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Using Bacteria to Eliminate the Glycerin Glut

Jonathan Kilroy
University of Alabama in Huntsville

Javier Sanchez
University of Alabama in Huntsville

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Jonathan Kilroy, Javier Sanchez
Department of Chemistry

Abstract **–** Crude glycerol is produced in large quantities as a byproduct of biodiesel production. *Clostridium pastuerianum* is an anaerobic species of bacteria capable of using glycerol as its sole carbon source. *C. pasteurianum* is known to convert glycerol into n-butanol, which is useful as a fuel additive. In this study, we measured the concentrations of glycerol along with compounds produced by *C. pasteurianum*, including n-butanol, under varying growth conditions. We found that doubling the initial concentration of glycerol had no noticeable effect on the ability of the bacteria to grow or produce butanol. Based on this finding, we intend to move forwards with a higher concentration of glycerol in order to sustain a continuous culture of bacteria in a bioreactor.

I. Introduction

Biodiesel-derived crude glycerol is produced as a byproduct of biodiesel production at 10% (w/w) [1]. An increase in the amount of biofuel production has led to an overabundance of crude glycerol. The production of more glycerol than there is a demand for, often referred to as the "glycerol glut", has led to an increased demand for an economically feasible method of disposing or using the glycerol [2].

The ability of *C. pasteurianum* to convert glycerol into butanol, a substance which can be used as a fuel additive, could be the key to eliminating unnecessary waste from current biofuel production methods [3, 4]. This species of Clostridium is of particular interest as it can utilize glycerol as its sole carbon source [1]. In addition, this bacteria can use crude glycerol directly, since the only compounds in crude glycerol that are toxic to this species are fatty acids, which can be easily removed from crude extracts [5]. *Clostridium pasteurianum* is an anaerobic, non-infectious soil bacterium. It is capable of producing a range of products from glycerol, including acetic acid, 1,3-propanediol (1,3- PDO), ethanol, butyric acid, and butanol, as part of its regular metabolism [4]. Butanol in particular is of special interest due to its miscibility in petroleum, lower vapor pressure, and higher energy content compared to current fuel additives, specifically ethanol [1]. The potential butanol has for improving the efficiency of biodiesel and other forms of biofuel makes it a desirable product. The purpose of this project is to determine trends in the concentration of butanol and other substances generated by *C. pasteurianum* to aid in the eventual design and implementation of a bioreactor capable of sustaining a continuous culture of this species that can consistently convert glycerol to butanol.

II. Materials and Methods

Growth of Bacteria

C. pasteurianum ATCC 6013 strain was obtained from American Type Culture Collection. Frozen samples were thawed and then cultured in glucose media. Glucose media is composed of 3.74 g/L K_2HPO_4, 1.43 g/L KH_2PO_4, 2.2 g/L $(NH_4)_2SO_4$, 80 g/L dextrose, and per 100mL solution: 1mL $MgSO_4$/$FeSO_4$ solution (22g/L $MgSO_4$, 0.55 g/L $FeSO_4$) per 100mL total solution, and 200 microliters trace metal solution SL7 per 100mL total solution. Trace metal solution SL7 is composed of 10mL of 25% HCl solution per liter, 1.5g/L $FeCl_2 \cdot 4H_2O$, 190.0 mg/L $CoCl_2 \cdot 6H_2O$, 100.0 mg/L $MnCl_2 \cdot 4H_2O$, 70.0 mg/L $ZnCl_2$, 62.0 mg/L H_3BO_3, 36.0 mg/L $Na_2MoO_4 \cdot 2H_2O$, 24.0 mg/L $NiCl_2 \cdot 6H_2O$, 17.0 mg/L $CuCl_2 \cdot 2H_2O$. 5mL of $CaCO_3$ were added to 100mL of the glucose media inside of the glove box.

Cultures were grown in a glove box under anaerobic conditions until an Optical Density (OD) of 0.6 and then transferred to glycerol media. Glycerol media has the same composition as glucose media, but 25g/L of glycerol are used instead of 80g/L of dextrose. Flasks were shaken continuously for both growth and fermentation at 37°C.

