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Improved Fermentation Design and Screening Devices for Biobutanol Production

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Abstract

A worldwide increase in demand for renewable fuels has revived interest in fermentatively produced butanol. However, butanol fermentation suffers from low product yields and productivity. The work presented in this thesis addresses part of these research and development needs at three levels: innovative fermentation process design; genetic manipulation for strain enhancement; and the development of a new tool for anaerobic process characterization and optimization.

Product yield could be increased through traditional fermentation engineering. Co-fermentation of butyric acid with glycerol increased the butanol yield from 0.45 mol/mol (mols C in butanol / mol C in substrates) to 0.51 mol/mol. In building on this concept, and capitalizing on the unique metabolism of *Clostridium pasteurianum*, an optimized glycerol to molasses (co-substrate) ratio was identified. *C. pasteurianum* produces butyric acid from the molasses sugars for later re-assimilation when consuming glycerol, resulting in a final product yield of 0.48 mol/mol.

A sample of *C. pasteurianum* putative mutants was obtained from a collaborating researcher, who had generated the sample using random mutagenesis techniques. The growth and product profiles were characterized, displaying higher growth rates and an altered product profile when compared to the wild-type strain. The DNA was isolated and sequenced, which confirmed that it is a novel mutant population, and will allow for directed mutagenesis techniques to be used to replicate and characterize the mutations.

Finally, it was found that the gas production of the fermentation yielded valuable data only observed at the reactor scale, and not during screening in shake flasks. To remedy this gap in data acquisition, a novel screening device was developed which collected off gas data from multiple shake flasks operating in parallel. The fermentations conducted at the shake flask scale matched previously reported results at the reactor level.

In conclusion, this thesis shows possible ways to increase butanol yields through fermentation engineering, and to increase butanol production rates through strain development. It further led to development of a highly flexible screening device suitable to further optimization of this or other anaerobic fermentation processes.

Keywords

Fermentation, butanol, optimization, *Clostridium*, screening, anaerobic, shake-flask, mutation

Summary for Lay Audience

While the most commonly known biofuel is currently ethanol, research has been ongoing to develop ways to produce another biofuel known as butanol. Butanol is similar to ethanol in that it is also an alcohol; however, butanol is a far more suitable biofuel for use with current engines and fuel infrastructure. In order to produce butanol in an environmentally friendly fashion, most research has turned to using bacteria to convert inexpensive wastes through fermentation. This approach comes with several obstacles though, as butanol fermentations will generally be slow, taking a long time to convert the waste to butanol, and require extremely large amounts of raw material in order to make the process worthwhile.

The research conducted for this thesis addressed these shortcomings in two different ways: increasing how much butanol can be made from the wastes by adding specific compounds to the fermentation, and by using mutant strains of the bacteria that can produce butanol at a faster rate. For the first, an acid was added in small amounts to the fermentation, which had the effect of "pushing" the bacteria towards making more butanol and less by-products. Butyric acid from various sources was used to find a possible inexpensive source for the process. Regardless of the source, the presence of the acid increased how much butanol was produced. For the second shortcoming, a sample of bacteria was obtained from a collaborating researcher, who attempted to mutate the bacteria to create a population with faster growth in the fermentation. To prove these were indeed mutants, DNA sequencing was done to pinpoint where the mutations happened, and attempt to explain why they improved the bacteria.

For both of these approaches, many test fermentations were conducted. To help with this, a new device was created that allowed for multiple tests to be conducted simultaneously, using small flasks containing a small amount of materials while still collecting large amounts of data. This prevented the need for large, expensive tests to be done one-at-a-time, and greatly increased the rate at which tests were conducted.

Co-authorship Statement

This thesis was completed under the supervision of Dr. Lars Rehmann. Parts of the research were conducted in collaboration with the BioVT chair at the RWTH-Aachen in Aachen, Germany, where I was able to spend 14 months to pursue the research outlined in Chapter 4. While in Aachen, I worked under the supervision of Dr. Rehmann, as well as Dr. Jochen Büchs and Dr. Lars Regestein. The extent of the collaboration for all chapters is stated below.

Chapter 2

Title: Increasing selectivity for butanol in *Clostridium pasteurianum* fermentations using butyric acid directly and in a dual substrate fermentation

Current Status: In preparation for submission to Fermentation

Garret Munch: Designed and conducted experiments, conducted analysis of samples, interpretation of data, and writing of manuscript.

Justice Mittler: Assisted with fermentation preparation, conducted preliminary fermentations and generation of xylose-derived butyric acid

Dr. Lars Rehmann: Oversaw project and funding, assisted in statistical analysis, data interpretation, figure generation, drafting/editing of manuscript. Acting as corresponding author

Chapter 3

Title: Characterization and sequencing of highly productive *Clostridium pasteurianum* mutant cultures.

Garret Munch: Designed and conducted experiments, extracted and sequenced DNA of mutant population, interpretation of data, and writing of results for report.

Dr. Erin Johnson: Generated the mutant population and provided samples for characterization and DNA extraction and sequencing, provided training, expert advice, and guidance with fermentations and equipment.

Dr. Lars Rehmann: Oversaw project and funding, assisted in data interpretation and analysis, figure generation, and drafting/editing of chapter.

Chapter 4

Title: Online measurement of CO_2 and total gas production in parallel anaerobic shake flask cultivations

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Garret Munch: Established anaerobic work capabilities at the BioVT, converted device for anaerobic work, adapted MATLAB code for HTR calculation, designed experiments, processed and interpreted data, wrote manuscript

Andreas Schulte: Prepared initial MATLAB code for calculation of CTR, aided with anaRAMOS design

Marcel Mann: Conducted confirmation experiments with anaRAMOS

Robert Dinger: Prepared long-form calculation of CTR and HTR for reviewers

Dr. Lars Regestein: Was instrumental in coordinating the collaboration, secured funding, provided technical and professional advice, oversaw revisions to manuscript, aided with experimental design.

Dr. Lars Rehmann: Oversaw collaboration and securing funding, as well as technical and professional advice. Produced Figures for publication, and gave revisions and edits for manuscript.

Dr. Jochen Büchs: Gave technical advice regarding the anaRAMOS, oversaw the BioVT chair and my time in Aachen, proposed experimental design, gave revisions for manuscript drafts.

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I don’t even know where to begin. This is somehow the most difficult part of this document to write. It is a massive understatement to say that I would not have been able to do this on my own.

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List of Abbreviations used in text:

1,3 PDO – 1,3 Propanediol

OD – Optical Density

PI – Post-Inoculation

RAMOS – Respiratory Activity Monitoring System

anaRAMOS – Anaerobic Respiratory Activity Monitoring System

Nomenclature, units of constants and variables used in text:

RCY – Relative Carbon Yield (mol carbon in butanol / mol carbon in consumed substrate)

CTR – Carbon Dioxide Transfer Rate (mmol CO_2 L^{-1} h^{-1})

HTR – Hydrogen Transfer Rate (mmol H_2 L^{-1} h^{-1})

TGTR – Total Gas Transfer Rate (mmol gasses L^{-1} h^{-1})

RGTR – Remaining Gas Transfer Rate (mmol gasses L^{-1} h^{-1})

V_g – Volume of headspace in the anaRAMOS flask (L)

V_l – Volume of process fluid being used in fermentation (L)

R – Ideal gas constant (8.3145 J mol^{-1} K^{-1})

T – Temperature of fermentation (^oC)

Chapter 1

1) Introduction

1.1) Background

The overwhelming majority of current transportation infrastructure is dependent on petroleum-based fuels (Fulton *et al.* 2015). Advancements in battery technology have made electric vehicles a more viable option in the future, but in the intermediate timeframe a solution to move away from petroleum-based transportation fuels being used in internal combustion engines must be found. Currently, the most widely used biofuel is ethanol, for use blended with gasoline (Azadi *et al.* 2017). However, butanol is a superior biofuel when compared to ethanol, and can be produced via fermentation using inexpensive feedstocks such as glycerol and the microorganism *Clostridium pasteurianum* (Gautam and Martin 2000; Biebl 2001). Fermentation of glycerol to butanol could serve as an effective method for butanol production for use either as a platform chemical or biofuel.

Fermentative production of butanol was traditionally conducted using sugars as the carbon source, but even with switching to glycerol as a substrate, the process still suffers from several complications, primarily low productivity and unwanted by-products reducing the butanol yield. One strategy to overcome these complications is the addition of butyric acid directly to the fermentation medium, resulting in increased metabolic flux towards butanol production (Regestein *et al.* 2015). This butyric acid could be chemically pure, or in an effort to reduce costs, butyric acid could be generated by a different fermentative organism also using an inexpensive carbon source (Zhu and Yang 2004). This could allow for increased productivity and yield while avoiding costly pure substrates and chemicals.

An alternate strategy that could achieve similar goals would be to utilize the natural metabolism of *C. pasteurianum* in a dual-substrate fermentation, first producing butyric acid from one substrate subsequently using that butyric acid when fermenting glycerol (Sabra *et al.* 2014; Kao *et al.* 2013). When fermenting sugars, *C. pasteurianum* produces butyric acid as the main product rather than butanol. It is possible to have a small amount of sugars to introduce butyric acid into the fermentation medium, which is then subsequently taken up by the cells (Sabra *et al.* 2014; Kao *et al.* 2013). This strategy would eliminate the need to use

chemically pure butyric acid, or even the requirement for introduction of chemicals to a process already under way, thus reducing the cost and operational complexity of butanol fermentations while raising the butanol yield and productivity. However, such a system would require a delicate ratio between the substrates in order to prevent excess butyric acid from being produced and inhibiting the later fermentation.

The interest in butanol fermentation with *C. pasteurianum* has resulted in mutagenic work to be undertaken, either using random mutagenesis and selection, or directed mutagenesis to target genes within the glycerol-butanol metabolic pathway (Pyne, Sokolenko, *et al.* 2016; Gallardo, Alves, and Rodrigues 2017; T. Ø. Jensen *et al.* 2012). Random mutagenesis can produce highly productive strains with a mutation in a metabolic pathway previously not thought to have a significant effect on the product profile (Sandoval *et al.* 2015). Directed mutagenesis allows for specific investigation of the consequences of knocking out genes along the glycerol-butanol pathway, as well as confirmation of effects seen in randomly generated mutants (Pyne, Sokolenko, *et al.* 2016).

Investigations with the goal of optimization or screening fermentation conditions will require a high number of fermentations to be conducted to determine conditions with highest yield and productivity. To this end, many initial screening experiments will be conduced in shake flasks, to limit the amount of material required while allowing ease of handling. However, this also limits the amount of online monitoring possible, notably in the gas phase (Payne, Davison, and Tate 1990). Online off-gas analysis allows for directly following the fermentation progress, and often will reflect fermentation conditions such as substrate or product inhibition, oxygen limitation, substrate limitation (Anderlei *et al.* 2004). Unfortunately, the majority of small scale off gas analysis systems are not suited for anerobic fermentations like those using *C. pasteurianum*. This lack of online signals results in certain phenomena not being detected until fermentations have been scaled up to the reactor scale, or missed completely if the fermentation is not scaled up.

1.2) Literature Review

1.2.1) Butanol as a biofuel and platform chemical

Interest in producing chemicals using renewable resources and environmentally friendly methods has increased dramatically in recent years, largely due to concerns regarding climate change and volatility of petroleum products (Ranjan and Moholkar 2012). Butanol is both a promising biofuel alternative and a valuable industrial chemical. As a biofuel, butanol has many superior qualities over the current most common biofuel, ethanol. Butanol is a 4-carbon chain alcohol, which gives a higher heating value over ethanol, a 2-carbon alcohol. As well, butanol is less volatile and corrosive when compared to ethanol, and can be used in both gasoline and diesel (30% butanol 70% diesel blend) powered vehicles without modification to the engine (Campos-Fernández *et al.* 2012; Rakopoulos *et al.* 2010; Dernotte *et al.* 2010).

Butanol is also used as an industrial solvent and platform chemical, allowing for butanol to be sold at a higher price than when sold as a fuel (Durre 2008). Butanol is a precursor for butyl acrylate, used in adhesives and paper and textile finishes to the scale that in 2008 almost half of butanol produced worldwide was converted into acrylates (Zeng, Kuo, and Chien 2006; Durre 2008). Other industrial uses for butanol range from polymer production for surface coatings to flocculants (Durre 2008).

The main industrial method for production of butanol has evolved since production began. Originally conducted via the Weizmann process in the early 1900's, the process converted starch substrates into acetone and butanol using *Clostridia acetobutylicum*, known as Acetone-Butanol-Ethanol (ABE) fermentation (Weizmann and Rosenfeld 1937). The Weizmann process was second only to ethanol fermentation in terms of industrial importance at the beginning of the 20th century. However, the availability of cheap petrochemical by-products allowed for chemical methods to become the economically superior method of butanol production (Rose 1961). Initially, condensation of acetaldehyde, and subsequent dehydration/hydration steps was used to produce butanol. However, development of the oxo synthesis, in which propene is upgraded to butyrlaldehyde (or isobutyrlaldehyde), and subsequently hydrogenated to butanol (and isobutanol), allowed for this process to become dominant for butanol production (Uyttebroek, Van Hecke, and Vanbroekhoven 2015). In

previous decades, the volatility of the petrochemical market has led to a renewed interest in the biological production of butanol, as the price of butanol is directly impacted by the price of crude oil (Green 2011b). In 2012, the worldwide market was approximately 350 million gallons of butanol sold per year, with the international price being approximately $4 per gallon (Ranjan and Moholkar 2012). The renewed interest in biological butanol production has resulted in multiple areas of study being developed to increase the productivity and selectivity of butanol fermentation processes (Ranjan and Moholkar 2012; Kießlich *et al.* 2017; Groeger *et al.* 2017). In addition, China has heavily invested in producing butanol by biological means, with several plants having opened in order to attempt to reach 1 million tons butanol produced per year (Ni and Sun 2009). Mainly, research explores how to overcome ABE fermentation complications like high substrate costs and low fermentative productivity.

1.2.2) Traditional Acetone-Butanol-Ethanol fermentations

As mentioned in Section 1.2.1, butanol has historically been produced using ABE fermentation. Organisms such as *C. beirerinckii,* and *C. saccharobutyrilcum* have been used in addition to *C. acetobutylicum* in ABE fermentation processes. However, ABE fermentations suffer from major obstacles for high production such as product (butanol) inhibition of the fermentative organisms, and displaying a biphasic growth patterns that limit the range of fermentation strategies which can be employed (Zhang *et al.* 2013). Biphasic growth begins with an initial growth phase which is acidogenic and produces compounds such as acetic, butyric, and lactic acid. The second growth phase is solventogenic, in which these acids are assimilated into the cells and the main products are butanol, ethanol, and acetone (Buendia-Kandia *et al.* 2018). The eventual drop in pH (pH < 5) from the acid production signals the beginning of the solventogenic phase. This biphasic growth creates novel difficulties when attempting to operate a continuous fermentation, and can generally limit the use of ABE fermentations to batch or fed-batch operations unless the cells have been fixed to a support (Friedl, Qureshi, and Maddox 1991). Most plants operate in a semi-continuous manner, using several large fermenters simultaneously. At the end of the fermentation, the products are distilled to recover acetone, butanol, and ethanol in an approximately 3:6:1 ratio (Green 2011b).

The second major obstacle for fermentatively produced butanol is product inhibition due to the toxicity of the butanol itself to the microorganism (Bowles and Ellefson 1985). Due to this, most fermentations without active butanol removal reach close to 13 g/L butanol as a final concentration. At higher butanol concentrations, cells lose the ability to uptake sugars from the medium (Jones and Woods 1986). Due to its hydrophobic nature, butanol increases membrane fluidity, which destabilizes it and disrupts the effectiveness of the membrane bound proteins (Jones and Woods 1986).

These two major obstacles for ABE fermentation require process modifications to overcome which can increase the overall cost of the processes, and as a result, using an inexpensive carbon feedstock is critical to keep costs low.

1.2.3) Butanol Fermentations on cellulose derived sugars

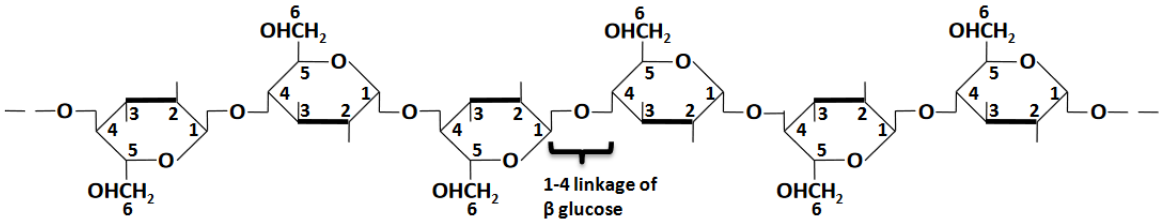

Figure 1.1: Molecular structure of cellulose (adapted from O'Sullivan 1997)

Considerable research has been done, and continues to be done, on developing methods which utilize inexpensive lignocellulosic-derived sugars as the carbon source for ABE fermentations (Jang *et al.* 2012; Gottumukkala, Haigh, and Görgens 2017; Nasib Qureshi and Ezeji 2008). Deriving sugars from the lignocellulosic biomass has proven difficult, as the products are mixed with inhibitory compounds, as well as difficulty splitting the β1-4 link between glucose monomers found in cellulose (Figure 1.1). A brief overview of some work using sugars extracted from various biomass substrates, using various methods of extraction and further treated by enzymatic saccharification summarized in Table 1.1.

As can be seen in Table 1.1, the results of ABE fermentations using lignocellulosic sources can vary significantly in productivity and butanol yield. For example, Wang and Chen matched the sugar composition extracted from corn stover with chemically pure sugars, and were able to compare the fermentation products between the lignocellulosic sugars and the chemically pure sugars (Wang and Chen 2011). When fermentations were conducted using *C. acetobutylicum* and the model solution (30 g/L dextrose, 15 g/L xylose, and 5 g/L cellobiose) the fermentation produced 10.9 g/L total solvents and 4.13 g/L total acids (Wang and Chen 2011). However, when the hydrolysate mixture was used, the presence of the lignin compounds produced an inhibitory effect on the organism, slowing growth and reducing the solvent production in favour of acid production. At the conclusion of the fermentation, significantly less of the sugars were consumed (only 62% of dextrose was consumed), and only 3.71 g/L total solvents were produced, with 7.25 g/L acids.

