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Conversion of Glycerol to Butanol Using Continuous Culture of Clostridium pasteurianum

Jonathan Michael Kilroy

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by

Jonathan Michael Kilroy

An Honors Capstone
submitted in partial fulfillment of the requirements
for the Honors Diploma

to

The Honors College

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April 24, 2019
Honors Capstone Director: Dr. Carmen Scholz
Professor of Chemistry

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Abstract

Crude glycerol is a significant byproduct of biodiesel production. Although glycerol is a useful compound, purifying crude glycerol is not cost effective. Fortunately, *Clostridium pasteurianum* has the inherent ability to utilize glycerol as it's sole carbon and energy source. *C. pasteurianum* preferentially converts glycerol into butanol, which can be used as a fuel additive. This project tests a bioreactor designed to indefinitely sustain a culture of *C. pasteurianum* that continuously converts glycerol into butanol. A continuous culture is achieved by pumping fresh glycerol media into the reactor at the same rate used media is being pumped out. Keeping the two flow rates constant allows the volume of the reactor to remain constant while the bacteria continue to grow and ferment. Three dilution factors were tested and the effects on bacterial growth and butanol fermentation were determined. Bacterial growth was measured using optical density at 600 nm. Concentrations of glycerol and butanol were measured, along with other fermentation products, using High Performance Liquid Chromatography (HPLC). Butanol production is consistently higher, and production of minor fermentation products is consistently lower in continuous cultures than in batch cultures of the same volume. A continuous culture of *C. pasteurianum* is capable of producing high yields of butanol from glycerol while maintaining a large bacterial population.

Introduction

Crude glycerol is produced at 10% (w/w) as a byproduct of biodiesel production (Venkataramanan, 2011). This glycerol contains fatty acids which are toxic to bacteria, but all other impurities show no impact on bacterial growth. While it is necessary to remove these fatty acids from crude glycerol, *Clostridium pasteurianum* can grow in presence of all other impurities (Bothun, 2016). *C. pasteurianum* has been the subject of study for glycerol conversion using ABE fermentation which is characterized by the production of acetone, butanol, and ethanol and is typical of the genus *Clostridium* (Moon, 2016). *C. pasteurianum* is of particular interest because of its ability to rely solely on glycerol, even crude glycerol, for carbon and energy (Venkataramanan, 2014). This species produces n-butanol from crude glycerol as a major product. Butanol is useful as a fuel additive similar to ethanol. Butanol has a higher heat of combustion, better miscibility in petroleum, and a lower vapor pressure than ethanol (Venkataramanan, 2012). Several minor products are also produced, namely, ethanol, 1,3-propanediol (PDO), butyric acid, and acetic acid. These solvents have been shown to be toxic to the bacteria, especially butanol, which partitions the phospholipid bilayer of the cell membrane (Venkataramanan, 2014).

Batch cultures of bacteria exhibit traditional exponential growth. There is a lag phase after inoculation, followed by an exponential growth phase which ends once carrying capacity is reached. The culture subsequently dies off once all resources are depleted (Monod 1949). For useful conversion of glycerol to butanol, batch cultures are insufficient. Monod Kinetics are better observed in continuous culture than in batch cultures (Egli 1998). Continuous cultures naturally tend towards a steady state that allows fast enough growth to counteract dilution of the culture by the influx of fresh media that is still fast enough to allow replenishment of the used

nutrient or substrate (Button 1985). This steady state is reached during the exponential phase of bacterial growth where growth is more or less constant (Monod 1949). The replenishment of resources in continuous cultures potentially allows cells to develop higher maximum growth rates (Egli 1998).

In this paper, the use of a continuous culture is tested to promote cell growth of *C. pasteurianum* and stimulate butanol production. A continuous culture is achieved by pumping fresh media consisting of energy and carbon sources and trace nutrients required for bacterial growth into a reactor while pumping the reactor contents out at equal flow rates. The flow rate corresponds to a dilution factor, which is the measure of how quickly the volume in the reactor is replaced. The change in biomass in the reactor is a function of the specific growth rate of the bacteria and the rate at which bacteria are being diluted out. Once the reactor reaches a steady state, biomass levels are constant causing the change in biomass to equal zero. When this occurs, the specific growth rate becomes equal to the dilution factor which remains constant (Microbial Growth 2014)

Methods

Bacterial Culture

C. pasteurianum ATCC 6013 strain was obtained from American Type Culture Collection. Frozen *C. pasteurianum* stocks were thawed in a 37°C water bath for 2 minutes and then cultured in glucose media.