Fermentations

All fermentations were done in a flask designed to simulate a reactor in a glove box under anaerobic conditions at 37°C. The atmosphere in the

glove box consisted of approximately 5% hydrogen and 95% nitrogen. A pair of palladium catalysts was used to remove excess oxygen from the air by catalyzing the reaction:

$$2H_2 + O_2 \rightarrow 2H_2O$$

A second pair of catalysts was rotated with the first pair weekly, and the catalysts regenerated. Fans were used to mix the air to optimize the removal of oxygen by the palladium catalyst. The pH was measured each day to determine growth using a pH meter. The optical density (OD) was also measured at 610nm each day.

Taking Samples

Samples were taken in 24-hour intervals from the reactor. These samples were centrifuged at 10,000 X g for 10 minutes. The supernatant was filtered through syringe filtration, and then frozen overnight. The next day samples were thawed and 25 microliters of each sample were run through High Performance Liquid Chromatography (HPLC). Another sample from each flask was taken and the pH measured to be certain the bacteria were still alive.

HPLC

HPLC media was prepared regularly by mixing 570 microliters of concentrated sulfuric acid (H_2SO_4) in 2 liters of distilled water (diH_2O). 25 microliters of sample were loaded into an Aminex® HPX-87H Column for HPLC and run for a minimum of an hour. Each sample was run twice. Samples whose peak heights were significantly different were run a third time.

The column was calibrated with varying concentrations of glycerol, acetic acid 1,3-PDO, ethanol, butyric acid, and butanol. The average retention time was determined, along with the slope of the line of the graph of concentration (g/L) vs. area under each peak (µV*min) for each substance. This is recorded in Table 1.

Table1: Calibration of HPLC Machine

Substance	Retention time (min)	Slope (µV*min/g/L)
Glycerol	19.7	11444
Acetic Acid	22.8	6790.1
1,3-PDO	25.9	8227
Ethanol	31.8	4593.5
Butyric Acid	33.5	9466.1
Butanol	55.5	8767.1

Different Growth Conditions

Multiple flasks were used to determine the trends in glycerol consumption and butanol production. Variations included the size of the flask and the initial concentration of glycerol. The concentration of initial glycerol was doubled from 25g/L to 50g/L in two flasks to determine if the bacteria could survive at higher concentrations of glycerol. Two control flasks at 25g/L initial concentration were also grown.

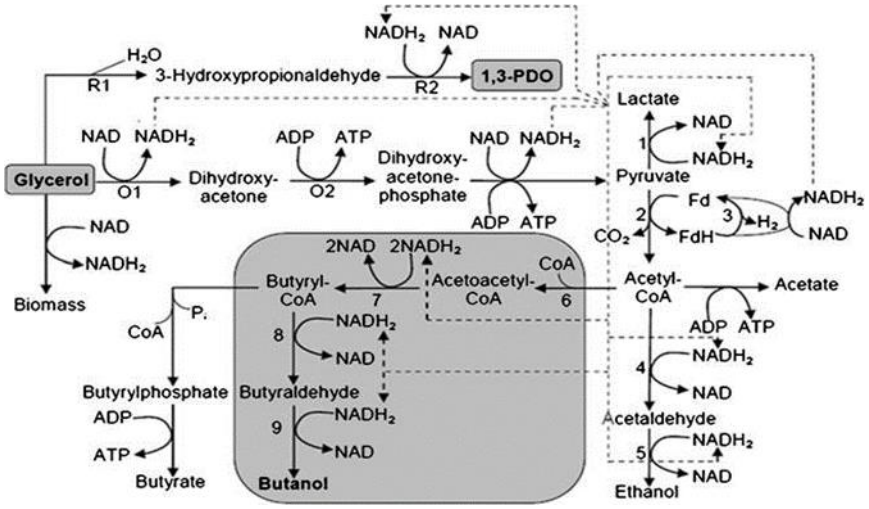

Figure 1. Overview of metabolic pathways of *C. pasteurianum*. Dotted lines represent movement of electrons. R1 glycerol dehydratase, R2 PDO dehydrogenase, O1 glycerol dehydrogenase, O2 dihydroxyacetone kinase, 1 lactate dehydrogenase, 2 pyruvate–ferredoxin oxidoreductase, 3 hydrogenase, 4 acetaldehyde dehydrogenase, 5 ethanol dehydrogenase, 6 thiolase, 7 butyryl-CoA dehydrogenase, 8 butyraldehyde dehydrogenase, 9 butanol dehydrogenase The boxed area indicates the solventogenesis pathway of butanol formation. [1]