Table 1.1: Sample of ABE fermentations using sugars obtained from lignocellulosic sources with various pretreatments

Organism	Substrate	Biomass Pretreatment	Total Sugars (g/L)	Final butanol concentration (g/L)	Solvent Productivity (g/L/h)	Reference
C. beijerinckii P260	Barley Straw	Dilute H_2SO_4	58.8	4.0	0.1	(Nasib Qureshi, Saha, Dien, *et al.* 2010)
C. beijerinckii P260	Switchgrass	Dulute H_2SO_4	60.0	0.97	0.01	(Nasib Qureshi, Saha, Hector, *et al.* 2010)
C. saccharobutylicum DSM 13864	Corncobs	Dilute NaOH / autoclaving	55.3	12.36	0.34	(Gao and Rehmann 2014)
C. saccharoperbutylacetonicum N1-4	Eucalyptus	Steam explosion	48.9	8.16	0.07	(Zheng *et al.* 2015)
C. acetobutylicum SE-1	Corncob	Wet Disk Milling	39.7	9.0	0.13	(Zhang *et al.* 2013)
C. *acetobutylicum* ATCC 824	Corn Stover	Steam Explosion	53.0	0.36	0.005	(Wang and Chen 2011)

The pretreatment of the lignocellulosic biomass can result in inhibitory compounds being produced, resulting in significant reduction in productivity and growth rates (Gao *et al.* 2014). These inhibitors are generally broken into two categories, those from the degradation of carbohydrates (furan aldehydes, aliphatic acids) and those from lignin (phenolic compounds) (Jönsson, Alriksson, and Nilvebrant 2013). These inhibitors and their effects continue to be a major obstacle for ABE fermentations using sugars from lignocellulosic biomass sources (Baral and Shah 2014; Yujia Jiang *et al.* 2019).

While utilizing sugars from biomass sources allows for use of inexpensive feedstocks, the variability of lignocellulosic biomass requires several expensive and time-consuming processing steps, which limits the feasibility of these processes. In addition, the presence of inhibitory compounds resulting from the processing steps can have unpredictable effects (N Qureshi *et al.* 2012; Baral and Shah 2014; Jönsson, Alriksson, and Nilvebrant 2013).

Research is continuing into addressing the complications of using sugars derived from lignocellulosic biomass, and work is also being conducted into finding alternate inexpensive carbon sources for butanol fermentations.

1.2.4) Butanol fermentation from glycerol using *Clostridium pasteurianum*

A carbon source which is becoming increasingly abundant and has been used successfully for fermentations is glycerol (Ciriminna *et al.* 2014; D. T. Johnson and Taconi 2007). The increase in worldwide biodiesel production has inevitably led to an increase in the availability of the main by-product of waste glycerol. Glycerol is produced as a by-product from the transesterification of fatty acids in biodiesel production, accounting for 10% wt of the total biodiesel produced (Figure 1.2) (Ziyai *et al.* 2019). However, this glycerol, often referred to as either ‘waste glycerol’ or ‘biodiesel-derived waste glycerol’ contains impurities from the biodiesel production process that are costly to remove. The saturation of the market with this waste glycerol has impacted the prices of chemically pure (or ‘technical grade’) glycerol as well, making purifying the waste glycerol a revenue negative stream (Ziyai *et al.* 2019). In 2012, it was estimated that technical grade glycerol sold for approximately $0.20/lb, and biodiesel-derived waste glycerol for $0.02 – 0.05/lb, with the result that direct

combustion was one of the more economically viable options for the waste glycerol produced (Roberts 2012).

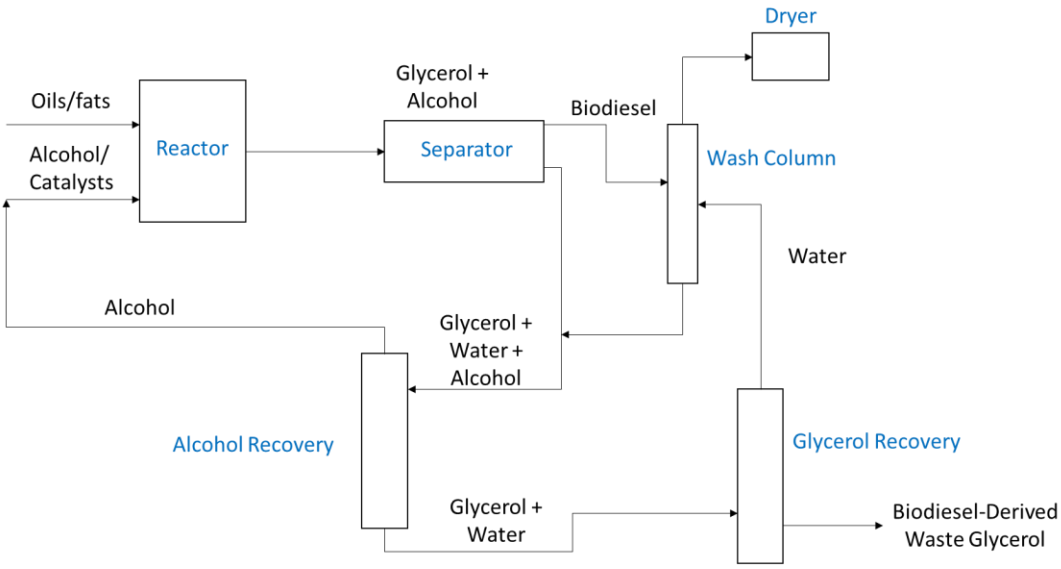

Figure 1.2: Flow diagram overview of a standard conversion of oil and fats to biodiesel

Many organisms used in ABE fermentations are unable to utilize glycerol as the sole carbon source for butanol production, which has led to a considerable amount of research into organisms capable of metabolizing glycerol to valuable products (Sestric *et al.* 2014; Munch *et al.* 2015; Dabrock, Bahl, and Gottschalk 1992; Biebl 2001). The ability to metabolize glycerol as the sole carbon source is found in multiple anaerobic organisms, including *Klebsiella pneumoniae* and *K. oxytoca*, *Enterboacter aerogenes*, and several species within the *Clostridia* genus (Petrov and Stoyanov 2012; Metsoviti *et al.* 2013; Chatzifragkou *et al.* 2011; Yadav *et al.* 2014). These glycerol metabolizing organisms also share several proteins with high degrees of similarity (Pyne, Liu, *et al.* 2016). As glycerol is a small, uncharged molecule its passage through the cell membrane can happen passively, though *Clostridia* species contain the gene for an integral membrane protein which can selectively diffuse glycerol into the cell (glycerol facilitator (GlpF)) (Fu *et al.* 2000).

In *C. pasteurianum*, glycerol is among many substrates that can be metabolized (Mitchell *et al.* 1987). The substrate has a significant effect on the products of the fermentation. In the case of glycerol, it can be metabolized both oxidatively and reductively, while not displaying any biphasic growth patterns typical of ABE fermentations (da Silva, Mack, and Contiero 2009; Pyne, Liu, *et al.* 2016). The reductive pathway is a conversion of glycerol to 1,3 propanediol (1,3 PDO), which is believed to serve as a method for balancing the reducing equivalents within the cell (Pyne, Liu, *et al.* 2016). While the central metabolism is redox-neutral, glycerol is more reduced than cell mass, leading to an imbalance. The reductive metabolic pathway serves to consume excess NADH and allow the cell to maintain redox homeostasis (Pyne, Liu, *et al.* 2016). When the genes required for the reduction of glycerol to 1,3 PDO were knocked out, the resulting mutant cultures were unable to grow in minimal medium with only glycerol as the carbon source (Schwarz *et al.* 2017).

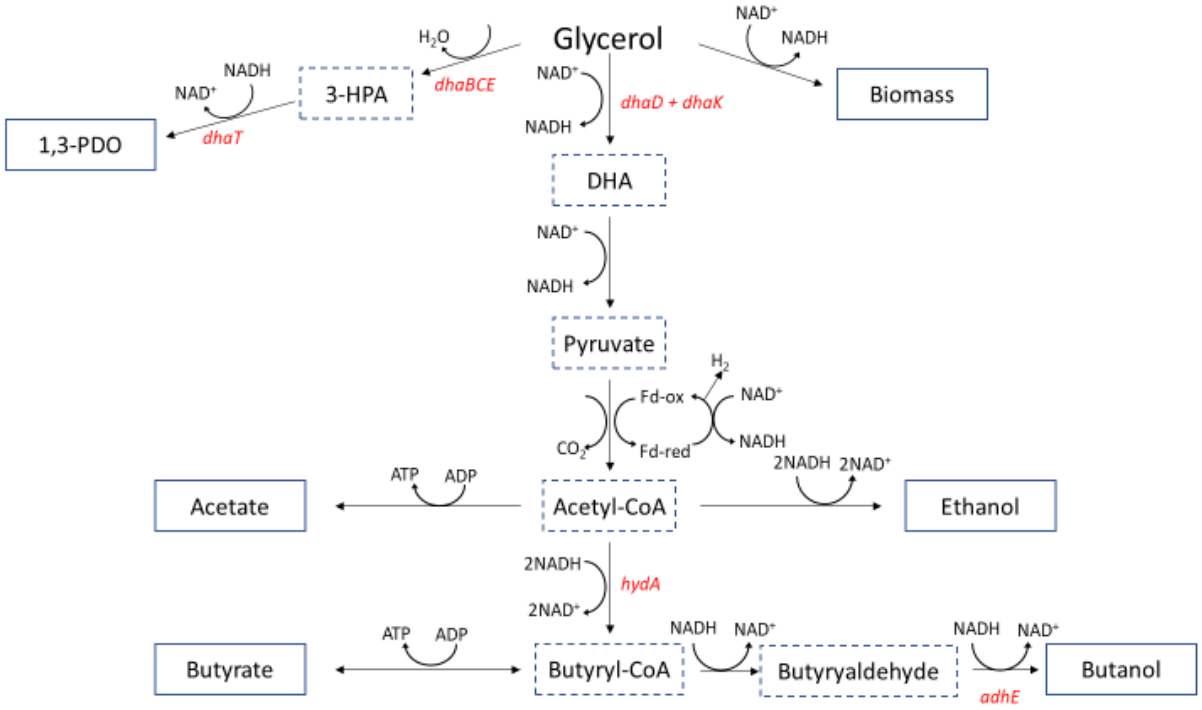

Figure 1.3: Overview of glycerol metabolism in *Clostridium pasteurianum*. Text in red highlights genes of interest controlling aspects of the metabolism. 3-HPA: 3-hydroxypropionaldehyde. 1,3-PDO: 1,3-propanediol. DHA: dihydroxyacetone. (Figure adapted from (Gallardo, Alves, and Rodrigues 2017) and (E. Johnson *et al.* 2016))

The oxidative metabolism of glycerol by *C. pasteurianum* initially converts the glycerol to CO_2, H_2, reducing equivalents (NADH), and pyruvate. Following the conversion to pyruvate, the metabolism becomes highly branched and able to produce several different alcohols and acids, depending on the fermentation conditions (Moon *et al.* 2011a). Under most circumstances, the main product is butanol, though yield can vary depending on fermentation conditions. Variation of the iron, the nitrogen source, and the amount of yeast extract can favour 1,3-PDO over butanol production (Moon *et al.* 2011a; Dabrock, Bahl, and Gottschalk 1992). Initial pH of the medium was also found to be an important condition in pH-uncontrolled batch studies, while the process pH during pH-controlled studies was found to have a significant effect on product yields (Khanna, Goyal, and Moholkar 2013; Erin Johnson and Rehmann 2016). This high degree of variation allows optimization for each process using a different carbon source, medium, and fermentation strategy (Sarchami, Johnson, and Rehmann 2016b; Sarchami and Rehmann 2014).

1.2.5) Economics of butanol and glycerol over time

Worldwide demand for butanol has been increasing steadily the past several years, with a corresponding increase in the selling price (N-Butanol Market by Application and Region - Global Forecast to 2022). The overall market is expected to reach a value of 5.58 billion USD by 2022, largely driven by expansion of industries which use butanol as a solvent or intermediate substrate (construction, textile, agrochemical, or pharmaceutical industries). While the price of butanol is largely tied to the petroleum market, and the economy overall, the price has recovered from economic fluctuations such as the 2008 Great Recession to return to a high selling price (Figure 1.4). Diversification of butanol use to include the increasing demand for biofuels and ‘green’ solvents will further expand the market, and could result in more processes becoming economically viable due to the higher and more stable market and demand.

In 2015, it was calculated that while the petrochemical production route was less expensive ($1.52/kg n-butanol via upgrading petrochemical products vs. $1.87/kg n-butanol produced via ABE fermentation), the potential feedstocks and carbon utilization yield for ABE

fermentation was far superior to petrochemical feedstocks (Yu Jiang *et al.* 2015). While the instability of the petroleum market could allow for periods of time when petrochemical precursors are reduced in price, utilization of an abundant and inexpensive feedstock could allow fermentatively produced butanol to compete with petrochemical means, regardless of the condition of the economy or the price of crude oil.

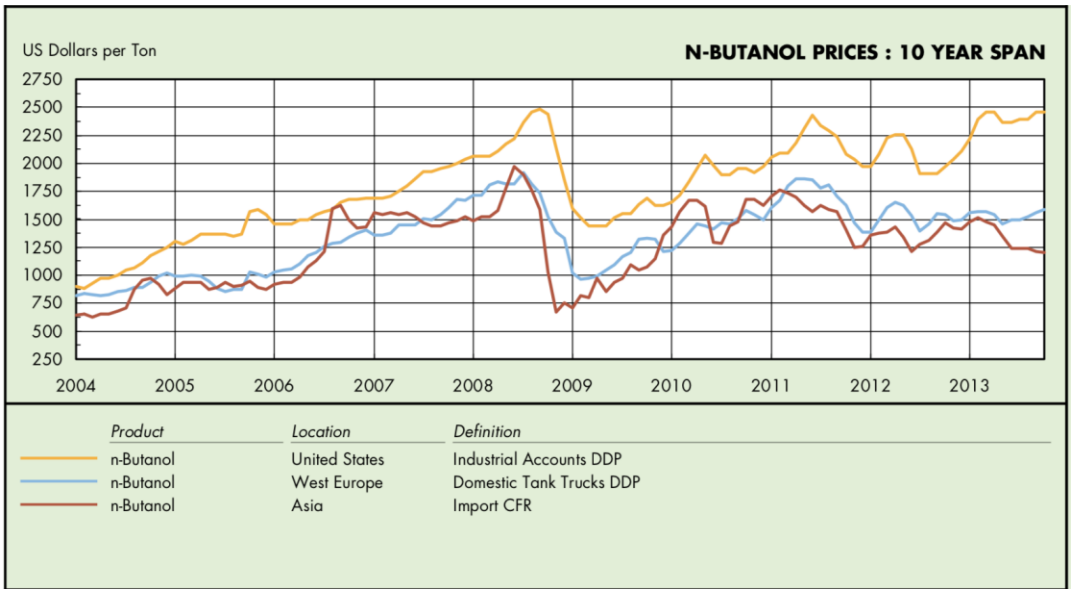

Figure 1.4: Prices of butanol over a ten-year span encompassing the Great Recession of 2008 and subsequent recovery (Figure obtained online: Tecnon OrbiChem – Chemical market insight and foresight; n-butanol).

As mentioned in Section 1.2.4, the price of glycerol, as well as the availability, has made it an attractive possible carbon source for butanol production via fermentation. As can be seen in Figure 1.5, the global glycerol production has followed similar patterns as the global biodiesel production, resulting in a significant increase (Nomanbhay, Hussein, and Ong 2018; *OECD-FAO Agricultural Outlook 2015* 2015). As a result of this excess of glycerol, the price steadily decreased over time. A brief plateau in the drop of glycerol prices is observed corresponding to the 2008 economic recession mirrors a decrease observed in butanol prices; however, the subsequent increase in production furthered the decrease in

glycerol prices. The recovery of butanol prices, and the further decrease of glycerol prices, following the 2008 recession indicates that this specific market pairing (butanol from glycerol) could be well-suited for stability despite disruption of external markets.

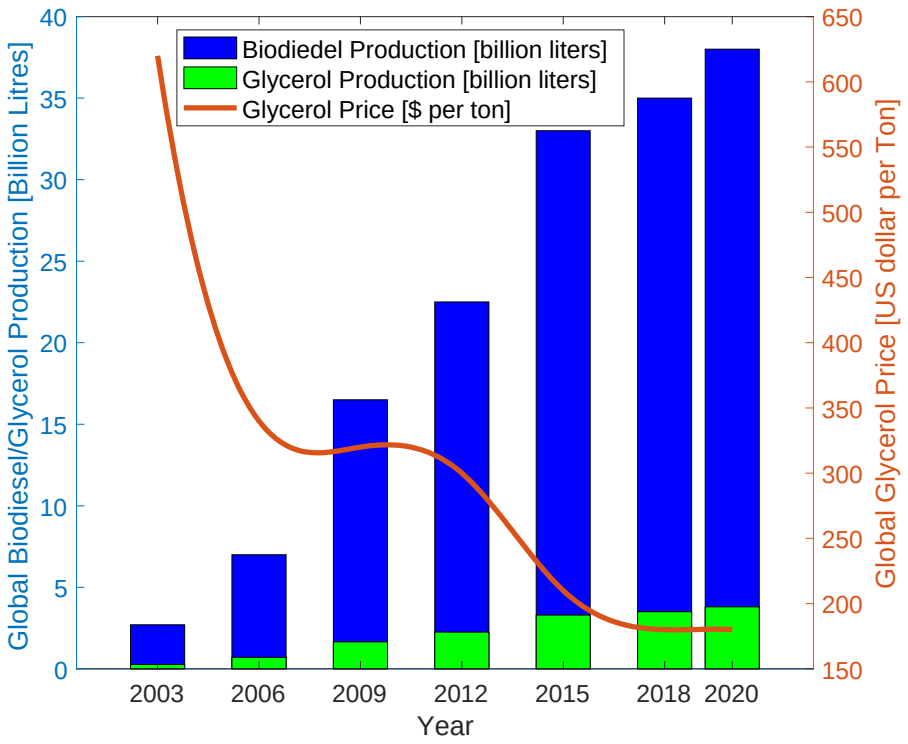

Figure 1.5: Production of biodiesel and glycerol over time, compared with the price of glycerol over the same period (Nomanbhay, Hussein, and Ong 2018; *OECD-FAO Agricultural Outlook 2015*).