Media components

Cultures were started in glucose media and subsequently transferred to glycerol media for each experiment. Note that each concentration is given in grams of solute per liters of diH_2O unless otherwise noted. Glucose media and glycerol media are both composed of 3.74 g/L K_2HPO_4, 1.43 g/L KH_2PO_4, 2.2 g/L $(NH_4)_2SO_4$, and per 100mL diH_2O: 1mL $MgSO_4$/$FeSO_4$ solution (22g $MgSO_4$ per liter solution, 0.55 g $FeSO_4$ per liter solution) per 100mL diH_2O, and 200 microliters trace metal solution SL7 per 100mL diH_2O. In glucose media, 80 g/L dextrose is added. The dextrose is measured and added to only 50 mL of diH2O while the other components are added to a separate 50 mL. The two solutions are autoclaved separately and then combined in an anaerobic glove box. Glycerol media is prepared at full volume with 25 g/L glycerol added along with the other components. 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 $CuCl_2 \cdot 2H_2O$. Each component of trace element solution is measured per liter of total solution. Each individual solution was autoclaved in autoclave bottles. Each bottle was sealed immediately upon removal from the autoclave and allowed to cool. Once cool enough to handle without insulating gloves, the solutions were transferred to the anaerobic glove box, and 0.5 mL of $CaCl_2$ solution added for approximately every 100 mL of media. $CaCl_2$ solution was prepared

by adding 22g $CaCO_3$ to approximately 40 mL concentrated hydrochloric acid and then bringing the total volume up to 100 mL with diH_2O for a final concentration of 24.4g $CaCl_2$ per liter of solution. In later experiments, $CaCl_2$ solution was prepared directly by dissolving 24.4 g of $CaCl_2$ in enough water to produce 100mL total solution. There was no discernable difference in bacterial growth between the two methods.

Fermentations

All fermentations occurred under anaerobic conditions in a glove box with atmosphere of a mix of nitrogen and hydrogen gas at 37°C. A pair of palladium catalysts was used to remove excess oxygen from the air by catalyzing its reaction with hydrogen gas to produce water. These catalysts were rotated with a second pair weekly and regenerated in an oven at 125°C. 100 mL of glucose media was prepared for each experiment per methods listed under media composition. 25-30 mL of glucose media was transferred into a culture flask which was continuously shaken. A frozen 1mL stock of *C. pasteurianum* was heat shocked for 2 minutes in a hot water bath at 37°C and immediately transferred into the glove box. This stock was inoculated in the glucose media and allowed to grow for approximately 24 hours. For batch culture experiments, 1 mL of glucose culture was transferred into a culture flask containing 25-30 mL glycerol media. For continuous experiments, 5 mL of glucose culture was transferred to 500 mL of glycerol media in the bioreactor. Cell growth was measured using optical density (OD) at 600 nm and fermentation was measured using a pH meter.

Continuous Culture

Peristatic pumps were used to regulate flow of fresh media into the bioreactor and the flow of used media out of the reactor. Fig 1. shows the diagram of the reactor setup.