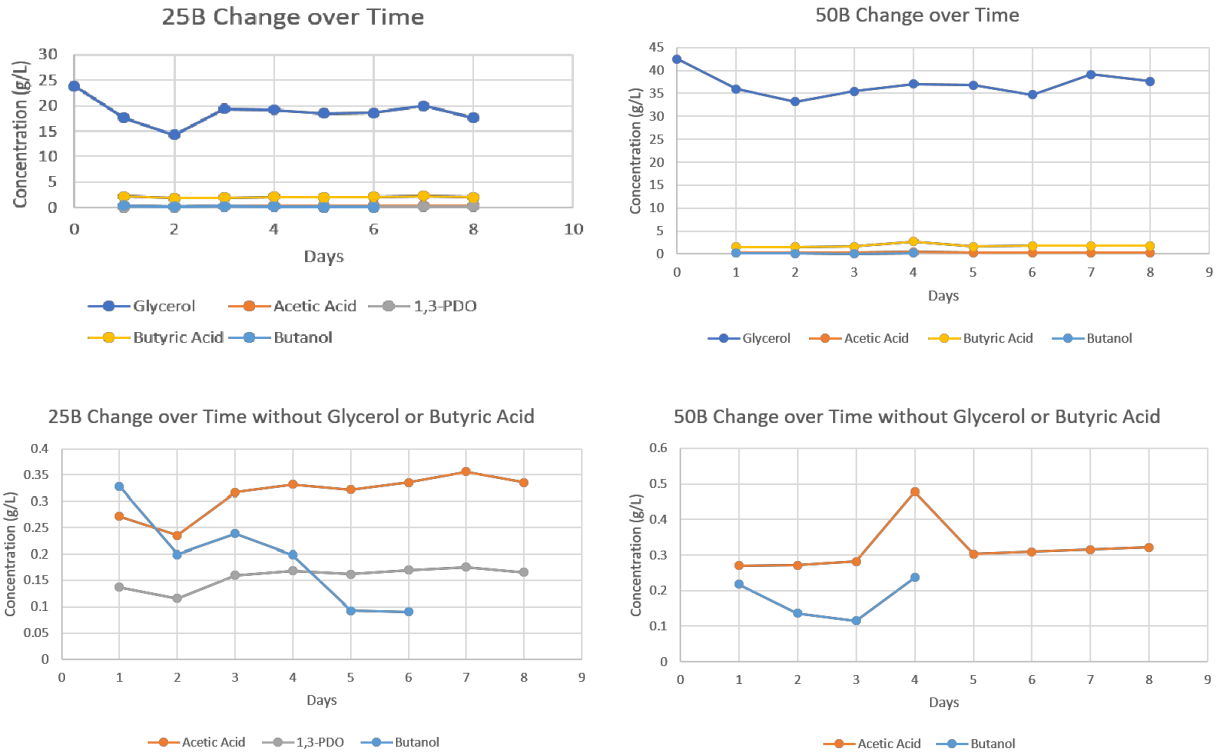

Figure 2. Comparison of 25g/L growth and 50g/L growth. 25B started with approximately 25g/L and 50B started with 50g/L. The two charts without glycerol or butyric acid do not include the measurements from glycerol or butyric acid included in the top two charts to better visualize the remaining products.

III. Results and Discussion

HPLC data was compiled for each flask tested. The calibration table was used to determine the concentration represented by each peak. An average concentration was determined for each sample. A chart was made of concentration of each substance over time. The charts for a 25g/L glycerol control and a 50g/L sample are included in Figure 2. These charts are a sample of the type of data collected.

No correlation was found between the size of the flask and the rates of butanol production. The size of the flask ranged from 100.0 ml to one liter. The only notable difference was that the cultures in larger flasks were sustained longer than those in larger flasks. This is primarily due to less space and less total resources available. However, the concentrations of butanol, glycerol, and other compounds were similar across all sizes of flask. As such, the focus of the data is more on the concentration of each substance and less on the total mass or volume.

