applied Environmental Microbiology 44, 1318-1324.

Phillips JA, & Humprey AE. (1982). An overview of process technology for the production of liquid fuels and chemical feedstocks via fermentation. In: Wise DL (ed) Organic chemicals from biomass. Benjamins/Cummings Publishing, Menlo Park, CA, 249-30.

Qureshi N, & Blascheck HP. (2001). Recent advances in ABE fermentation: hyper-butanol producing Clostridium beijerinckii BA101. J Ind Microbiology Biotechnology 27:292-297.

Renewable fuel standard: Potential economic and environmental effects of U.S. biofuel policy (L. B. Lave, Comp.). (2011). National Academics Press.