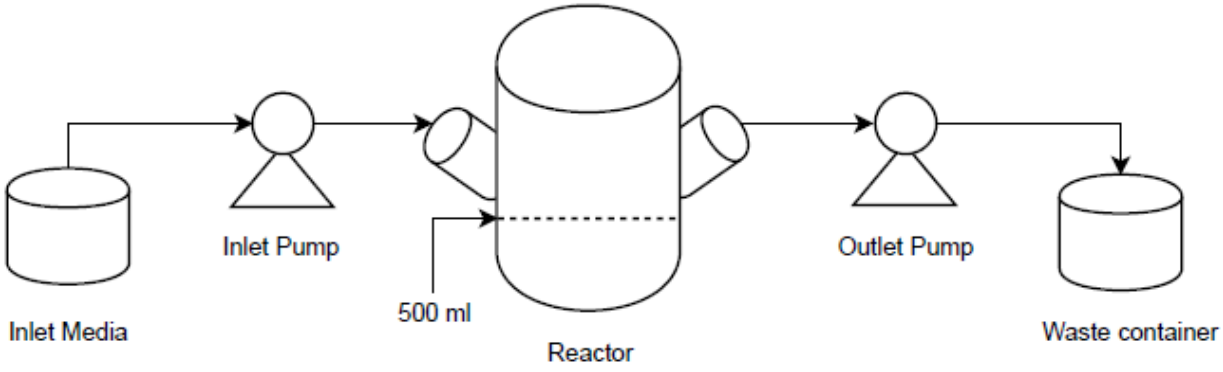

Fig 1. Diagram of the Bioreactor system.

The inlet and outlet pumps were run at the same flow rate to maintain constant volume in the reactor. 5 mL of glucose culture was used to inoculate the 500mL glycerol media in the reactor. The culture was allowed to grow for 24 hours before the pumps were started. Three dilution factors were tested sequentially: 0.072 h^{-1}, 0.12 h^{-1}, and 0.18 h^{-1}, which corresponded to flow rates of 0.6 mL/min, 1.0 mL/min, and 1.5 mL/min, respectively. These dilution factors were chosen based on literature values and limitations of the pumps available (Biebl, 2001). Samples were taken at the mathematically calculated 98% turnover time for each dilution factor and again two hours later. Table 1 contains the 98% turnover times and Equations 1, 2, and 3 in the appendix show the calculations. After 24 hours of growth, the pumps were started at 0.6 mL/min for the first dilution factor. After the second sample for that dilution factor was taken, the pump speed was increased to 1.0 mL/min, and then increased to 1.5 mL/min after the two samples were taken for that dilution factor. Each sample consisted of 1 mL for pH and OD analysis, 1mL for HPLC analysis, and in some cases 1mL for NMR analysis.

Table 1: Dilution Factors, Flow Rates, and 98% Turnover Times

Flow rate	DF (specific growth rate)	98% Turnover time
0.6 mL/min	0.072 h^{-1}	13.6 hours
1.0 mL/min	0.12 h^{-1}	8.17 hours
1.5 mL/min	0.18 h^{-1}	5.44 hours

High Performance Liquid Chromatography (HPLC) Analysis

HPLC media was prepared regularly by mixing 570 microliters of concentrated sulfuric acid in enough diH_2O to prepare two liters of media. Approximately 900mL of this media was degassed daily for use by the HPLC. An Aminex® HPX-87H Column for HPLC was attached to a Varian ProStar 355 RI detector which was used for quantitation. Samples taken from batch and continuous cultures were centrifuged at 10,000 X g for 10 minutes. The supernatant was filtered through syringe filtration then frozen overnight. Samples were thawed and 25 microliters of sample run through HPLC for a minimum of one hour. Each sample was run at least twice. If the readings between the two separate runs of the same sample differed by more than 5%, the sample was run a third time. A set of standard serial dilutions for each compound of interest was prepared and used to determine the linear relationship between area under each peak and concentration. The area under each peak for each sample was used to calculate the concentration of each compound in grams solute per liter solution.

OD and pH protocols

Culture samples taken for pH and OD were placed in 14mL Falcon tubes. The pH was measured using a pH meter. Once pH was determined, the sample was analyzed using Thermo-Genysys machine at 600nm and the absorbance recorded as OD.