Large scale production of butanol via ABE fermentation has been largely conducted in China, where the 2008 economic downturn resulted in the closure of several of the existing butanol production plants using ABE fermentation (Yu Jiang *et al.* 2015). However, the butanol market recovered, and existing plants were re-fitted and resumed production, and new butanol production plants were created outside of China. Two such plants which were proposed and begun in the early-2010's, when crude oil had reached over $100/barrel, were The Saudi Butanol Company (Saudi Arabia) and Butamax (North America) (Kujawska *et al.* 2015). These plants were planned to capitalize on the high price of petroleum and the high demand for butanol in China. However, only a few years following construction, these

companies are no longer active in butanol production after their parent companies decided against continuing in the butanol market, as the high production costs prevented the process from becoming economically viable.

An advantage afforded to ABE fermentations using glycerol as the carbon source could be the proximity to existing infrastructure. Glycerol is currently largely produced as a by-product for biodiesel production, meaning that an ABE fermentation plant could be designed as an 'add-on' to the existing biodiesel plant. Such an arrangement would eliminate transportation costs associated with gathering the carbon feedstock (as is the case for ABE plants using first- or second-generation cellulosic biomass), and allow the butanol produced to be handled and distributed using the existing infrastructure for biodiesel. This would allow any existing biodiesel plant to potentially turn a value-negative stream (waste glycerol disposal) into a value-positive stream (waste glycerol conversion to butanol) without the construction of a new processing plant – merely the modification of and addition to existing plants.

1.2.6) Work conducted with waste glycerol and *C. pasteurianum*

This now abundant carbon source has attracted significant research for its use in fermentations, despite the impurities present in the waste glycerol having negative effects on the organism, most often affecting the productivity of the fermentation (Sarchami *et al.* 2016). Analysis of these impurities and their effects on *C. pasteurianum* have been conducted by groups looking to determine the precise effect of each inhibitor commonly found in biodiesel-derived waste glycerol (T. O. Jensen *et al.* 2012; Venkataramanan *et al.* 2012). Venkataramanan *et al.* found that the presence of free fatty acids in the waste glycerol contributed the most to inhibition effects observed, with inhibitory effect increasing with the degree of saturation (Venkataramanan *et al.* 2012). It was also determined that the presence of excess salt and trace methanol had no negative effect on the fermentation. When the free fatty acids were removed from the waste glycerol, glycerol consumption and butanol yields increased to be comparable to those observed with pure glycerol. When completely unpurified waste glycerol was used as the carbon source, the fermentation time increased from 4 days with pure or partially-purified waste glycerol to 14 – 24 days, and the yield was the lowest (0.21 g/g, compared to 0.28 g/g with pure glycerol) (Venkataramanan *et al.* 2012).

Another investigation into the effect of the impurities, and comparing different treatment strategies to remove the impurities, found that with effective treatment there was no significant difference between treated waste glycerol and technical grade (T. O. Jensen *et al.* 2012). Four treatment methods were tested: carbonization (precipitating calcium carbonate which removes impurities as well), electrodialysis (exposing the crude glycerol to anion/cation exchange membranes), activated stone carbon (adding activated carbon directly to crude glycerol and allowing to incubate at room temp), and storage (storing for 10 months at 20^{o}C). Ultimately, a combination of the activated stone carbon with long-term storage was found to have the most significant effect on increasing the yield and cell dry weight, increasing the glycerol consumed at high initial concentrations, though the glycerol consumption rate was still 16% lower than observed on technical grade glycerol. The effect of the activated carbon may be attributed to its acting as an adhesion surface for *C. pasteurianum*, and providing some form of physical protection from external inhibitors (T. O. Jensen *et al.* 2012).

While the availability and price point of biodiesel-derived waste glycerol make it an attractive option as a carbon source, the inhibitory effect will have to be eliminated in a cost-effective manner in order to allow for an economically viable fermentation to be designed.

1.2.7) Optimization studies with *C. pasteurianum*

The highly branched and substrate dependent metabolism of *C. pasteurianum* has allowed for a variety of substrates and conditions to be used during fermentations (Mitchell *et al.* 1987; Biebl 2001). This variation requires optimization for the conditions in order to maximize yield and productivity, and as each change in substrate can have a significant effect on the process, each individual *C. pasteurianum* fermentation should undergo optimization.

A straightforward optimization of conditions would be one factor at a time, as displayed by Gallardo *et al.* or by Johnson and Rehmann (Gallardo, Alves, and Rodrigues 2017; Erin Johnson and Rehmann 2016). Gallardo *et al.* first analyzed the effect of iron concentration by adding 0, 0.6, 2, 10, and 20 mg/L of iron in the form of $FeSO_4 \bullet H_2O$ to the medium and analyzed the effect. It was found that supplementing the medium with 2 mg/L of iron increased butanol production by 163% over the medium with no additional iron (Gallardo,

Alves, and Rodrigues 2017). Iron is necessary for the function of the pyruvate:ferredoxin oxidoreductase enzyme, which oxidizes pyruvate to acetyl-CoA. The reduced form of ferredoxin is subsequently oxidized by Hydrogenase while producing H_2 and restoring the oxidized state of the ferredoxin, allowing for another round of oxidation of pyruvate. Too low a concentration of iron in the medium does not allow for function of ferredoxin, while too high a concentration can have inhibitory effects (Lee *et al.* 2001). Next the group examined the effect of inoculum age on the overall fermentation, inoculating with precultures which had varying incubation times. It was found that 12 hours (the shortest incubation time) was the optimal age for the preculture, resulting in faster growth and higher productivity, though results involving preculturing timing are highly subjective on several factors unique to each laboratory setup. Johnson and Rehmann altered the pH of *C. pasteurianum* fermentations and found that the product profile altered significantly when the fermentation is held at pH values between 4.7 and 5.9, finding that as pH values increased, the amount of butanol produced would decrease (Erin Johnson and Rehmann 2016). Here, it was determined that the pH of 5.0 produced the highest yield of butanol and lowest amount of 1,3-PDO.

While this approach has indeed led to increased butanol yields and productivity, other groups have chosen more robust statistical methods to account for interactive effects of multiple factors (Sarchami, Johnson, and Rehmann 2016a; Sarchami and Rehmann 2014; Moon *et al.* 2011a; Sarchami and Rehmann 2019). Using Response Surface Methodology, a fermentation can be optimized taking into account several factors and interactions simultaneously. Moon *et al.* initially attempted a one-factor-at-a-time approach, but found that in fermentations with the same glycerol concentration the product pattern differed when other medium components were altered (Moon *et al.* 2011a). By investigating the effects of the buffer, co-factors, and yeast extract, the investigators were able to identify that the significant factors were $FeSO_4 \bullet 7H_2O$, $(NH_4)_2SO_4$, and yeast extract, and the concentrations favoring butanol production differed significantly from those favoring production of 1,3-PDO. Fermentations conducted at optimized conditions for butanol production increased productivity from 0.71 g/L/h in the unoptimized medium to 1.01 g/L/h, showing that there is indeed an optimal point, and that the concentration of the medium components has a highly significant effect on the products and productivity of the fermentation (Moon *et al.* 2011a).

Sarchami *et al.* used response surface methodology to optimize the butanol productivity with a waste stream of glycerol from biodiesel production (Sarchami, Johnson, and Rehmann 2016a). The factors investigated did not involve the medium components, but rather the fermentation conditions (inoculum age, initial cell density, initial pH, and fermentation temperature). The initial concentration of glycerol in the medium was found to not be a significant factor in this model. The optimum point with an initial glycerol concentration of 50 g/L was found to have an inoculum age of 16 hours, a cell density of 0.4 g dry cell weight/L, an initial pH of 6.8, and a temperature of 30^{o}C (Sarchami, Johnson, and Rehmann 2016b). These conditions resulted in a fermentation with a molar yield of 0.34 mols butanol / mol waste glycerol consumed.

In a similar study, Sarchami and Rehmann used statistical methods to optimize a dual-substrate fermentation using two inexpensive waste substrates – Jerusalem artichokes, as a source of fermentable sugars, and biodiesel-derived crude glycerol (Sarchami and Rehmann 2019). The fermentation of sugars by *C. pasteurianum* primarily produces butyric acid, and the effect it can have on glycerol fermentation is discussed in further detail in Section 1.2.9. This study found the optimized amounts of the two substrates to be 54 g/L glycerol and 12 g/L total sugars in the medium (Sarchami and Rehmann 2019). This ratio increased the butanol productivity and yield, and was the first reported instance of utilizing two waste carbon streams in a co-substrate butanol fermentation with high efficiency.

1.2.8) Genes controlling butanol fermentation

The central glycerol metabolism (both the oxidative and reductive pathways) of *C. pasteurianum* have been described as unique in nature, as it combines metabolic pathways usually found elsewhere and independent of one another in other *Clostridia* species (Pyne, Liu, *et al.* 2016).

As mentioned in Section 1.2.4, the reductive pathway to produce 1,3 PDO is necessary for the growth of *C. pasteurianum* on glycerol. The conversion begins when glycerol dehydratase, encoded by the gene *dhaBCE,* coet nverts glycerol to 3-hydroxypropionaldehyde, which is subsequently reduced to 1,3 PDO by 1,3-propanediol dehydrogenase (*dhaT*) (Luers *et al.* 1997). Both of these enzymes are located on a single

regulon, which differs in organization from other glycerol consuming organisms in that the regulon is completely read in the same direction (Sun *et al.* 2003). These two enzymes are also highly conserved between other glycerol consuming organisms and *C. pasteurianum,* sharing up to 81% similarity with enzymes found in species of *Citrobacter* and *Klebsiella* (Sun *et al.* 2003).

The oxidative pathway begins with the conversion of glycerol first to dihydroxyacetone, and then to dihydroxyacetone phosphate, where it subsequently follows the standard glycolytic pathway (Pyne, Liu, *et al.* 2016). The enzymes for these steps are glycerol dehydrogenase and dihydroxyacetone kinase, encoded by the genes *dhaD* and *dhaK*, respectively (Pyne *et al.* 2013). It is interesting that the *C. pasteurianum* genome contains 5 putative genes for *dhaD*, which may be responsible for the organisms ability to tolerate high glycerol concentrations with little to no inhibition (Dabrock, Bahl, and Gottschalk 1992; Pyne, Liu, *et al.* 2016). The metabolism continues until the glycerol has been fully converted to pyruvate, at which point the metabolism becomes highly branched.

The pyruvate is converted first to acetyl-CoA via pyruvate:ferredoxin oxidoreductase, which couples this oxidation with a reduction of the protein ferredoxin, which subsequently gets oxidized (in order to repeat the redox reaction) by hydrogenase. The protein ferredoxin has become a model electron transfer protein, was first isolated in *C. pasteurianum*, and is controlled in part by the gene *hydA* which encodes for ferredoxin dehydrogenase (Schwarz *et al.* 2017). This step results in a molecule of acetyl-CoA being produced, along with the evolution of H_2 gas and, if iron is not limited, an oxidized form of ferredoxin ready to oxidize another pyruvate molecule. In order to continue the production of butanol, the four-carbon intermediate butyryl-CoA must be generated by condensing together two aceteyl-CoA molecules. The initial step is catalyzed by the enzyme Thiolase, which condenses two aceteyl-CoA molecules into a single acetoacetyl-CoA molecule (Meng and Li 2006). This condensation reaction is followed by redox reactions which ultimately produce one butyryl-CoA molecule and the oxidation of two NADH molecules from the two acetyl-CoA molecules (Pyne, Liu, *et al.* 2016).

The butyryl-CoA intermediate is of considerable interest for the fermentative production of butanol from glycerol, as this is a branching point between the products of butyric acid and

butanol (Figure 1.3). Two genes control the conversion from butyryl-CoA to butyric acid, and are located on a single operon, and the corresponding proteins are highly similar (between 73% and 80%) to those found in *C. acetobutylicum* (Pyne, Liu, *et al.* 2016). The reaction catalyzed is reversable under standard conditions, and allows for the butyric acid to be converted directly to butanol when in the presence of an external carbon source (Regestein *et al.* 2015).

The further reduction of butyryl-CoA to butanol begins with further dehydrogenation of butyryl-CoA to butyraldehyde, then finally butanol. This step is catalyzed by alcohol dehydrogenase enzymes, of which there are several encoded within the *C. pasteurianum* genome (Pyne, Liu, *et al.* 2016). These enzymes have been shown to play a direct role in butanol formation in *C. acetobutylicum*, and knock-out mutations with *C. acetobutylicum* have identified the gene *adhE* as having the highest impact on butanol formation (Cooksley *et al.* 2012; Walter, Bennett, and Papoutsakis 1992). While a perfect homolog to the *adhE* gene is not present in *C. pasteurianum*, there are at least four protein products produced by *C. pasteurianum* with similarities to the AdhE protein product in *C. acetobutylicum* (Pyne *et al.* 2013).

1.2.9) Mutagenesis and DNA recombination

Genetic manipulation of *C. pasteurianum* has become an area of research following the sequencing of the genome (Poehlein *et al.* 2015). The highly branched nature of the metabolism, with each branch having its own distinct genetic controls, allows for a variety of possible gene targets for manipulation (Pyne, Liu, *et al.* 2016). Multiple tools have been developed for manipulation, including electrotransormation, gene deletion and integration, antisense RNA gene knockdown, and recently a successful transformation using a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system (Pyne, Liu, *et al.* 2016; Pyne *et al.* 2013; Schwarz *et al.* 2017; T. Ø. Jensen *et al.* 2012; Malaviya, Jang, and Lee 2012; Pyne *et al.* 2014; Pyne, Bruder, *et al.* 2016).

1.2.9.1) Work using random mutagenesis to improve butanol fermentation

Prior to the development of these targeted genetic tools, the mutagenic work was conducted via random mutagenesis (Pyne *et al.* 2014). Using ethane methyl sulfonate, a well-known

mutagen, Jensen at al. were able to produce a mutant strain of *C. pasteurianum* which displayed increased tolerance to biodiesel-derived crude glycerol (up to 105 g/L), and had an increased butanol productivity of 1.8 g/L/h vs. 1.3 g/L/h in the wild-type strain (T. Ø. Jensen *et al.* 2012). Two different groups have reported using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to introduce random mutations to a population of *C. pasteurianum*, and subsequently select for highly productive mutants, while another has reported using N-Ethyl-N-nitrosourea as the mutagen (Malaviya, Jang, and Lee 2012; Sandoval *et al.* 2015; Gallardo, Alves, and Rodrigues 2017).

Malaviya *et al.* produced a mutant strain which increased the productivity from 0.25 g butanol / g glycerol with the wild-type organism to 0.30 g butanol / g glycerol, as a result of decreased production of by-products (Malaviya, Jang, and Lee 2012). Unfortunately, the highly productive mutant was not sequenced, and as such the genotypic cause of the productive phenotype was not determined. Similar work was conducted by Gallardo *et al.* who used the mutagen N-Ethyl-N-nitrosourea to create randomly generated mutants of *C. pasteurianum* (Gallardo, Alves, and Rodrigues 2017). They were able to optimize a process with the mutant, and the result of the optimized conditions increased the butanol production by 22% and the yield by 17% over the wild-type strain in identical conditions, and both strains consumed similar amounts of glycerol over the course of the fermentation. The mutant strain was also able to tolerate higher concentrations of butanol in the medium, up to 10 g/L butanol added to the medium, while the parent strain was unable to grow at 5 g/L (Gallardo, Alves, and Rodrigues 2017). This work mirrors similar work that showed between 5 and 7.5 g/L of added butanol to an culture will cause complete inhibition (Munch *et al.* 2020a).

However, Sandoval *et al.* took the work a step further, and were able to determine the mutation which resulted in the highest producing variant in a population of cells growing on biodiesel-derived crude glycerol following treatment with the mutagen (Sandoval *et al.* 2015). The mutant, designated M150B, showed higher glycerol consumption and butanol production when compared to the wild-type organism, regardless if grown on biodiesel-derived or pure glycerol. Subsequent genetic analysis showed a deletion mutation which resulted in complete inactivation of the gene which controls sporulation, *SpooA* (Sandoval *et*

al. 2015). The phenotype was re-created via deletion of 200 base pairs within the *SpooA* gene, including the start codon and ribosomal binding site of the gene, indicating that the deletion genotype was responsible for the increase in productivity. It was speculated that while the *SpooA* gene may not directly influence butanol production, alteration to growth-related genes can have a significant effect. This work highlighted that while the majority of genetic manipulation would focus on the genes directly responsible for glycerol metabolism (Figure 1.3), there are many possible avenues to increase the productivity of butanol fermentation.

1.2.9.2) Work using directed mutagenesis to improve butanol fermentations

In contrast to using random mutagenesis, highly specific transformation tools have been developed for use with *C. pasteurianum* (Schwarz *et al.* 2017; Pyne *et al.* 2013). Pyne *et al.* have developed several tools specifically for use with *C. pasteurianum* (Pyne *et al.* 2013; Pyne, Liu, *et al.* 2016; Pyne *et al.* 2014). A protocol to allow for high level of DNA transfer into *C. pasteurianum* via electrotransformation with methylate plasmid DNA resulted in an increase of three orders of magnitude successful transformants per µg of plasmid DNA (Pyne *et al.* 2013). The same group also produced a mutant *C. pasteurianum* which eases metabolic engineering by removing processing requirements of the plasmid DNA to be introduced (Pyne *et al.* 2014). They then used this method to create a knock out mutant lacking a functional *dhaT* gene, which codes for the enzyme 1,3-propanediol dehydrogenase, responsible for catalyzing the conversion from 3-hydroxypropionaldehyde to 1,3-PDO (Pyne, Sokolenko, *et al.* 2016). The result was that the reductive pathway which results in 1,3-PDO production was nearly completely eliminated, and the selectivity of butanol increased from 0.51 g butanol / g total solvents produced (total solvents was the combined amount of butanol, ethanol, 1,2-PDO and 1,3-PDO) in the wild-type to 0.83 g butanol / g total solvents in the mutant (Pyne, Sokolenko, *et al.* 2016). Interestingly, the production of 1,2-propanediol was significantly increased in the mutant strain, indicating that the mutant utilized a secondary metabolic pathway in order to balance the reducing equivalents in the absence of the 1,3-PDO pathway (Pyne, Sokolenko, *et al.* 2016).

In similar work, Schwarz *et al.* introduced specific knockout plasmids to *C. pasteurianum* in a targeted technique known as Allele-Coupled Exchange, where the desired traits (knocked

out gene or selection marker) are put onto a plasmid and electroporated into the cells (Schwarz *et al.* 2017). This technique was used to knock out the entire *dhaBCE* operon (encoding for glycerol dehydratase), which resulted in the elimination of 1,3-PDO, but did not have a significant effect on the production of other solvents (Schwarz *et al.* 2017). The same group also used this technique to knock out the *hydA* gene, thus removing a functional ferredoxin dehydrogenase. The result was a mutant which produced higher amounts of acetate, lactate, and ethanol, and less butyrate and 1,3-PDO compared to the wild-type, though there was only a modest increase in butanol titer (Schwarz *et al.* 2017). These results agree with a previous report which used anti-sense RNA mediated repression of *hydA* gene expression, though this report used dextrose as the carbon source in the medium, preventing a direct comparison (M. Pyne *et al.* 2015).