It was found that in most cases butanol levels spiked between days 1 and 3 and then decreased steadily thereafter. This phenomenon is more easily observed in the 25B chart than the 50B chart. In the 50B sample, the butanol levels spiked on day one, decreased until day 3, and then spiked once on day 4 and disappeared entirely. More samples that were tested followed the trend of an initial spike followed by decreasing levels. The decreasing concentrations of butanol over time are believed to be due to its reabsorption back into the cellular membrane of dead bacteria cells [3]. Butanol, as with all alcohols, is known to be absorbed by the cell membrane thereby increasing membrane fluidity. Bacteria counteract this often toxic effect by altering the composition of their membranes and the ratios of unsaturated to saturated fatty acids. This response is known as the homeoviscous response [3]. If the butanol produced and excreted by the bacteria is reabsorbed over time, both by living and deceased cells, then the concentration of butanol would appear to go down, as our results show.

Based on this hypothesis, it is the intention of this lab group to test methods for removing butanol from solution. This will not only harvest the butanol, but also shift equilibrium towards butanol diffusing out of the cells. We intend to test pumping fresh glycerol media into the reactor and pumping used media out to replenish glycerol and remove butanol. Liposomes will also be used to remove butanol from the used media in order to harvest it. These methods are designed to aid continuous growth of a bacterial culture and prevent reabsorption of butanol by cells.

Overall, it was found that *C. pasteurianum* can grow at higher concentrations of glycerol than had been previously tested and produce butanol. The results of 50g/L glycerol were incredibly similar to those of 25g/L. Initial butanol concentrations were similar, within 0.1g/L of each other, and decreased at similar rates. However, butanol was only detected in one of the four flasks by day six. Despite the apparent lack of change in doubling the glycerol concentration, these results are promising. The bacteria is capable of surviving at higher concentrations of glycerol. Further testing of higher concentrations of glycerol may yield larger amounts of butanol produced and higher rates of production.

IV. Conclusion

Although our initial results do not show that an increase in glycerol concentration leads to an increase in butanol production, it is highly plausible that such an increase could lead to a higher rate of butanol production. Our theory that the butanol is reabsorbed by dead bacterial cells would account for the lack in apparent change in butanol levels from 25g/L of glycerol to 50g/L. It is possible that once this effect is compensated for, there will be a correlation between initial glycerol concentration and butanol production rate. At the very least, the preliminary results here have shown that the bacteria are capable of surviving at 50g/L glycerol. Further testing is necessary to determine if the rate of butanol production and the amount of butanol produced can be increased by increasing available glycerol. Pumping fresh media into the reactor and pumping old media out will be the next step in this project, followed shortly with the use of liposomes to remove butanol from the used media. Both of these processes should provide a more accurate way of measuring butanol production.

References

[1] Venkataramanan, Keerthi P. et al. “Impact of Impurities in Biodiesel-Derived Crude Glycerol on the Fermentation by Clostridium Pasteurianum ATCC 6013.” *Applied Microbiology and Biotechnology* 93.3 (2012): 1325–1335. Web.

[2] Miodrag Belosevic, Mohamed Gamal El Din Zengquan Shu James R. Bolton. “Degradation of Alizarin Yellow R Using UV / H 2 O 2 Advanced Oxidation Process.” *Environmental science & technology* 33.2 (2014): 482–489. Web.

[3] Venkataramanan, Keerthi P. et al. “Homeoviscous Response of Clostridium Pasteurianum to Butanol Toxicity during Glycerol Fermentation.” *Journal of Biotechnology* 179.1 (2014): 8–14. Web.

[4] Biebl, H. “Fermentation of Glycerol by Clostridium Pasteurianum--Batch and Continuous Culture Studies.” *Journal of industrial microbiology & biotechnology* 27.1 (2001): 18–26. Web.

[5] Bothun, Geoffrey D. et al. “Cooperative Effects of Fatty Acids and N-Butanol on Lipid Membrane Phase Behavior.” *Colloids and Surfaces B: Biointerfaces* 139 (2016): 62–67. Web.