Results and Discussion

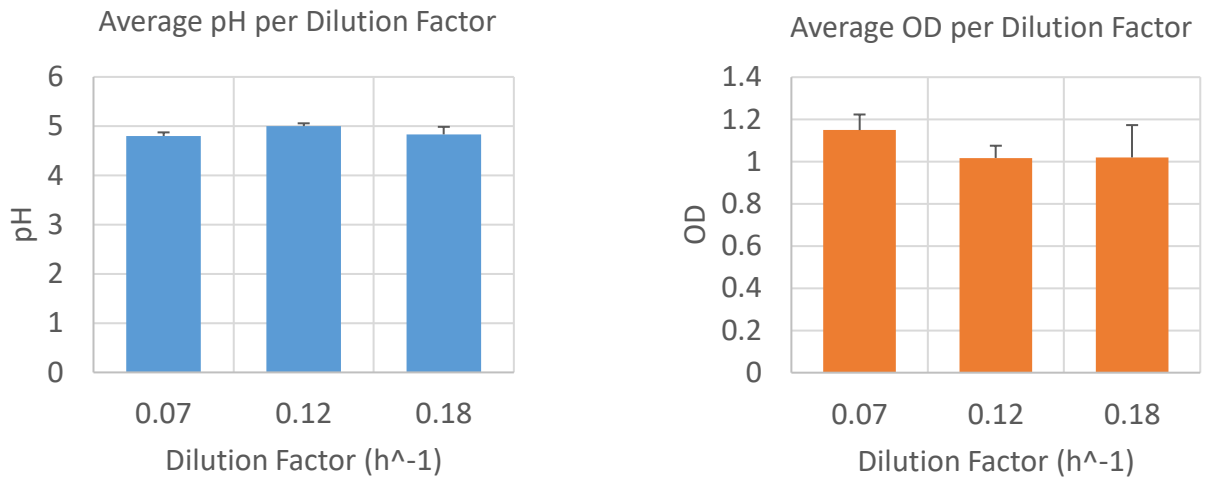

Fig 2: Average pH per Dilution Factor (left) contains the averaged results from all samples across three runs of the experiment described under continuous culture methods section. Average OD per Dilution Factor (right) contains averaged OD results from the same three runs of the experiment.

The OD measurements for all three runs of the continuous culture were averaged together and the results included in Fig 2. The OD remains reasonably consistent across all three runs and all three dilution factors, ranging between 1.0 and 1.15. The pH measurements were similarly averaged, and the pH found to be between 4.8 and 5.0 for all three dilution factors. In batch cultures, the pH typically drops to 4.5 and remains there. Note that uninoculated media has a pH between 6.5 and 6.7. The drop in pH results from fermentation of glycerol into acidic products. In continuous culture, these acidic products are diluted out by the outlet pump keeping the pH above 4.5.