1.2.10) Addition of butyric acid to glycerol fermentations

When grown on glycerol as the sole carbon source, *C. pasteurianum* produces organic acids in addition to alcohols. An interesting acid which is produced in small amounts is butyric acid, which plays a role in the production of butanol (Regestein *et al.* 2015; Lin *et al.* 2015). In biphasic *Clostridia* species, it has been demonstrated that addition of butyric acid to the initial medium can increase butanol productivity throughout the fermentation, even during the acidogenesis phase (Tashiro *et al.* 2004). It has been suggested that butyric acid is a precursor for the formation of butanol in many ABE fermentative organisms, signaling the metabolic change from acidogenesis to solventogenesis, though the effect is not consistent (Zigová and Šturdík 2000). With *C. pasteurianum*, which does not exhibit biphasic behavior, addition of low concentrations of butyric acid to the fermentation medium can increase the yield of butanol, while addition of higher concentrations (greater than ~6 g/L) will cause inhibition (Regestein *et al.* 2015; Tashiro *et al.* 2004; Kao *et al.* 2013; Heyndrickx *et al.* 1991).

Regestein *et al.* determined that in order to ease the uptake of butyric acid into the cells, the acid must be in its completely protonated form (Regestein *et al.* 2015). As the pKa of butyric acid is 4.82, a pH value of 5.3 was selected, as this allowed for 25% of the butyric acid to be in the fully protonated form, while also preventing pH inhibition of *C. pasteurianum*. Using this strategy, Regestein *et al.* achieved a yield of 0.38 g butanol / g glycerol + g butyric acid

with the addition of 4 g/L butyric acid to a fermentation containing 30 g/L glycerol, an increase from a yield of 0.31 g butanol / g glycerol + g butyric acid in fermentations without additional butyric acid added. The uptake of butyric acid was found to only occur in the presence of a carbon source. With higher concentrations of glycerol present in the medium, higher uptake of butyric acid was observed. Increasing the initial glycerol available from 30 g/L to 45 g/L, with 4 g/L butyric acid in the medium as well, the uptake was increased from 0.8 g/L butyric acid to 2.8 g/L. However, the increased uptake did not result in increased butanol yield, resulting in only 0.34 g butanol / g glycerol + butyric acid. It was also determined that higher concentrations of butyric acid in the presence of lower glycerol concentrations resulted in inhibition (Regestein *et al.* 2015).

Lin *et al.* incorporated butyric acid addition to a *C. pasteurianum* fermentation with vacuum membrane distillation as an *in situ* butanol removal system, creating a fermentation system which simultaneously increased the butanol productivity and prevented product inhibition (Lin *et al.* 2015). The conditions selected (temperature, pH, glycerol concentration, butyric acid addition, and medium composition) were initially optimized via response surface methodology, though the vacuum membrane distillation was not factored into the optimization. The optimized starting butyric acid concentration was determined to be 6 g/L, which is significantly higher than the highest concentration used by Regestein *et al.* (4 g/L) (Lin *et al.* 2015; Regestein *et al.* 2015). However, Lin *et al.* used a starting glycerol concentration of 100 g/L, more than double that used by Regestein *et al.*, as well as the vacuum membrane system, making a direct comparison between the systems difficult. The effect of the added butyric acid was still evident, as its addition increased butanol yield from 0.24 mol butanol / mol glycerol to 0.39 mol butanol / mol glycerol, though this number does not include the butyric acid taken up by the cells as a carbon source (Lin *et al.* 2015).

It must also be noted that these studies used chemically pure butyric acid, which would be prohibitively expensive for large scale industrial production of butanol. In order to make an economically viable process utilizing butyric acid to increase butanol yield, expensive pure chemicals would need to be avoided in lieu of lower cost chemicals and substrates.

1.2.11) Microbial production of butyric acid

A possible source of inexpensive butyric acid for use in butanol fermentations could be from microbial fermentations. When produced using chemical synthesis, butyric acid is production uses the same precursors as butanol production – butyraldehyde produced by upgrading propene (Green 2011). Also similar to butanol production, the use of fermentatively derived butyric acid would decouple butyric acid production from the volatility of the petroleum market by providing a stable and possibly renewable source.

Several organisms are capable of producing butyric acid, some which are potentially dangerous to humans and thus not a strong choice for large-scale processes, like *Butyrivibrio* species (Ha *et al.* 1991; Kopecny *et al.* 2003). However, *C. tyrobutyricum* has emerged as a possible organism for butyric acid production from C5 and C6 sugars (Figure 1.6) (Baroi *et al.* 2015).

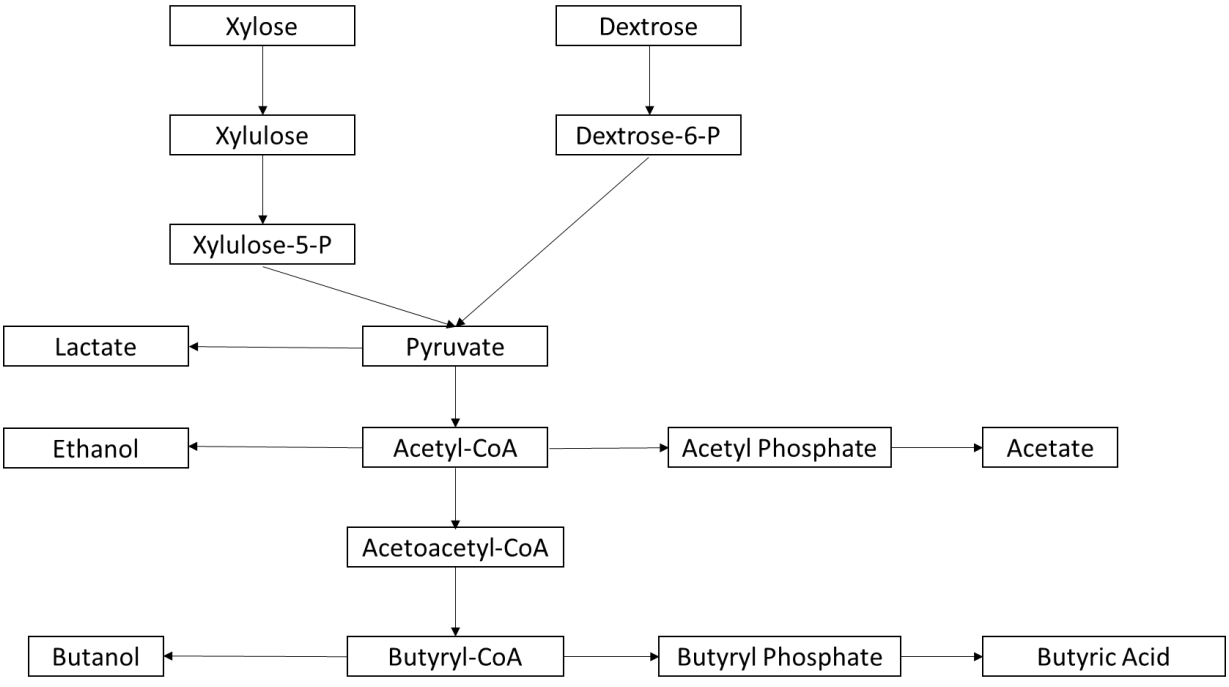

Figure 1.6: Overview of xylose and glucose metabolism in *C. tyrobutyricum*. Figure adapted from H. Luo *et al.* 2018.

C. tyrobutyricum has been used to produce butyric acid from both dextrose and xylose, which has opened an avenue for butyric acid production using lignocellulosic or other waste sources. Several lignocellulosic carbon sources, for example corn fibers or Jerusalem artichokes, have been used following acid hydrolysis pretreatments with success to produce butyric acid (Zhu, Wu, and Yang 2002; Huang *et al.* 2011). Both substrates were tested with a fed-batch fermentation, resulting in productivities of 2.91 and 1.14 g/L/h and yields of 0.47 and 0.38 g/g yield, respectively, indicating that *C. tyrobutyricum* can convert both dextrose and xylose efficiently to butyric acid effectively when both substrates are present. The low concentrations of secondary products (acetate, lactate, and ethanol) could allow for ease of recovery for use in a second butanol producing fermentation, if separation is needed at all.

1.2.12) Co-culture fermentations

The idea of a microbial co-culture, in which one organism makes use of a carbon source inaccessible to a second organism, and converting it into a compound usable by that second organism, has been explored previously (Chen 2011; Hanly and Henson 2011; Bader *et al.* 2010). In anaerobic co-cultures, a highly explored co-culture utilizes an organism capable of degrading cellulose into sugars, which can then be used by a second organism (Geng *et al.* 2010). Other work has been conducted to explore using a microbial co-culture to provide butyric acid for butanol producing organisms (Lin Li *et al.* 2013). In this study, a co-culture of *C. tyrobutyricum* and *C. beijerinckii* was used to increase the productivity of butanol. What was of interest was that the fermentations were operated in a continuous manner, with the cells immobilized in separate reactors and the medium circulating between the two reactors using a peristaltic pump. The overall butanol productivity was increased, reaching 0.96 g/L/h, compared to 0.10 g/L/h using only *C. beijerinckii* with no additional butyric acid or co-culture (Lin Li *et al.* 2013). Though both organisms were grown in individual reactors, this work shows the possibility of using a co-culture to increase butanol productivity by providing butyric acid in the medium.

However, for butanol production using *C. pasteurianum*, options for co-culturing are limited. The work conducted with *C. pasteurianum* as a part of co-cultures or consortiums has been focused on biohydrogen production rather than butanol (Masset *et al.* 2012; Hsiao *et al.* 2009; Ozmihci and Kargi 2011). The specific condition requirements for *C. pasteurianum* for

high butanol productivity limit the range of organisms capable of growing and producing butyric acid under identical conditions. The most likely candidate for use is *C. tyrobutyricum*, as it is capable of converting xylose (which *C. pasteurianum* is unable to metabolize) to butyric acid while *C. pasteurianum* consumes glycerol (which *C. tyrobutyricum* is unable to use). However, *C. tyrobutyricum* requires a much higher pH for butyric acid production than *C. pasteurianum* requires for high butanol production (Zhu and Yang 2004; Erin Johnson and Rehmann 2016). This discrepancy would require extensive genetic or process engineering to allow for a process in which *C. tyrobutyricum* and *C. pasteurianum* will grow in the same fermentation vessel. However, this does not prevent use of *C. tyrobutyricum* to produce the butyric acid which can be added to the *C. pasteurianum* fermentations with little to no processing.

1.2.13) Dual substrate fermentations with *C. pasteurianum*

An interesting component of the metabolism of *C. pasteurianum* is that when sugars are the carbon source the primary product is butyric acid (Dabrock, Bahl, and Gottschalk 1992). Combined with the knowledge that small amounts of butyric acid can increase butanol productivity and yield, this has led to work exploring a dual-substrate strategy for butanol production using dextrose and glycerol. In such a strategy, a single *C. pasteurianum* culture can use the dextrose to initially produce butyric acid, then subsequently re-assimilate the butyric acid with the glycerol, thus increasing the butanol yield and productivity without using chemically pure butyric acid (Sabra *et al.* 2016, 2014; Kao *et al.* 2013).

This strategy has been used by Kao *et al.*, who initially repeated the work of Lin *et al.* and reached a similar conclusion, that with 100 g/L glycerol at the onset of the fermentation, 6 g/L of butyric acid addition resulted in yield increasing from 0.200 mol butanol / mol glycerol to 0.307 mol/mol (Kao *et al.* 2013). Subsequent work in this study investigated the use of a dual-substrate strategy to avoid the need for chemically pure butyric acid. Initially, a sequential carbon source addition strategy was attempted, in which 40 g/L dextrose was first used, and upon its depletion, 100 g/L glycerol was added to the medium. When consuming the dextrose, the primary product was butyric acid, with smaller amounts of acetate produced (Kao *et al.* 2013). Upon the addition of the glycerol, a significant lag phase was observed before glycerol was consumed. No butyric acid uptake was observed, and the overall

productivity rate was lower than observed with pure butyric acid, which was 0.14 g/L/h. In order to attempt to limit the lag phase during the substrate switch, and subsequently increase the productivity, a strategy in which both substrates were added at the beginning of the fermentation was utilized. A ratio of 20:60 g/L dextrose to glycerol in order was found to result in the highest yield, and increase the butanol yield from 0.22 mol butanol / mol glycerol in a fermentation with only glycerol, to a yield of 0.38 mol butanol / mol glycerol (Kao *et al.* 2013). The productivity of this dual substrate strategy was 0.19 g/L/h, which was higher than both productivities from the sequential addition and pure butyric acid strategies. However, it must be noted that the yield calculated here only takes into account the carbon from the glycerol consumed, not accounting for the dextrose present, which was completely consumed and would represent a significant amount of carbon towards the yield calculation. This work also used sugars derived from lignocellulosic biomass and crude glycerol as the carbon sources, which resulted in a yield of 0.33 mol butanol / mol glycerol and a productivity of 0.14 g/L/h, which were still high, though once again the yield calculation did not include the carbon from the sugars present from the biomass (Kao *et al.* 2013).

In a similar study, Sabra *et al.* first studied the effect of direct addition of butyric acid to the initial medium, and found that concentrations higher than 5 g/L butyric acid resulted in greatly diminished cell growth (Sabra *et al.* 2014). However, adding butyric acid later in the fermentation, or added continuously to a pH-controlled (pH = 6) fermentation with 75 g/L glycerol as the carbon source, which resulted in some uptake of the butyric acid, and increased butanol/1,3-PDO ratio. The authors of this study tested multiple ratios of dextrose and glycerol to find the most effective ratio to have the highest amount of butanol produced at the end of the fermentation (ratios tested were 1:0, 4:1, 1:1, and 1:4 dextrose:glycerol). The ratio of 1:1 dextrose to glycerol (50 g/L of each) achieved an extremely high final concentration of butanol of 21.1 g/L, the highest concentration reported (without any *in situ* product removal) in literature to date (Sabra *et al.* 2014). However, despite the high final concentration reported, the process had a yield of only 0.286 mol butanol / mol glycerol + dextrose, which would need to be increased to be considered for large scale production. The same study also used a fermentation strategy using biomass hydrolysate from spruce tree biomass to provide the sugars, and pure glycerol in a 1:1 ratio at 50 g/L each. The

hydrolysate sugars and pure glycerol resulted in in a titer 17 g/L butanol, lower than with pure substrates but still higher than observed with only pure glycerol (Sabra *et al.* 2014).

These studies show that while there is considerable optimization work to be undertaken before a fermentation involving butyric acid derived from sugars in the medium could be a viable option for increasing yield and productivity when producing butanol from glycerol. However, the need for an inexpensive source of sugars is vital for the economic viability of the process.

Table 1.2: Studies using glycerol and dextrose to increase yield and productivity of butanol fermentations with *C. pasteurianum.*

Substrate	Glycerol utilized	Time to completion (h)	Max butanol yield[a] (mol/mol)	Butanol Productivity (g/L/h)	Reference
Glycerol (100 g/L)	70%	80	0.220	0.13	(Kao *et al.* 2013)
Glycerol + Dextrose (60 + 20 g/L)	71.6%	80	0.380[b]	0.19	(Kao *et al.* 2013)
Waste glycerol + Biomass hydrolysate (60 + 25 g/L)	69.8%	80	0.330[b]	0.14	(Kao *et al.* 2013)
Glycerol + Dextrose (ratio 1:1)	91.7 g/L	50	0.286	0.69	(Sabra *et al.* 2014)
Glycerol + Biomass Hydrolysate	87 g/L	N/A	0.248	0.62	(Sabra *et al.* 2014)

[a]Yield calculated based on glycerol consumed
[b]Yields were calculated based solely on the glycerol consumed, not including dextrose

A possible source of inexpensive sugars for use in fermentations, that does not require enzymatic digestion like lignocellulosic biomass, is molasses. Molasses is a by-product of sugar production, containing sucrose, fructose, and dextrose that does not precipitate to be sold as pure sugar. Molasses has been used as a substrate for butyric acid production using *C.*

tyrobutyricum (L. Jiang *et al.* 2009), butanol production using *C. saccharobutylicum* (Ni, Wang, and Sun 2012), and hydrogen production (Hsiao *et al.* 2009). To the best of the authors knowledge, no research using molasses as a carbon source for *C. pasteurianum* has been yet conducted. The inexpensive carbon source could be well-suited for use in a dual-substrate fermentation, using high amounts of glycerol as the second (primary) carbon source for butanol production.

1.2.14) Online process monitoring of fermentations

Online monitoring of fermentative processes allows for real-time measurements of important parameters for the productivity and yield of the process, and can provide a powerful tool for the optimization, control, and assessment of a process (Cervera *et al.* 2009). This can include monitoring the health of fermentations, identify times for sampling, and identify periods of metabolic changes in the population indicating exhaustion of a nutrient or an imbalance in population (Cervera *et al.* 2009). For example, in anaerobic biomass digestion, a balance between the consortium of organisms is required to prevent digestor collapse and maintain a high yield of methane (Rudnitskaya and Legin 2008). However, the process conditions are difficult to monitor without resorting to time-consuming laboratory tests requiring skilled personnel. To combat this, advanced techniques such as near-infrared spectroscopy, a non-invasive light based technique which can provide valuable information without requiring sampling (Rudnitskaya and Legin 2008).

Near-infrared spectroscopy is one of many tools used for measuring multiple parameters during a fermentation in a multi-sensor system. Common parameters monitored, using probes and other sensors not necessarily based on optics or near-infrared principles, are optical density, pH, temperature, and gasses produced (Ge, Zhao, and Bai 2005; Herweg *et al.* 2018). Recent work has been conducted to allow estimates of individual biomass levels for each species in a co-culture fermentation by analyzing the scattered light spectrum of the co-culture, and using statistical methods to determine the individual biomass from the collective spectra (Geinitz *et al.* 2020). This work was conducted using microtiter plates, creating a powerful screening device for aerobic fermentations. In a larger, more industrial application of online monitoring using spectroscopic techniques, fluorescent spectroscopy was used at multiple stages of *Bordetella pertussis* vaccine production, and was able to successfully

correlate the fluorescence data obtained during production with the protein content in the final product (Zavatti *et al.* 2016).