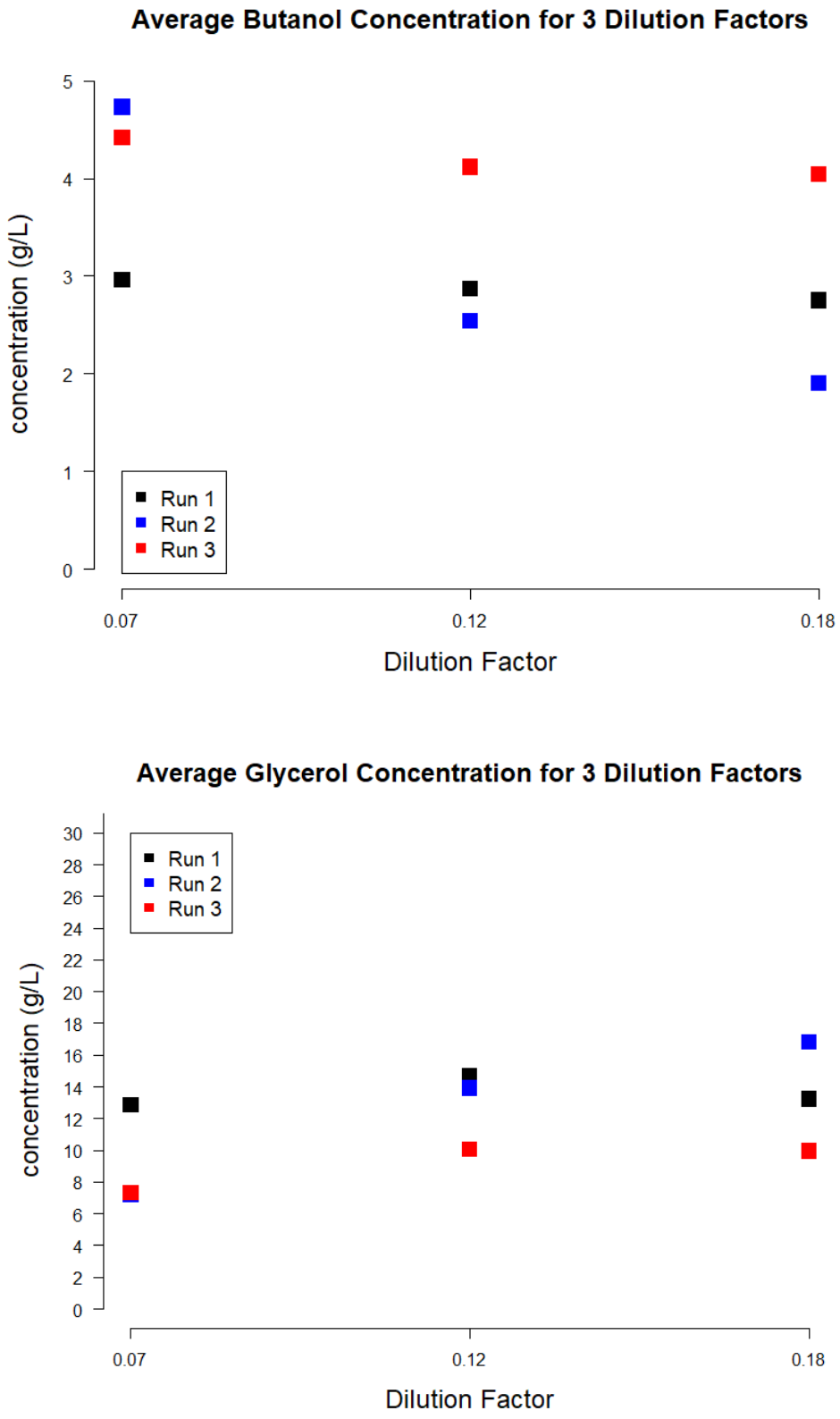

Fig 3: The average concentration of butanol (top) and average glycerol concentration (bottom) in the bioreactor at each dilution factor for all three runs of continuous culture. Each point is the result of the average between samples taken at the 98% turnover time and samples taken two hours later.

Three tests of the reactor yielded the averages reported in Figure 3 and in the appendix. At smaller dilution rates, more glycerol was consumed on average and more butanol was produced. However these trends are not consistent across runs of the reactor. There is a rough correlation between glycerol and butanol levels. Butanol levels are higher when glycerol levels are lower and vice versa. Due to this relationship, the product yield was used to provide a better analysis. Product yield is determined by dividing the mass of each product by the mass of glycerol consumed. Yield is a measure of the proportion of glycerol used to make each product. The amount of glycerol consumed was calculated by subtracting the concentration of glycerol present in the media from the concentration of glycerol in the inlet media at the same point in time.

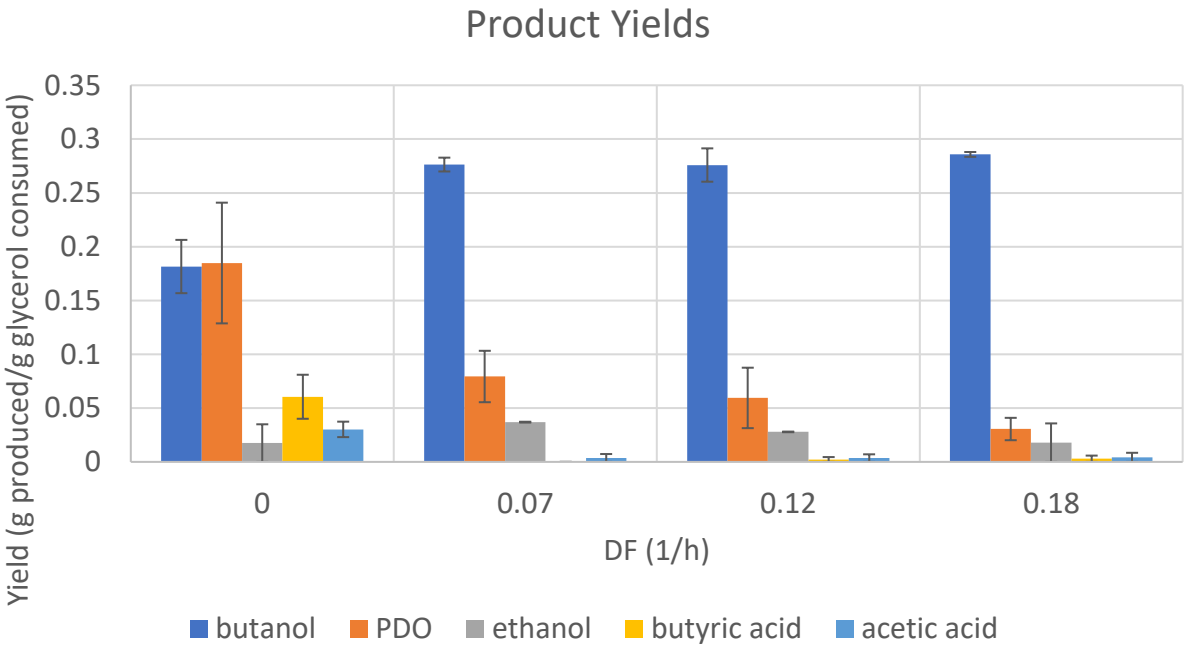

Fig 4. Product yield for each of 5 products measured as mass of product produced over mass of glycerol produced. Values are determined from the average of concentrations from three runs of the experiment described under continuous culture in the methods section of this report. DF = 0 column describes conditions after 24 hours of growth in 500mL batch reactor with no pumps and no flow into or out of reactor. The remaining three columns correspond to the three DFs in Table 1.

The PDO pathway regenerates NAD from $NADH_2$. The butanol pathway generates 2 $NADH_2$ and 1 ATP through breakdown of glycerol to pyruvate. Pyruvate is cleaved into carbon dioxide and acetyl to which coenzyme A is added, producing 1 NADH. This process mimics glycolysis in eukaryotic cells. Conversion of Acetyl CoA to butanol requires 4 $NADH_2$, resulting in a net loss of one $NADH_2$ and a net gain of 1 NAD and 1 ATP. Glycerol conversion to biomass requires NAD, so both the butanol and PDO pathways favor biomass formation (Venkataramanan, 2011). Figure 5 in the appendix shows the metabolic pathways of *Clostridia*. During batch culture, the quicker but less beneficial PDO pathway is relied on alongside the butanol pathway. Once the culture is switched to continuous, the PDO pathway is either reduced or shuts off completely and butanol production is favored. PDO levels reach a maximum yield right before the pumps start. If the pathway is shut off, then the PDO measured at higher dilution factors is left over from batch growth and has not yet been completely diluted out. If the pathway is still used, then PDO is produced at low levels. Shifting from batch to continuous favors butanol production so biomass generation and reproduction can increase. The extra ATP generated by the butanol pathway makes it more efficient than PDO which is important when growth is induced by increased dilution factors. In batch, the bacteria multiply but lack the external stimulation of dilution factors. Thus, they do not need to grow as efficiently in batch as in continuous culture. Once they are being diluted out, efficient growth becomes necessary for continued survival of the population. At higher dilution factors, butanol yield remains effectively constant. The yields of PDO and other minor products, however, decrease with increasing dilution factor. This suggests that at higher dilution factors, more glycerol is used for biomass production at the expense of PDO and minor products. Therefore, bacterial cells grow at higher

rates with higher dilution factors which fits expected results based on literature and confirms that no dilution factor tested exceeds the maximum growth rate for this species.

Conclusion

A continuous culture of *Clostridium pasteurianum* can be used to convert glycerol into butanol. Biomass production is stimulated by applied dilution factors which encourage the bacteria to grow at higher specific growth rates. This increased need for effective cell growth in turn promotes butanol production over PDO and other pathways. The additional ATP produced by butanol encourages growth and makes it a more efficient pathway than PDO. The yield of butanol is higher in continuous culture than in batch, but does not increase with increasing dilution factor. Further work will involve determining the optimal dilution factor for growth and conversion and testing a method of extracting butanol from the reactor for use as a fuel additive.

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Appendix

Table 2: Average pH and OD for Each Dilution Factor			
	DF 0.072 h^{-1}	DF 0.12 h^{-1}	DF 0.18 h^{-1}
pH	4.8	5.0	4.8
OD	1.15	1.02	1.02

Table 3: Average Glycerol and Butanol Concentrations for Each Dilution Factor							
		DF 0.072		DF 0.12		DF 0.18	
Time (hours)*	24	37.6	39.6	48.0	50.0	55.4	57.4
Butanol (g/L)	2.23	3.95	3.91	3.32	3.36	3.23	3.48
Glycerol (g/L)	15.65	9.10	10.76	12.87	12.47	13.32	13.86

*time since inoculation.