However, many fermentation online monitoring methods generally suffer from two major drawbacks: first, they generally require a large or complicated fermentation vessel, such as a bioreactor, and second, they are generally applied to aerobic fermentations (Meissner *et al.* 2015; Wewetzer *et al.* 2015). The probes for pH, redox, optical density, and temperature are subject to drift and require recalibration during extended fermentations, while the same can be true for several off-gas sensors. Regardless, the off-gasses can give extremely important information regarding the fermentation, including effects such as oxygen limitation, pH inhibition, substrate limitation, and product inhibition (Anderlei and Büchs 2001; Munch *et al.* 2020b). Combining these tools with smaller fermentation vessels, such as shake flasks, would allow for robust data collection while keeping the demands for space and materials low.

1.2.15) Online monitoring of shake flask fermentations

Online analysis of off-gasses often requires large sensors and high volumes of gasses produced, necessitating the use of bioreactors in lieu of the more simple and economic shake flask model (Anderlei *et al.* 2004). As a method to address the lack of off-gas analysis available at the shake-flask scale, a device has been developed which allows for the online monitoring of off-gasses in multiple parallel shake flasks (Anderlei and Büchs 2001; Wewetzer *et al.* 2015). This device is known as the Respiratory Activity Monitoring System (RAMOS), and has become a robust tool for screening fermentations at the small scale while still obtaining the maximum amount of data possible. The RAMOS allows for eight parallel flasks to be operated simultaneously, with each flask operating under the same conditions (temperature, shaking rate, air flow) and collecting off-gas data from each individual flask. Each flask can hypothetically test a different condition, such as presence of vitamins, excess carbon, or varying concentration of inhibitors, while keeping the physical conditions between flasks constant. The RAMOS utilizes specialized flasks, for which the volume has been accurately determined, that allow for the sterile flow of gasses through the headspace (Anderlei and Büchs 2001). Over the course of the fermentation, the RAMOS uses control of the airflow by opening and closing inlet and outlet valves in a repeating pattern (Anderlei *et*

al. 2004). The majority of the fermentation will have the air flow through the headspace at a defined rate, allowing free transfer of gasses. This is referred to as the 'rinse phase'. At defined times, the flow of gasses through the headspace is stopped by closing the inlet and outlet valves. During this time, referred to as the 'measurement phase', oxygen sensors placed on each flask record the amount of oxygen consumed, while a pressure sensor on the outlet records the change in the total pressure within the headspace (Anderlei *et al.* 2004). Assuming a linear change in the partial pressures of the gasses, the oxygen and carbon dioxide transfer rates can be calculated (Anderlei *et al.* 2004). Following the measurement phase, the inlet and outlet valves are opened and the headspace is purged of accumulated gasses, and free respiration resumes. This device has become an invaluable tool in fermentation research, and has been used in several studies (Meissner *et al.* 2015; Anderlei and Büchs 2001; Anderlei *et al.* 2004; Herweg *et al.* 2018; Buchenauer *et al.* 2009; Wewetzer *et al.* 2015).

However, though the RAMOS has advanced significantly since it's construction, a similar device for anaerobic fermentations is still lacking. At the shake flask level, anaerobic fermentations are currently conducted in sealed flasks with no constant gas transfer, which allows for gas measurements by sampling in a gas chromatograph, and could lead to build-up of pressure or headspace gasses, or in shake flasks in anaerobic chambers, which allow for gas exchange with the chamber atmosphere but no measurement of the gasses produced (Munch *et al.* 2020a). Some devices measure the total gasses produced over the course of the fermentation in the shake flask by measuring the number of discrete bubbles through tubing with a measured diameter, but are unable to differentiate the gasses produced. Addressing this gap in screening technology would allow for increased data to be collected at the shake-flask scale in anaerobic fermentations, reducing the need for more expensive and cumbersome bioreactor experiments.

1.3) Identified gaps in literature

The previous section outlined a portion of the work done in the area of fermentation, butanol production, strain and process development, and online process monitoring. Throughout this review, the need for further development of butanol fermentations has been identified. While there has been success using butyric acid or dual-substrate strategies, the current reports have

focused on high concentrations of butanol with little regard for the yield of the process. The development of a process focusing on yield would provide valuable information for the advancement of butanol production by limiting the waste of the carbon substrate. Further, use of mutagenic compounds and spontaneous mutations has allowed for improvement of butanol fermentation through strain improvement. However, the possible improvements through random mutagenesis have not yet been exhausted, and could still yield improved strains for butanol production and yield. Finally, while online monitoring of anaerobic fermentations has allowed for off-gas data collection in fermentations operating at the benchtop reactor scale, fermentations in shake flasks lack the ability to collect off-gas data. Development of a device for off-gas data collection from shake flasks would create a powerful medium-throughput screening device for anaerobic fermentations.

1.4) Research Objectives

1.4.1) General Objective

The overall objective of this research was to increase the productivity of butanol fermentations using glycerol as the primary carbon source and develop online off-gas monitoring capabilities for anaerobic fermentations at the shake-flask scale.

1.4.2) Specific Objectives

Objective 1: Increase selectivity for butanol by addition of butyric acid to glycerol fermentation to maximize butyric acid uptake and butanol yield

Butyric acid was used to increase the selectivity for butanol during glycerol fermentations via addition in the initial medium, and delayed addition to the fermentation medium. The initial addition resulted in slight increase in selectivity, but an inhibitory effect was observed. Delaying the addition of butyric acid increased the uptake of butyric acid while limiting any inhibitory effect. Chemically pure butyric acid was used in parallel with sterilized exhausted medium from a fermentation in which the main product being butyric acid.

Objective 2: Increase butanol yield in glycerol fermentations by optimizing addition of a sugar substrate

The metabolism of *C. pasteurianum* can be used to produce butyric acid when fermenting sugars. This was utilized to create a dual-substrate fermentation strategy in which sugars were initially consumed to produce butyric acid, which was subsequently re-assimilated by the cells during consumption of glycerol, increasing the selectivity for butanol. The ratio between the substrates was optimized for high levels of butyric acid re-assimilation and butanol selectivity.

Objective 3: Investigate a highly productive mutant of *C. pasteurianum* to determine locations of variants by DNA sequencing analysis

A putative mutant strain of *C. pasteurianum* that displayed a phenotype with higher productivity than the original wild-type strain was generated by and obtained from E. Johnson. The DNA for the putative mutant was isolated, sequenced, and compared to both the laboratory wild-type stock strain and a published reference genome. The mutations were found and located on an annotated genome, giving insight into the product of the gene containing the mutation.

Objective 4: Create a screening device to obtain off-gas data from anaerobic shake flask fermentations

The lack of online off-gas monitoring for anaerobic fermentations at the shake flask scale resulted in missed phenomena and sampling points of interest. This technological gap was addressed by modifying equipment for aerobic fermentation off-gas monitoring to work anaerobically, collecting online data for CO_2 and H_2 gas transfer rates. This allowed for several fermentations to operate in parallel while online data collection for each individual fermentation was conducted, identifying key phenomena in the fermentations such as inhibition and exhaustion of substrates.

Chapter 2

Increasing selectivity for butanol in *Clostridium pasteurianum* fermentations using butyric acid or a dual substrate fermentation

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This chapter explores the effect that butyric acid can have on glycerol fermentations by *C. pasteurianum*. Addition of butyric acid in the medium has been shown previously to have an effect on the selectivity for butanol, though its presence from the onset of the fermentation can also have an inhibitory effect on the organism (Regestein *et al.* 2015). In this study, the addition of butyric acid to the fermentation medium was delayed to various timepoints following inoculation in order to increase the uptake and minimize the inhibitory effect. Chemically pure butyric acid was used in parallel with the process fluid from a butyric acid producing fermentation (xylose fermentation by *C. tyrobutyricum*). This allowed direct comparison of a possible low-cost source of butyric acid with chemically pure to assess any effects of other compounds present in the process fluid.

Additionally, a dual-substrate fermentation was optimized using molasses sugars (as a possible low-cost carbohydrate source) and pure glycerol. The initial metabolization of the sugars resulted in butyric acid production, which was subsequently taken up by the cells when consuming glycerol in a manner similar to pure and fermentation-derived butyric acid added directly to the medium. The ratio of molasses to glycerol required optimization to ensure the proper amount of butyric acid was produced: too high a concentration would result in inhibition and lack of uptake, and too low a concentration would result in no significant effect on butanol yield and selectivity. The optimized conditions resulted in the highest molar yield of carbon being utilized to produce butanol, 0.4 mol carbon consumed / mol carbon butanol. This yield was higher than observed with either the fermentation derived or the chemically pure butyric acid.

The study described in this chapter fulfilled the first two of the four objectives of this thesis. The delayed addition of butyric acid, regardless of the source or presence of additional fermentation products, increased the yield of butanol from all carbon substrates. Achieving this objective demonstrated the efficacy of delaying the addition of butyric acid in increasing

yield while avoiding inhibition. The second objective built on the first, using molasses as the sugar source, allowing *C. pasteurianum* to produce butyric acid, which was subsequently taken back up by the cells. The ratio of glycerol to molasses was optimized to allow high butyric acid uptake, and subsequent high butanol yield.

Abstract: Volatility of the petroleum market has renewed research into butanol as an alternate fuel. In order to increase the selectivity for butanol during glycerol fermentation with *Clostridium pasteurianum*, butyric acid can be added to the medium. In this manuscript, different methods of extracellular butyric acid addition are explored, as well as self-generation of butyric acid fermented from sugars in a co-substrate strategy. Molasses was used as an inexpensive sugar substrate, and the optimal molasses to glycerol ratio was found to allow the butyric acid to be assimilated into the cells and increase the productivity of butanol from all carbon sources. When butyric acid was added directly into the medium, there was no significant difference between chemically pure butyric acid, or butyric acid rich cell free medium from a separate fermentation. When low concentrations of butyric acid (1 or 2 g/L) were added to the initial medium, an inhibitory effect is observed, with no influence on the butanol selectivity. However, when added later to the fermentation, over 1 g/L butyric acid was taken into the cells and increased the relative carbon yield from 0.449 to 0.519 mols carbon in product / mols carbon in substrate. An optimized dual substrate fermentation strategy in a pH-controlled reactor resulted in the relative carbon yield rising from 0.439 when grown on solely glycerol, to 0.480 mols C product / mols C substrate with the dual substrate strategy. An additional benefit is the utilization of a novel source of sugars to produce butanol from *C. pasteurianum.* The addition of butyric acid, regardless of how it is generated, under the proper conditions can allow for increased selectivity for butanol from all substrates.

2.1) Introduction

The increasing costs of fossil fuels, and the volatility of the petroleum market has led to increased worldwide research into renewable sources of fuels and platform chemicals. Butanol, a 4-carbon alcohol, has attracted interest due to its superior fuel properties when compared to the current most widespread biofuel, ethanol (J. Lee *et al.* 2012; S.-M. Lee *et al.* 2008). In addition, butanol is a valuable chemical in many industrial applications.

Butanol was originally produced at a large scale via fermentation using *Clostridium acetobutylicum* in the early 1900's, with the main products of the fermentation being acetone, butanol, and ethanol (Jones and Woods 1986). The petroleum boom provided access to inexpensive precursor chemicals allowing for butanol to be produced from these petroleum industry by-products. However, environmental and economic considerations have resulted in a renewed interest in fermentatively produced butanol using renewable or waste carbon sources (Sarchami, Johnson, and Rehmann 2016b; Gallardo, Alves, and Rodrigues 2014). Work has been conducted using various *Clostridium* species, including *C. acetobutylicum, C. beijerinckii,* and *C. pasteurianum* (W. Luo *et al.* 2018; Hou *et al.* 2017; Sarchami, Johnson, and Rehmann 2016a; Erin Johnson and Rehmann 2016).

In particular, *C. pasteurianum* has attracted considerable attention due to its ability to rapidly metabolize glycerol to butanol, 1,3 propanediol (1,3 PDO), and ethanol, with only trace amounts of the by-products (Sabra *et al.* 2014; Biebl 2001; Gallazzi *et al.* 2015; Regestein *et al.* 2015; Erin Johnson and Rehmann 2016). More importantly, when grown on glycerol *C. pasteurianum* doesn't exhibit the typical biphasic growth patterns seen with other *Clostridium* species. Work has been done to enhance the selectivity or productivity of this process by altering the conditions of the fermentation (Dabrock, Bahl, and Gottschalk 1992; Moon *et al.* 2011a; Sarchami, Johnson, and Rehmann 2016a).

Interestingly, when *C. pasteurianum* is grown with sugars as the main carbon source, the main product is butyric acid (Kao *et al.* 2013). This metabolic pathway has been explored as a possible method to increase the selectivity of butanol in *C. pasteurianum* fermentations using glycerol as the carbon source through the addition of butyric acid to the medium (Regestein *et al.* 2015; Gallardo, Alves, and Rodrigues 2014; Sabra *et al.* 2014; Kao *et al.* 2013). Regestein et al (2015) added chemically pure butyric acid to the fermentation medium to increase the butanol yield from 0.31 to 0.38 g butanol / g substrate, though an inhibitory effect on the fermentation was observed (Regestein *et al.* 2015). It was also determined that the uptake of butyric acid from the fermentation medium required the presence of an external carbon source. The inhibition effect, when combined with the high cost of chemically pure butyric acid, makes this strategy for enhancing butanol production not economically viable.

For increased clarity, for the remainder of the manuscript we will be referring to the combined butyrate and butyric acid solely as butyric acid.

Work has been done to utilize the metabolism of *C. pasteurianum* when grown in a dual-substrate fermentation on sugars and glycerol (Sabra *et al.* 2016, 2014; Kao *et al.* 2013; Dabrock, Bahl, and Gottschalk 1992). The initial consumption of the sugars allows production of butyric acid, which can be beneficial to production of butanol from glycerol. Kao *et al.* used high concentrations of both glucose and glycerol (20 and 60 g/L, respectively) to achieve a molar yield of 0.38 mol butanol / mol glycerol, though this calculations does not include the glucose consumed (Kao *et al.* 2013). Sabra *et al.* used another glucose and glycerol dual fermentation (50 g/L of each) to produce a high concentration of butanol of 21 g/L, and while the final concentration of butanol is high, the overall molar yield from both substrates remains low (Sabra *et al.* 2014). While both of these fermentation strategies were successful in producing high amounts of butanol, the amount of substrate required would prevent the process from becoming economically viable.

In this chapter, the different strategies for the addition of butyric acid to enhance the selectivity of butanol during glycerol fermentations by *C. pasteurianum* are outlined. As an alternative to chemically pure butyric acid, the supernatant from a separate fermentation using *C. tyrobutyricum*, a butyric acid producing bacteria capable of consuming xylose, was used in parallel with experiments using pure butyric acid. Finally, a dual-substrate fermentation using molasses and glycerol was optimized to result in significantly increased selectivity for butanol from all carbon sources.

2.2) Materials and Methods

2.2.1) Chemicals

Yeast extract, peptone, ammonium sulfate, KH_2PO_4, K_2HPO_4, and glycerol were purchased from Fischer Scientific. Beef extract was obtained from BD-Becton, Dickinson and Company (New Jersey, USA). Xylose, butyric acid, soluble starch, sodium acetate, resazurin, and thiamine were purchased from Alfa Aesar (Massachusetts, USA). Dextrose was from Amresco (Ohio, USA) and $CaCl_2$ was from EMD Millipore (Massachusetts, USA). $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, were purchased from Caledon (Ontario, Canada).

Pure glycerol, $FeSO_4 \cdot 7H_2O$, NaCl and l-cysteine were obtained from BDH (Georgia, USA). Commercially available molasses was purchased and diluted to a working concentration of 200 g/L using ddH_2O before use. All other chemicals were purchased from Sigma-Aldrich.

2.2.2) Organisms and Media

Clostridium pasteurianum (DSM 525) and *Clostridium tyrobutyricum* (DSM 2637) were purchased from the DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cultures were revived using Reinforced Clostridium medium (RCM) containing (per liter): 10 g peptone, 10 g beef extract, 3 g yeast extract, 10 g dextrose, 5 g NaCl, 1 g soluble starch, 0.5 g l-cysteine, and 4 ml resazurin, at pH 6.8 until reaching a high density, before 1 mL aliquots were stored at -80^{o}C with 20% v/v glycerol added to the medium.

2.2.2.1) *C. pasteurianum* preculturing and fermentation conditions

For preculturing *C. pasteurianum*, thawed 1 mL aliquots were added to 9 mL fresh RCM and incubated at 35^{o}C for 20 hours in 10 mL sterile tubes. Following this, 10% v/v inoculum was added to fresh RCM containing 10 g/L pure glycerol as the carbon source in a 150 mL Erlenmeyer flask closed with a cotton plug and wrapped in aluminum foil. Cultures were grown for 8 hours before being used for inoculation of experimental flasks if being used for fermentations at the shake flask scale. Experiments investigating the effects of butyric acid addition to fermentations were inoculated with a starting culture volume of 10% v/v. Experiments conducted for the RSM model creation and validation were inoculated with a specific volume to result in an initial OD of 0.02. For reactor scale experiments, the culture was transferred once again at 10% v/v to fresh RCM with 10 g/L pure glycerol in a 1 L glass bottle. Pre-cultures were allowed to grow another 8 hours before being used for inoculation of a 7 L reactor (Labfors, Infors, Quebec, Canada) containing 4.5 L uninoculated medium and 500 mL of inoculum. Reactor temperature control was conducted through water jacket, and equipped with Rushton impellers and baffles for agitation.

Fermentations were conducted using a modified medium published by Biebl (2001) and containing in g/L (unless stated otherwise): glycerol, 20; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4$, 0.05; yeast extract, 1.0. 2 mL of

trace element solution was added per liter of medium, the trace element solution containing (in g/L): $FeCl_2 \cdot 4H_2O$, 1.5, dissolved in 25% HCl solution; $CoCl_2 \cdot 6H_2O$, 0.19; $MnCl_2 \cdot 4H_2O$, 0.1; $ZnCl_2$, 0.07; H_3BO_3, 0.062; $Na_2MoO_4 \cdot 2H_2O$, 0.036; $NiCl_2 \cdot 6H_2O$, 0.024; $CuCl_2 \cdot 2H_2O$, 0.017. All experiments had an initial pH of 5.3, adjusted using 5 M H_2SO_4 or 5 M KOH. Experiments done in flasks used 0.05 M citric acid buffer to maintain the pH in this region, while experiments at the reactor scale maintained the pH at 5.3 using 5 M H_2SO_4 and 5 M KOH.

2.2.2.2) *C. tyrobutyricum* preculturing and fermentation conditions

All preculturing steps were conducted in an anaerobic chamber at 35°C (environment 10% CO_2, 5% H_2, rest N_2), on a shaking plate at 200 RPM with a 50 mm shaking diameter, as described in Section 2.2.2.1 when discussing preculturing of *C. pasteurianum*. A concentrated solution of xylose was filter sterilized using a 0.22 micron-filter and diluted in sterile water to a final concentration of 150 g/L.

A 1 mL *Clostridium tyrobutyricum* stock sample was added to 9 mL of fresh RCM containing 10 g/L dextrose and allowed to grow for 24 hours. A 10% v/v aliquot of actively growing culture was transferred to 45 mL of fresh RCM in a 125 mL flask containing 10 g/L xylose and grown for 19 hours. This was subsequently transferred to 450 mL of fresh RCM containing 10 g/L xylose, and allowed to grow for 15 hours before being used to inoculate 4.5 L fermentation medium.

The fermentation strategy to produce butyric acid was described by Zhu and Yang (2004). The medium was described by Huang et al (1998). The medium contained the following (per liter): 40 mL of solution A; 40 mL of solution B; 10 mL of trace metals solution; 10 mL of vitamin solution; 10 mL of 0.005% $NiCl \cdot 6H_2O$; 1 mL of 0.2% $FeSO_4 \cdot 7H_2O$; 0.5 mL of 0.1% resazurin; 2 g of trypticase; 2 g of yeast extract. The pH was controlled at 6.3 using 5 M H_2SO_4 and 5 M KOH.

Solution A contained 7.86 g/L $K_2HPO_4 \cdot 3H_2O$. Solution B contained (per liter): 6 g of K_2HPO_4; 6 g of $(NH_4)_2SO_4$; 12 g of NaCl; 2.5 g of $MgSO_4 \cdot 7H_2O$; 0.16 g of $CaCl_2 \cdot 2H_2O$. The trace metal solution contained (per liter): 1.5 g of nitrilotriacetic acid; 0.1 g of $FeSO_4 \cdot 7H_2O$; 0.5 g of $MnSO_4 \cdot 2H_2O$; 1.0 g of NaCl; 0.1 g of $CoCl_2$; 0.1 g of $CaCl_2 \cdot 2H_2O$; 0.1 g of

$ZnSO_4 \cdot 5H_2O$; 0.01 g of $CuSO_4 \cdot 5H_2O$; 0.01 g of $AlK(SO_4)_2$; 0.01 g of H_3BO_3; 0.01 g of $Na_2MoO_4 \cdot 3H_2O$. The vitamin solution contained (per liter): 5 mg of thiamine-HCl; 5 mg of riboflavin; 5 mg of nicotinic acid; 5 mg of capantothenate; 0.1 mg of vitamin B_{12}; 5 mg of *p*-aminobenzoic acid; 5 mg of lipoic acid. Concentrated xylose was added to result in a final concentration of 30 g/L xylose in the medium.

Fermentations were conducted in a 7 L reactor (Labfors 4, Infors HT, Switzerland), with a 5 L filling volume. Sampling and analytics conducted identically to those described in Section 2.2.2.1

Fermentations were monitored via off gas emission, and once the CO_2 began to decrease, indicating exhaustion of the available xylose, 500 mL of 150 g/L xylose solution was added to the reactor. Upon the exhaustion of this second quantity of xylose, the fermentation was terminated. The process fluid was centrifuged in 500 mL aliquots for 30 minutes at 2739 x g in a Sorvall ST 40R centrifuge (Thermo Scientific). The supernatant was collected and autoclaved before being stored at 4^{o}C prior to analysis and use.

2.2.3) Experiments with Butyric Acid

Experiments were conducted in 150 mL Erlenmeyer flasks containing 30 mL combined medium and preculture. Butyric acid was added to the initial medium prior to autoclaving for use in experiments requiring butyric acid to be present from the onset of fermentation. Experiments requiring the delayed addition of pure butyric acid used a concentrated (100 g/L) stock that had been adjusted to a pH of 5.3 before autoclaving. Experiments requiring the supernatant from the xylose fermentation using *C. tyrobutyricum* used supernatant that had been adjusted to a pH of 5.3 before autoclaving. For all experiments using butyric acid, the concentrations were confirmed via high performance liquid chromatography prior to use. All flask experiments were conducted in an anaerobic chamber (Plas-Labs, Michigan, USA, model: 855-ACB-EXP) in which an anoxic environment was confirmed prior to the beginning of each experiment.

2.2.4) Statistical Methodology and Analysis

A central composite design was designed using the carbon sources of glycerol and molasses as the two factors in order to establish the optimal ratio resulting in high butanol selectivity. The uncoded values [low star point, low central point, central point, high central point, high star point] for glycerol were [10.34 12 16 20 21.66] and for molasses were [3.76 5 8 11 12.24], in g/L. The experimental design was developed using Design Expert 8.0.7.1 (Statease, Inc., Minneapolis, MS, USA) and resulted in 8 conditions, plus 5 center points. Conditions were tested in duplicate, and the resulting 26 conditions were randomized.

Linear regression analysis was used to fit the experimental data with a second-order model. Experimental data was analyzed using Design Expert 8.0.7.1 (Statease, Inc., Minneapolis, MS, USA). Each term was tested for significance using analysis of variance (ANOVA). The significance of the main effects, the interaction effect, and quadratic effects was determined based on a α of 0.05 using the F-test. The adequacy of the model was evaluated using normal probability plots, R^2 and adjusted R^2, and lack of fit. Design Expert was determined the optimal ratio of substrate for maximum butanol selectivity, and the optimal point was validated by triplicate experiment at the predicted optimum.

2.2.5) Substrate Screening and Scale-up

Fermentations for defining and validating the model were conducted in 150 mL flasks with a filling volume of 30 mL, as outlined in Section 2.2. Molasses and glycerol were added from separate stock solutions (200 g/L each) to allow precise addition of the carbon sources. Experiments were conducted in an anaerobic chamber (Plas-Labs, Michigan, USA, model: 855-ACB-EXP) at 35^{o}C, and performed on a shaking plate with a shaking frequency of 200 rpm and 50 mm shaking diameter (Multitron, Infors HT, Switzerland). Samples (1 mL) were taken at regular intervals, and centrifuged at 16 300 x g, before being stored at -20^{o}C until analysis according to Section 2.6.

Reactor scale experiments were conducted in a 7 L stirred tank reactor (Labfors 4, Infors HT, Switzerland) with a filling volume of 5 L. The agitation rate was set to 200 rpm. The flow rate for nitrogen purging was kept constant at 0.6 L / h using high purity nitrogen gas.

2.2.6) Analytical Methods

High performance liquid chromatography (HPLC) was used to determine the components within the fermentation broth. Liquid samples were filtered through 0.2 µm cellulose acetate filters, before being diluted with mobile phase to within the operational range of the HPLC. A Breeze™ 2 HPLC System from Waters was used (Waters Corp. Milford, USA), including an autosampler (Waters model 2707), a refractive index detector (Waters model 2414) and an isocratic pump (Waters model 1515). The method used a combination with the Hi-Plex-H guard and column from Agilent Technologies, Santa Clara, USA. The injection volume was 30 µL, column temperature was 50^{o}C and the refractive index detector temperature was 35^{o}C. The flow rate was 0.45 mL/min using 5 mM H_2SO_4 as the mobile phase.

In reactor fermentations, the pH was monitored using a pH probe (Hamilton, Reno, USA). The redox potential was measured using a redox probe (Mettler-Toledo, Delaware, USA). Off-gas analysis was done using a BlueSens BlueVary (Herten, Germany) equipped with CO_2 and H_2 sensors (Sensor ID: CO_2 – 30783; H_2 – 31068). Biomass measurements were conducted by filtering 5 mL sample through a cellulose filter (VWR CA28333-129) which had been previously dried and weighed. Following filtration, samples were rinsed with 10 mL H_2O before being dried (temperature of 80^{o}C) until a constant weight was achieved. Biomass samples were taken in duplicate for every sample.

2.3) Results and Discussion

2.3.1) Generation of butyric acid rich process fluid using xylose and *C. tyrobutyricum*

To generate the fermentation-derived butyric acid, a fed-batch fermentation was conducted using xylose as the carbon source, which resulted in a process fluid containing 23.5 g/L butyric acid and 3.8 g/L acetic acid (Figure 2.1). HPLC analysis showed no other detectable products in the supernatant. These results agree with similar published experiments using *C. tyrobutyricum* and xylose (Zhu and Yang 2004). The lack of any significant products other than butyric acid should allow for the process fluid to be used following sterilization only, without extraction and purification of the butyric acid. It is also possible that the concentration could be increased further with subsequent xylose feeding steps, which could

further reduce the number of times this secondary fermentation needs to be conducted while still supplying butyric acid for enhancing butanol fermentations.

Unfortunately, the pH required for high selectivity for butyric acid from xylose with *C. tyrobutyricum* is 6.3, significantly higher than the pH value at which *C. pasteurianum* produces high amounts of butanol (Johnson and Rehmann, 2015). This difference in pH prevents the adoption of a co-culture fermentation strategy between these two organisms.

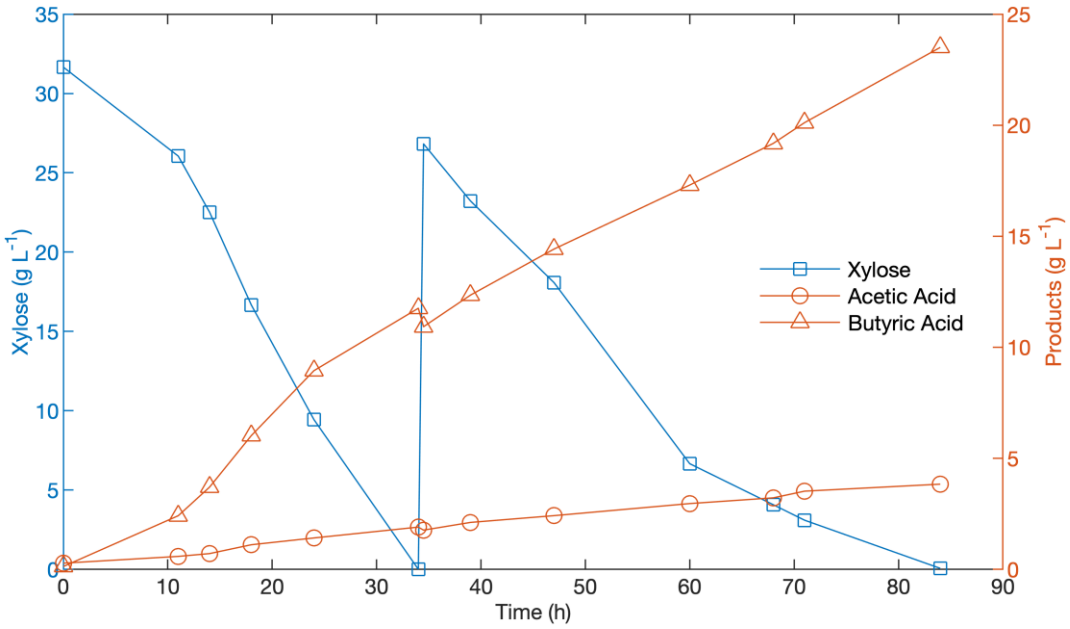

Figure 2.1: Xylose consumption and product formation in a fed-batch fermentation strategy of *C. tyrobutyricum* to produce butyric acid rich supernatant. Culture was grown in a 7L reactor with a 5L initial filling volume, and maintained at a pH of 6.3 to select for butyric acid production. Final values for detectable products were (in g/L): butyric acid = 23.5; acetic acid = 3.8.

2.3.2) Effect of butyric acid addition when added to the initial medium

The initial experiments were conducted to compare the effects of both chemically pure and xylose-derived butyric acid on the fermentation of glycerol to butanol. The volume of butyric acid rich supernatant was added to result in the desired final concentration of butyric acid in the experimental flask, without consideration of any additional compounds present.

When added in low concentrations (1 and 2 g/L) to the medium prior to inoculation, the butyric acid did not have a large effect on butanol selectivity. The ratio of mols carbon butanol / mols carbon consumed substrate for the sample without any butyric acid added was 0.449 mols C / mols C. For samples with 1 g/L butyric acid added, the ratios were 0.465 mols / mols C with pure butyric acid, and 0.448 mols C / mols C when using xylose-derived butyric acid. For samples in which 2 g/L butyric acid was added, the ratio was 0.454 mols C / mols C when using pure butyric acid, and 0.433 when using xylose-derived butyric acid. However, a distinct inhibitory effect on glycerol consumption and butanol production was observed, which increased with increasing concentrations of butyric acid. When no butyric acid was added to the medium, there was a small amount of butyric acid re-uptake (0.262 g/L); however, there was no observable uptake of butyric acid when added to the initial medium. Regestein et al found that the uptake of butyric acid occurred late in fermentations containing 30 and 45 g/L starting glycerol (Regestein *et al.* 2015). Similar results were reported by Gallardo et al (2014), with butyric acid uptake occurring only in samples containing 35 and 50 g/L crude glycerol, and a maximum of 28.18 g/L glycerol was consumed (Gallardo, Alves, and Rodrigues 2014). It is possible the lower starting glycerol concentration of only 20 g/L did not provide sufficient carbon to allow uptake of butyric acid.

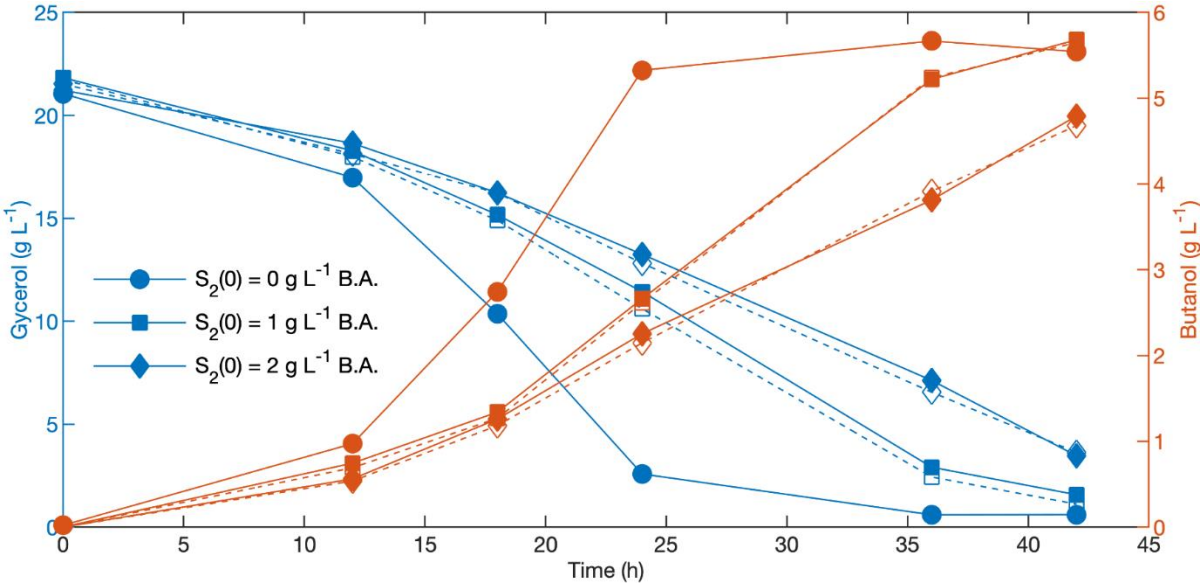

Figure 2.2: Growth of *C. pasteurianum* in medium containing differing amounts (0, 1 or 2 g/L) of chemically pure butyric acid (solid lines, filled symbols) or butyric acid rich supernatant from xylose fermentation (dotted lines, unfilled symbols). Blue line = substrate concentration; Orange line = butanol concentration. C-source: 20 g/L glycerol, T = 35°C, V_l = 30 mL, 0.05 M Citric Acid buffer added, pH_o=5.3. All conditions were tested in duplicate with the mean of the results presented. Deviation from the mean was less than 10% for all samples.

2.3.3) Effect of delayed addition of butyric acid to fermentation

As has been previously demonstrated in the literature, butyric acid uptake occurs at late stages in the fermentation providing an external carbon source is present (Regestein *et al.* 2015; Gallardo, Alves, and Rodrigues 2014). To that end, experiments were conducted in which butyric acid, either chemically pure or in the process fluid from xylose fermentation, was added at different times during fermentation of 20 g/L glycerol as a proof-of-concept for later experiments using delayed addition strategies.

When butyric acid was added while the concentration of glycerol in the fermentation remained high (approximately 16 and 13 g/L glycerol remaining), the butyric acid was not observed to be taken up by the cells (Figure 2.3). The concentration of butyric acid was actually observed to continue increasing after the addition of 1 or 2 g/L butyric acid, indicating that the cells continued to produce the acid despite the increased concentrations appearing in the medium. As there was no uptake of the butyric acid, the effect on the ratio of mols C product to mols C substrate consumed was minimal. When the butyric acid was added with 16 g/L glycerol remaining, samples with 1 g/L butyric acid added had ratios of 0.421 mols / mols C with pure butyric acid, and 0.427 mols C / mols C when using xylose-derived butyric acid. For samples in which 2 g/L butyric acid was added, the ratio was 0.447 mols C / mols C when using pure butyric acid, and 0.405 when using xylose-derived butyric acid. When the butyric acid was added at 18 hours PI, for all samples with 1 g/L butyric acid added, the ratios were 0.439 mols / mols C with pure butyric acid, and 0.439 mols C / mols C when using xylose-derived butyric acid (Table 2.1). For samples in which 2 g/L butyric acid was added at 18 hours post inoculation, the ratio was 0.430 mols C / mols C when using pure butyric acid, and 0.417 when using xylose-derived butyric acid. The difference between the

pure and xylose-derived butyric acid effects indicate that the xylose-derived butyric acid has a greater effect on the yield ratio, possibly due to the additional compounds present in the process fluid from the xylose fermentation with *C. tyrobutyricum*. As well, the presence of the added butyric acid reduced the amount of butyric acid produced by the cells when compared to samples without any added butyric acid, especially when 2 g/L xylose-derived butyric acid was added.