Dilution Factor Calculations:

Equation 1) $$DF\left(\frac{1}{h}\right) = \frac{Q\left(\frac{mL}{min}\right) \times 60\left(\frac{min}{h}\right)}{V(mL)}$$

Where DF is the dilution factor in 1/h, Q is the flow rate in mL/min, and V is the volume of the culture in the reactor. Dilution Factor is a function of flow rate per volume.

Equation 2) $$98\%\ time(h) = \frac{V(mL) \times 0.98}{Q\left(\frac{mL}{min}\right) \times 60\left(\frac{min}{h}\right)}$$

The 98% turnover time is the time required for 98% of the volume in the reactor to be replaced.

Equation 3) $$98\%\ time(h) = \frac{0.98}{DF\left(\frac{1}{h}\right)}$$

When the equations 1 and 2 are combined, equation 3 is produced where the 98% turnover time is a direct function of the dilution factor.

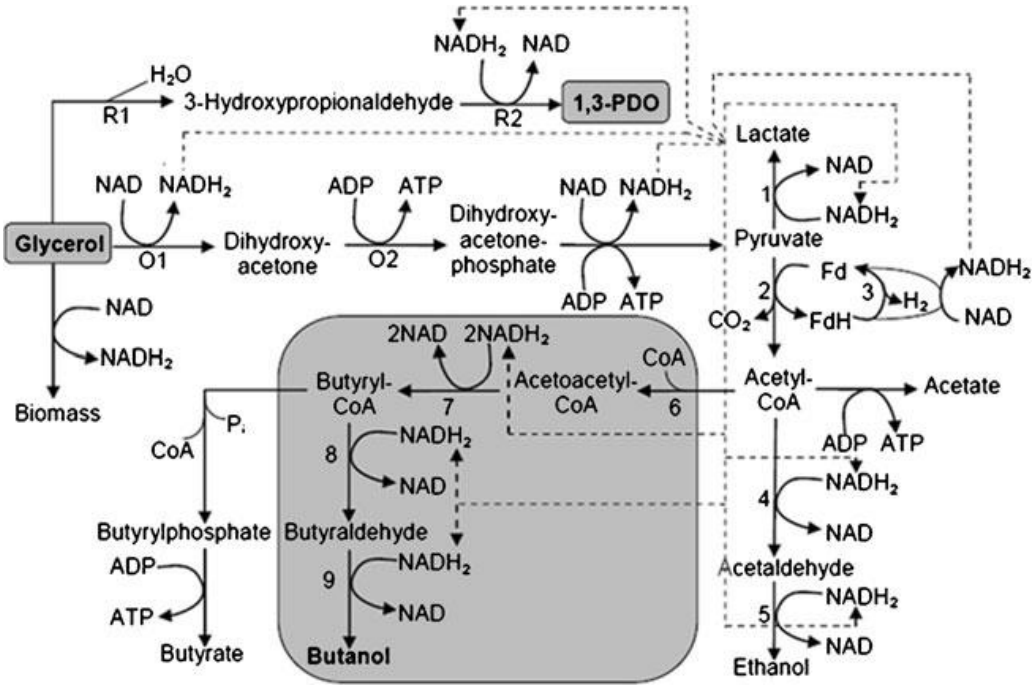

Fig 5: The metabolic pathway of glycerol in Clostridia. The dashed lines represent the electron flow. R1 glycerol dehydratase, R2 PDO dehydrogenase, O1 glycerol dehydrogenase, O2 dihydroxyacetone kinase, 1 lactate dehydrogenase, 2 pyruvate–ferredoxin oxidoreductase, 3 hydrogenase, 4 acetaldehyde dehydrogenase, 5 ethanol dehydrogenase, 6 thiolase, 7 butyryl-CoA dehydrogenase, 8 butyraldehyde dehydrogenase, 9 butanol dehydrogenase (adapted from Taconi et. al. 2007). The boxed area indicates the solventogenesis pathway of butanol formation, which is activated at lower pH obtained after the acidogenesis phase. Figure and description from Venkataramanan, 2011