When butyric acid was when the glycerol concentration had reached approximately 8 g/L and the fermentation was well-established, uptake of the butyric acid was observed. Samples in which 1 g/L butyric acid was added resulted in just over 1 g/L butyric acid being taken up (1.03 when pure butyric acid added, and 1.07 when supernatant from xylose fermentation was added), leaving only small amounts of butyric acid (0.20 and 0.28 g/L) remaining in the medium. The ratio of mols carbon in butanol / mols carbon in consumed substrate was increased to 0.518 mols C / mols C when pure butyric acid was used, and 0.515 mols C / mols C when xylose-derived butyric acid was used. The amount of carbon found in the butyric acid taken up by cells was added to the mols of carbon in the substrate, in order to demonstrate the overall effect of increased carbon being metabolized to butanol. Samples in which 2 g/L butyric acid was added showed slightly increased acid uptake (1.22 and 1.21 g/L of pure and xylose derived butyric acid, respectively), with some butyric acid remaining in the medium at the end of the fermentation. This lack of complete uptake could be explained by depletion of the primary carbon source. The carbon ratios for each sample were 0.516 and 0.498 mol C / mol C (pure butyric acid and xylose-derived, respectively).

Table 2.1: Comparison of the butyric acid production/consumption, butanol production, and relative carbon yield following addition of butyric acid to fermentations at different points in the fermentation.

	Glycerol consumed in fermentation prior to B.A. addition (g/L)											
	4				7				12			
	1 g/L Pure B.A. added	1 g/L ferm. B.A. added	2 g/L Pure B.A. added	2 g/L ferm. B.A. added	1 g/L Pure B.A. added	1 g/L ferm. B.A. added	2 g/L Pure B.A. added	2 g/L ferm. B.A. added	1 g/L Pure B.A. added	1 g/L ferm. B.A. added	2 g/L Pure B.A. added	2 g/L ferm. B.A. added
B.A. Produced / Consumed (g/L)	1.002	0.962	0.923	0.499	1.011	1.082	1.103	0.689	*-0.916*	*-0.935*	*-1.22*	*-1.084*
Butanol Produced at end of fermentation (g/L)	5.134	5.021	4.527	4.414	4.893	5.117	4.802	4.706	6.761	6.723	6.668	6.525
Relative Carbon Yield (mols C product / mols C substrate consumed)	0.421	0.427	0.447	0.405	0.439	0.439	0.430	0.417	0.518	0.515	0.516	0.498

B.A.: Butyric Acid
Ferm.: Fermentation-derived
Negative value: Indicating net uptake of butyric acid

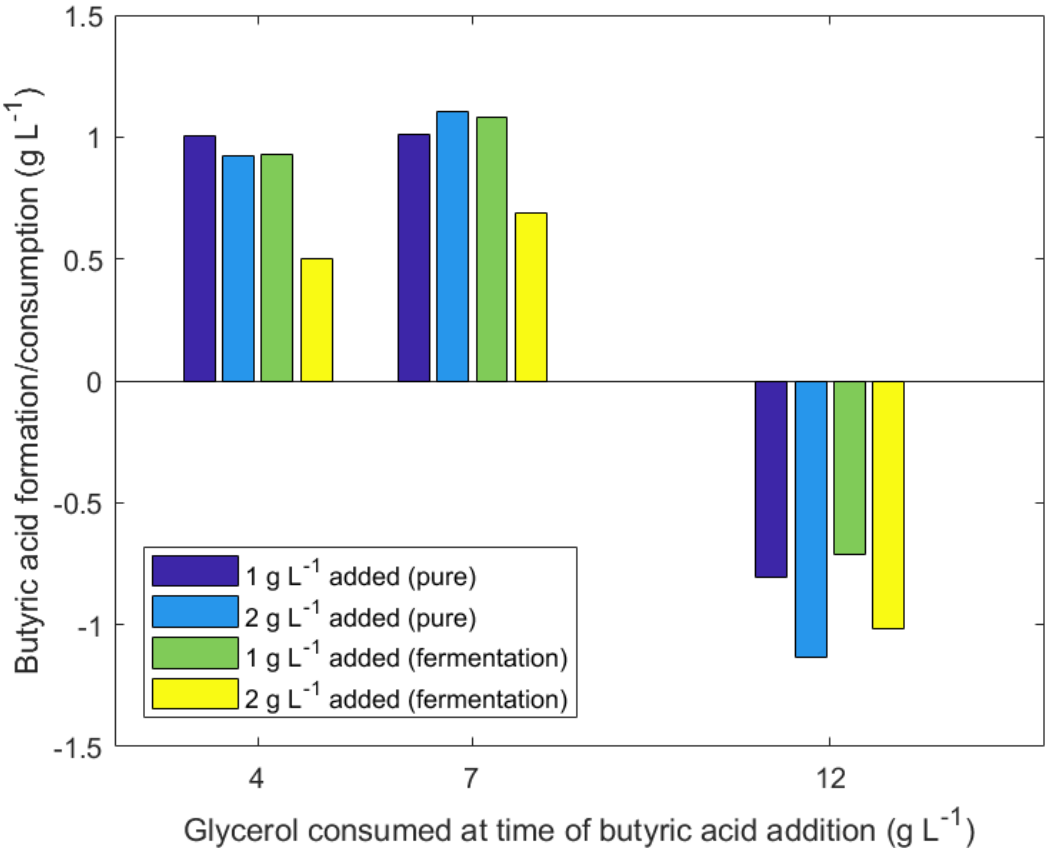

Figure 2.3: Comparison of the amount of butyric acid produced or consumed when added during fermentation with differing amounts of remaining glycerol.

2.3.4) Optimization of glycerol and molasses ratio

Direct addition of butyric acid, even produced using fermentation of waste xylose sources, could still result in high costs for butanol fermentation either from expensive materials or having to run a preliminary fermentation to create the butyric acid in a separate vessel. A preferred method would be for a single-vessel fermentation without a requirement for dosed addition of chemicals, which could be achieved through the introduction of an additional substrate to the fermentation. Dual-substrate fermentations have been conducted in which *C. pasteurianum* is grown on a mixture of sugar and glycerol, allowing the cells to first produce the butyric acid from the sugar, and subsequently uptake the acid to aid butanol productivity and selectivity (Sabra *et al.* 2014, 2016). Here, we utilized sugar beet molasses as a low-cost source of the sugars, rather than pure dextrose. An optimal ratio of molasses to glycerol is

required, as too much molasses results in inhibitory concentrations of butyric acid being produced, and too little would not significantly affect the relative carbon yield of butanol.

The ratio of carbon available from glycerol and molasses was chosen based on a central composite design, and the actual values for the variables, and the measured responses, are given in Table 2.2. Each run was conducted in duplicate and the averages are reported here.

Table 2.2: Relative Carbon Yield under conditions determined for identification of an optimum ratio. All conditions were tested in duplicate and the average values are reported here. Deviation from the mean was less than 10% for all samples.

Run	Glycerol	Molasses	Butanol	Relative Carbon Yield
1	16	8	6.108	0.458
2	16	8	6.089	0.458
3	16	8	6.231	0.463
4	20	5	6.588	0.445
5	16	3.75736	5.107	0.429
6	12	11	4.227	0.342
7	16	12.2426	6.211	0.389
8	16	8	6.122	0.457
9	21.6569	8	8.063	0.431
10	20	11	7.494	0.413
11	12	5	3.663	0.329
12	10.3431	8	3.537	0.296
13	16	8	6.101	0.453

As observed from these results, the selectivity for butanol is affected by the ratio between glycerol and molasses within the range of the variables.

The complete dataset was used with a fitted quadratic model, the results of which are in Table 2.3. The model constraints were to maximize both the final butanol concentration as well as the relative carbon yield. The *F* value of the model is 202.58, indicating the model is highly significant. The parameter coefficient for both glycerol and molasses, the interaction effect between glycerol and molasses, and the quadratic effects of glycerol and molasses had *p*-values below 0.05 and were all significant.

Table 2.3: ANOVA table of model for relative carbon yield.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-Value	p-value	Comment
Model	0.0378	5	0.0050	202.58	< 0.0001	significant
A-Glycerol	0.0179	1	0.0110	478.55	< 0.0001	significant
B-Molasses	0.0007	1	0.0002	19.13	0.0033	significant
AB	0.0005	1	0.0004	13.57	0.0078	significant
A^2	0.0161	1	0.0111	432.32	< 0.0001	significant
B^2	0.0045	1	0.0037	120.30	< 0.0001	significant
R-squared	0.993					
Adj-squared	0.988					
Adeq Precision	39.3					

Based on this model, the quadratic model including all significant and non-significant factors, with actual values for the factors, for the Relative Carbon Yield is:

Relative Carbon Yield = -0.777 + 0.116•Glycerol + 0.057•Molasses – 0.003•$Glycerol^2$ – 0.003•$Molasses^2$

Residuals can be judged as normally distributed on a normal probability.

A surface plot of the effects of the concentrations of glycerol and molasses on the relative carbon yield for this system is shown in Figure 2.4. The plots indicate that an optimum exists within the observable design space between these two variables.

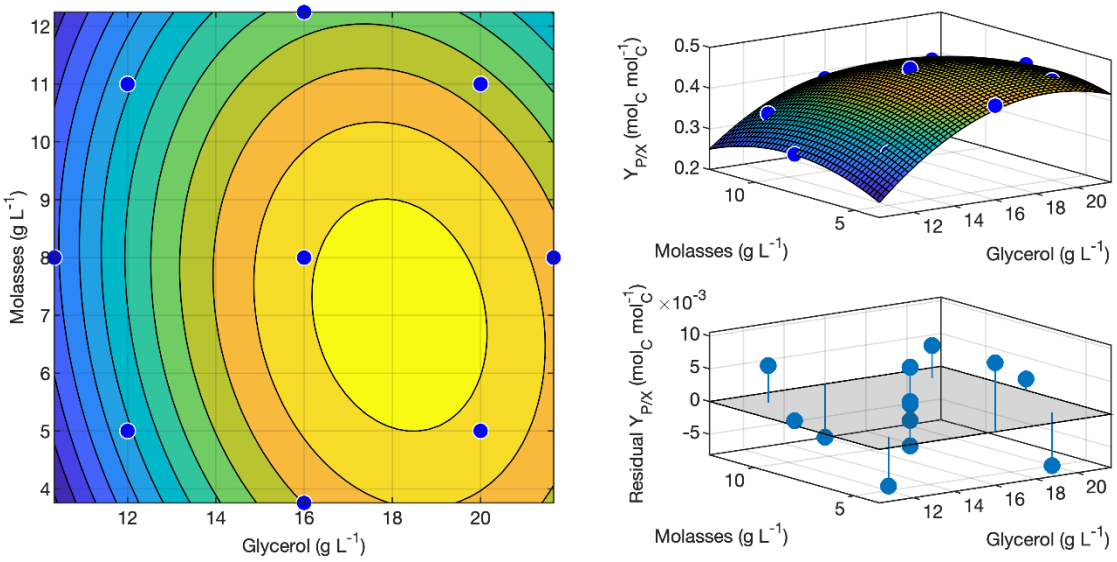

Figure 2.4: Surface and residual plots of effects of amount of glycerol and molasses in starting medium on the relative carbon yield.

2.3.5) Model Validation

Based on this model, numerical optimization was used to determine the ratio of glycerol and molasses that would result in the highest relative carbon yield as well as the highest butanol concentration. The optimum starting concentrations given were 19.32 g/L glycerol and 8.02 g/L molasses.

Experiments were conducted using these concentrations, and compared to the predicted values for the relative carbon yield. The final butanol concentration for these fermentations was on average 7.15 g/L, with 1.90 g/L butyric acid produced early in the fermentation from the consumption of molasses sugars. Butyric acid was taken back up by the cells, and the final concentration was 0.11 g/L butyric acid, meaning 1.80 g/L was taken back up between 12 and 24 hours Post-Inoculation (Figure 2.5). The results of these validations were within the bounds of the prediction interval, and were in close agreement with the predicted mean (0.460 mol C substrates / mol C butanol predicted vs. 0.459 mol C substrate / mol C butanol observed) and can be seen in Table 2.4.

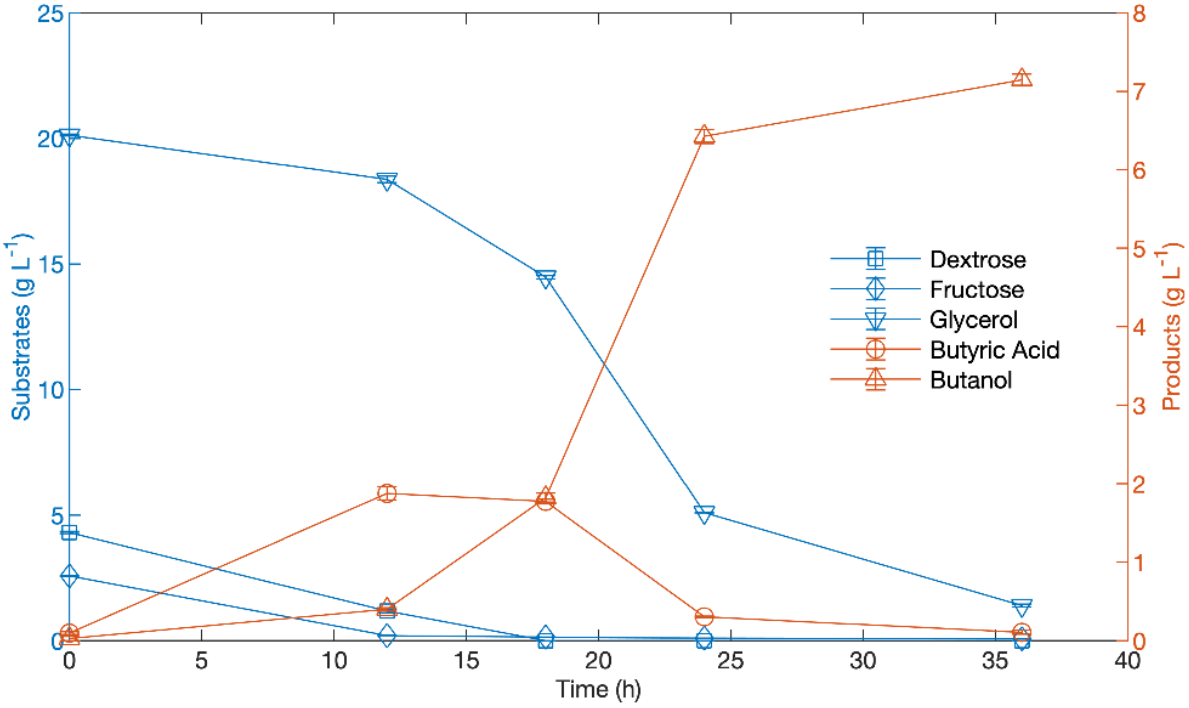

Figure 2.5: Fermentation products at the optimum glycerol:molasses ratio. Butyric acid is produced from the sugars present, then subsequently taken back up by the cells to increase the productivity of butanol. Conditions were tested in triplicate with the mean of the results presented. Error bars represent standard deviation from the mean.

Table 2.4: Comparison of the predicted and observed relative carbon yield at the optimum ratio point

	Predicted Mean	**Observed Mean**	**95% PI low**	**95% PI high**
Relative Carbon Yield	0.460	0.459	0.449	0.471

2.3.6) Testing dual-substrate at reactor scale with pH control

Following validation of the model, fermentation at the reactor scale was conducted to allow analysis of the gaseous products and greater process control, primarily control of the pH. The effects of pH on *C. pasteurianum* fermentations using glycerol as the carbon source have

been shown to alter the product profile observed (Johnson and Rehmann 2016). The pH was held at 5.3, as that would allow 25% of the butyric acid to be fully protonated and thus facilitate assimilation by the cells (Regestein *et al.* 2015).

The fermentation progressed in a similar fashion to fermentations conducted at the shake flask scale. The molasses sugars were consumed and converted largely to butyric acid in the first 8 hours PI (Figure 2.6), reaching a maximum concentration of 2.48 g/L. The butyric acid produced was subsequently taken up by the cells, reaching a final concentration of 0.172 g/L, an uptake of 2.3 g/L. The resulting final butanol concentration was 7.82 g/L, corresponding to a relative carbon yield of 0.480 mols C butanol / mols C substrate. This value is even greater than predicted, indicating that control of the pH throughout the fermentation had an overall positive effect. The fermentation time was shorter when compared to shake flask, with fermentations in the reactor being complete in 20 hours compared to those in flasks, in which not all the glycerol had been consumed after 24 hours. This can be attributed to growing pH inhibition within the flasks as the fermentation proceeds (Erin Johnson and Rehmann 2016). An identical fermentation at the reactor scale using only 20 g/L glycerol as the carbon source and no molasses sugars had a final butanol concentration of 5.62 g/L, and a relative carbon yield of only 0.439 mols C butanol / mols C substrate.

Off-gas analysis showed distinct decreases in the gas production at the depletion of the molasses sugars, followed by an increase of the CO_2 while the H_2 remained significantly lower. This decoupling behavior has been seen previously, and could be attributed to the downregulation of two non-ferredoxin-dependent hydrogenases following depletion of the sugars (Munch *et al.* 2020b; Sabra *et al.* 2016).

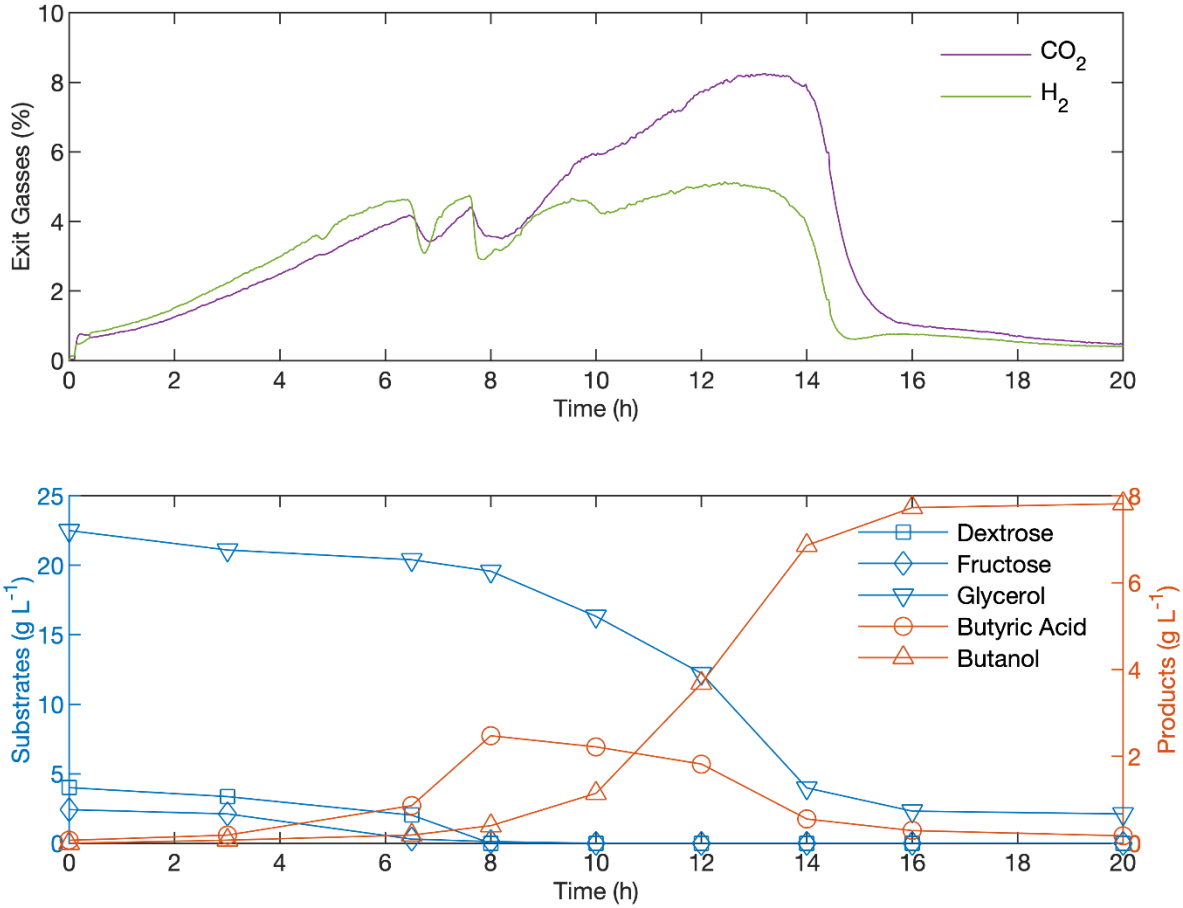

Figure 2.6: Top: Off gas (CO_2 and H_2) of the molasses/glycerol fermentation with *C. pasteurianum*. First drop in gas production corresponds to depletion of fructose, while second drop corresponds to depletion of dextrose. Bottom: Products of fermentation over time. Butyric acid is produced initially from metabolizing the sugars available, then subsequently taken back up to increase butanol productivity. Conditions were tested in duplicate, with the mean of the results presented. Deviation from the mean was less than 5% for all samples.

Table 2.5: Comparison of the relative carbon yield for dual-substrate fermentations (ratio of carbon in the available substrates over the carbon in the main product, butanol)

	Mols sugar substrate consumed	Mols glycerol consumed	Mols butanol produced	Relative carbon Yield (mols C consumed / mols C butanol)
Kao *et al.*	0.11	0.65	0.18	0.274
Sabra *et al.*	0.28	0.54	0.28	0.344
This work	0	0.23	0.076	0.439
This work	0.04	0.23	0.11	0.480

2.4) Conclusions

The effects of additional butyric acid from various sources increased the selectivity for butanol in fermentations using *C. pasteurianum* and glycerol as the main carbon source. When added in low concentrations (1 or 2 g/L), the effect was time-dependent. Early addition resulted in inhibition of the fermentation, without a large effect on the relative carbon yield. Addition at 24 hours post-inoculation resulted in the highest uptake of the butyric acid, and an increase in yield to a maximum of 0.512 mols carbon butanol / mols carbon substrate on average. This effect was consistent for both chemically pure butyric acid, as well as butyric acid rich supernatant from a separate fermentation. A dual-substrate fermentation strategy in which *C. pasteurianum* produced butyric acid using molasses in order to increase selectivity for butanol when glycerol was consumed was optimized. This system resulted in a calculated yield of 0.480 mols carbon butanol / mols carbon substrate, an increase from 0.425 mols carbon butanol / mols carbon substrate when using only glycerol as the carbon source, and a higher yield when compared to other dual-substrate fermentations in literature. Using a dual substrate process, centered around the production and re-uptake of butyric acid, could be used to create a highly selective butanol fermentation strategy and utilize a novel carbon source previously unused for butanol production with *C. pasteurianum*.

Chapter 3

Characterization and sequencing of highly productive *Clostridium pasteurianum* mutant cultures.

Garret Munch, Erin Johnson, Lars Rehmann

Portions of this research chapter appear in the successful Genomic Applications Partnership Program titled “Strain development for butanol process addition to existing biodiesel plants”, a partnership between researchers at the University of Western Ontario, Genome Canada, and World Energy Hamilton.

This chapter describes work using a putative mutant strain of *C. pasteurianum* that was generated in the lab via random mutagenesis in a continuous fermentation by E. Johnson in 2015. Batch fermentations were conducted to allow direct comparisons between the putative mutant and wild-type. The putative mutant displayed advantageous properties when compared to the wild-type *C. pasteurianum,* such as increased butanol productivity, faster growth rates, and a product profile more directed towards butyric acid and butanol production.

DNA was extracted from the putative mutant samples and the wild-type stock strain in the laboratory for comparison. The DNA was sequenced and analyzed to attempt to identify mutations of interest which could explain the advantageous phenotype of the putative mutant. DNA comparison between the putative mutant, the laboratory stock wild-type, and published reference genomes revealed clusters of mutations which were common between the putative mutants sampled and not found in either the laboratory wild-type or the published reference genome.

The research described in this chapter fulfills the third objective of this thesis. The growth behavior of the organisms was compared when grown in identical conditions in a bioreactor. The DNA was isolated, and variants on the mutants genomes were located and their products (where available) were identified.

Abstract: Novel genetic tools allow mutagenic work to be done to improve the performance of fermentative organisms. Work with *Clostridium pasteurianum* has produced mutants with

higher growth rates, increased butanol yield, increased glycerol uptake, and other beneficial characteristics superior to those found in the wild-type organism. A previous study conducted by E. Johnson resulted in a potential mutant strain of *C. pasteurianum* which displayed higher butanol production in continuous culture, with no synchronized oscillating metabolism (E. Johnson, personal communication, 2018). Samples of the putative mutant population was obtained from E. Johnson, and the growth characteristics of the culture was determined in batch fermentations. DNA was isolated for sequencing and identification of variants between the putative mutant and the wild-type. Growth characterization of the putative mutants showed increased growth rate and selectivity towards the butyric acid/butanol fermentative pathway. Variant analysis showed 29 common variants in the samples sequenced compared to the wild-type, with several unique variants being present in each sample. This non-homogeneity indicates that the samples are not of a pure culture, but of a community of genotypes, some of which display beneficial phenotypes.

3.1) Introduction

While butanol production from glycerol by *Clostridium pasteurianum* suffers from notable challenges, namely low productivity and yield, several strategies are under investigation to overcome these issues (Dabrock, Bahl, and Gottschalk 1992; T. Ø. Jensen *et al.* 2012; Khanna, Goyal, and Moholkar 2013; Zheng *et al.* 2013; Moon *et al.* 2011a). Research has been conducted into both up and downstream processes which can address the low overall productivity of the fermentation process. One upstream strategy to increase the productivity or yield of butanol production by *C. pasteurianum* previously and currently under investigation is via mutagenesis (Malaviya, Jang, and Lee 2012; T. Ø. Jensen *et al.* 2012; Pyne *et al.* 2013; Schwarz *et al.* 2017). Several studies have been conducted using either site-directed or random mutagenesis to create highly productive and selective strains of *C. pasteurianum* (Luers *et al.* 2006, 1997; Sun *et al.* 2003).

Malaviya *et al.* used non-specific mutagenesis via *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine to produce a several mutant strains of *C. pasteurianum,* which were then screened for heightened for increased butanol production (Malaviya, Jang, and Lee 2012). The most improved strain produced 10.8 g/L butanol compared to 7.6 g/L from the parent strain, when grown in batch culture on 80 g/L glycerol. This strain was also used for 710 hours in a

continuous fermentation, producing high amounts of butanol with little by-products, and displayed no strain degradation (Malaviya, Jang, and Lee 2012). Another study using ethane methyl sulfonate to induce random mutagenesis developed a strain which was better able to tolerate impurities found in biodiesel derived crude glycerol than the parent strain (T. Ø. Jensen *et al.* 2012; T. O. Jensen *et al.* 2012). In addition to this improved tolerance, the mutated strain displayed a higher butanol productivity compared to the parent strain in a continuous fermentation (1.80 g/L/h vs. 1.30 g/L/h, respectively).

In contrast to random mutagenesis, other studies have used targeted genetic methods in order to specifically target genes which are directly involved with the conversion of glycerol to butanol. These studies take advantage of the publication of the complete *C. pasteurianum* genome to find these gene sequences and create tools for altering them (Poehlein *et al.* 2015; Pyne *et al.* 2013; Pyne, Liu, *et al.* 2016). Pyne *et al.* recently provided an extremely detailed breakdown of the metabolism of *C. pasteurianum*, with identification of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) loci (Pyne, Liu, *et al.* 2016). Targeted gene deletion used by Schwarz *et al.* allowed for a complete knock-out of the 1,3 PDO pathway in *C. pasteurianum*, which ultimately resulted in poor growth of the mutant (Schwarz *et al.* 2017). However, by manipulating genes encoding for redox-dependent proteins, the authors were able to produce mutants which had widely different behavior and product ratios. Other genetic work has focused on increasing the hydrogen production through manipulation of a hydrogenase enzyme (Sarma *et al.* 2019). This same work also increased the glycerol uptake rates of *C. pasteurianum* by overexpressing two specific genes (*dhaD1* and *dhaK* – encoding glycerol dehydrogenase and dihydroxyacetone kinase, respectively), a phenotype that would be of interest in butanol production as well.

Previous attempts at establishing a continuous, steady state fermentation had only been achieved under conditions not desirable for industrial application (low glycerol feed concentration). Other conditions resulted in a self-synchronized oscillatory metabolism as shown by the off-gas production (CO_2 and H_2) and the redox potential during on-line monitoring in continuous fermentation (E. E. Johnson and Rehmann 2020). Samples of a *C. pasteurianum* population, following a mutagenesis in the continuous reactor was provided by E. Johnson for work conducted in this thesis (E. Johnson, personal communication, 2018).

Further study of this putative *C. pasteurianum* mutant was conducted, beginning with a direct comparison of the growth characteristics and product profile, and a complete sequencing of the genomes of the putative mutant and lab stock wild-type strain for identification of variations in the genomes. Sequences were also compared to the published reference genome.

3.2) Materials and Methods

3.2.1) Chemicals

Yeast extract, peptone, ammonium sulfate, KH_2PO_4, K_2HPO_4, and glycerol were purchased from Fischer Scientific. Beef extract was obtained from BD-Becton, Dickinson and Company (New Jersey, USA). Soluble starch, sodium acetate, resazurin, and thiamine were purchased from Alfa Aesar (Massachusetts, USA). Dextrose was from Amresco (Ohio, USA) and $CaCl_2$ was from EMD Millipore (Massachusetts, USA). $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, were purchased from Caledon (Ontario, Canada). Pure glycerol, $FeSO_4 \cdot 7H_2O$, NaCl and l-cysteine were obtained from BDH (Georgia, USA). All other chemicals were purchased from Sigma-Aldrich.

3.2.2) Organism and Medium

Clostridium pasteurianum DSM 525 was originally purchased from the DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cultures were revived using Reinforced Clostridium medium (RCM) containing (per liter): 10 g peptone, 10 g beef extract, 3 g yeast extract, 10 g dextrose, 5 g NaCl, 1 g soluble starch, 0.5 g l-cysteine, and 4 ml resazurin, at pH 6.8 until reaching a high density before 1 mL aliquots were stored at -80^{o}C with 20% v/v glycerol added to the medium. All samples of the putative mutant *C. pasteurianum* strain were provided by E. Johnson for use in this work.

For preculturing both the putative mutant and wild-type strains of *C. pasteurianum,* thawed 1 mL aliquots were added to 9 mL fresh RCM and incubated at 35^{o}C for 20 hours in 10 mL sterile tubes. Following this, 10% v/v inoculum was added to fresh RCM containing 10 g/L pure glycerol as the carbon source in a 150 mL Erlenmeyer flask. Cultures were grown for 8 hours before being transferred once again at 10% v/v to fresh RCM with 10 g/L pure glycerol

in a 1 L glass bottle with a 500 mL total volume. Pre-cultures were allowed to grow another 8 hours before being used for inoculation of 4.5 L medium in a 7 L fermenter.

3.2.3) Sequencing of putative mutant and laboratory wild-type strain

Four putative mutant samples were prepared for sequence in parallel with four laboratory wild-type cryogentic stocks. The putative mutant samples were denoted E53J_2 and E53J_3 (samples isolated by E. Johnson in July 2016) and E53D_1 and E53D_3 (samples isolated by E. Johnson in December 2015), while the wild-type samples were designated WT_1 through WT_4. Samples were revived according to Section 2.2.2.1, however following the 20- hour initial growth, 5 mL of pre-culture was used to inoculate 45 mL fresh RCM containing 20 g/L dextrose as the carbon source, taking samples regularly in order to perform a cell count. Once the count reached approximately $2x10^9$ cells / mL, the samples were centrifuged and the DNA extracted.

DNA extractions were conducted using an Invitrogen Purelink Genomic DNA minikit (ThermoFisher Scientific), following the protocol as outlined for gram positive bacteria. In brief, the cells were lysed using the lysozyme digestion buffer (25 mM Tris-HCL, pH 8.0, 2.5 mM EDTA, 1% Triton X-100, 20 mg/mL lysozyme) at 37^{o}C for 30 minutes. Proteinase K and the Genomic Lysis buffer were then added, and the mixture was incubated at 55^{o}C for 30 minutes before 100% ethanol was added and the solution was vortexed. The mixture was then added to a PureLink Spin Column and centrifuged at 10 000 x g for 1 minute to bind the DNA to the column. The bound DNA was washed twice, then the DNA was eluted into a sterile and DNAse-free microcentrifuge tube. Eluted DNA samples were stored at 4^{o}C. Prior to sequencing, the DNA was precipitated using 0.5x DNA sample volume of 7.5 M ammonium acetate and 2.5x sample volume of 100% ethanol, and chilling at -20^{o}C overnight. The samples were then centrifuged at 12 000 x g for 30 minutes at 4^{o}C, supernatant discarded, and the pellet washed twice with 80% v/v ethanol (centrifuging once again after washes). The final washed pellet was allowed to air-dry and resuspended in DNA/RNAse free water.

DNA was sequenced using a NextSeq Mid Output 150 cycle kit (Illumina, San Diego, USA) and the libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina,

San Diego, USA). This work was conducted with the London Regional Genomic Centre, a part of the Robarts Research Institute. Completed genome sequences were uploaded to Illumina BaseSpace Sequence Hub until required for further analysis.

3.2.4) Analysis and comparison of Genomes

Genome sequences were uploaded to the analysis software Partek Flow (Partek, St. Louis, USA) for comparison. Sequences were aligned using BWA-MEM aligner (version 0.7.12) using the published reference genome (NZ_CP009268.1) and subsequently the selected variant caller used was SamTools (version 1.4.1). The annotation model used was CP009268.1. Each putative mutagenic genome was compared to the laboratory wild-type genomes and searched for variants, with the subsequent genomic information being provided by the annotated genome.

3.2.5) Characterization of growth kinetics and product profile

Characterization experiments were conducted at the reactor scale in a 7 L stirred tank reactor (Labfors 4, Infors HT, Switzerland) with a filling volume of 5 L. The stirring rate was set to 200 rpm. The flow rate for aeration was kept constant at 0.6 L / h using high purity nitrogen gas. Pre-culturing was conducted as described in Section 3.2.2

Fermentations were conducted using a modified medium published by Biebl (2001) and containing in g/L (unless stated otherwise): glycerol, 30; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4$, 0.05; yeast extract, 1.0. 2 mL of trace element solution was added per liter of medium, the trace element solution containing (in g/L): $FeCl_2 \cdot 4H_2O$, 1.5, dissolved in 25% HCl solution; $CoCl_2 \cdot 6H_2O$, 0.19; $MnCl_2 \cdot 4H_2O$, 0.1; $ZnCl_2$, 0.07; H_3BO_3, 0.062; $Na_2MoO_4 \cdot 2H_2O$, 0.036; $NiCl_2 \cdot 6H_2O$, 0.024; $CuCl_2 \cdot 2H_2O$, 0.017. All experiments maintained the pH of 5.3, adjusted using 5 M H_2SO_4 or 5 M KOH.

3.2.6) Numerical analysis of growth kinetics

Growth associated product formation and constant yield coefficients were assumed for the conversion of glycerol to new cell mass and butanol. The formation and possible re-uptake of by-products and intermediates was not considered in the numerical analysis. The growth was

assumed to follow simple Monod kinetics and product inhibition was assumed not to be present over the investigated concentration range.

Hence substrate consumption, cell and product formation could be described using the following coupled differential equations:

$$\frac{dS}{dt} = -\frac{1}{Y_{\frac{X}{S}}}\frac{dX}{dt} \quad (3.1)$$

$$\frac{dX}{dt} = X\frac{\mu_{max}S}{K_S+S} \quad (3.2)$$

$$\frac{dP}{dt} = -Y_{\frac{P}{S}}\frac{dS}{dt} \quad (3.3)$$

Where S = glycerol (g/L), X = cell mass (g/L), P = butanol (g/L), $Y_{X/S}$ and $Y_{P/S}$ are the cell mass and butanol yield coefficients (g/g), μ_{max} (h^{-1}) is the maximum specific growth rate and K_S (g/L) the half-saturation constant. Equations 3.1-3.3 were solved numerically for the initial conditions X_0, S_0 and P_0 at t=0 based on the conditions in the respective reactor (`ode23`, Mathworks, MA). The yield coefficients were calculated based on the final values for X, S, and P while μ_{max} and K_S were estimated via non-linear regression of the time course data versus the numerical ODE solutions using the Levenberg–Marquardt algorithm (`fit`, Mathworks, MA).

3.2.7) Analytical Methods

High performance liquid chromatography (HPLC) was used to determine the components within the fermentation broth. Liquid samples were filtered through 0.2 µm cellulose acetate filters, before being diluted with mobile phase to within the operational range of the HPLC. A Breeze™ 2 HPLC System from Waters was used (Waters Corp. Milford, USA), including an autosampler (Waters model 2707), a refractive index detector (Waters model 2414) and an isocratic pump (Waters model 1515). The method used a combination with the Hi-Plex-H

guard and column from Agilent Technologies, Santa Clara, USA. The injection volume was 30 µL, column temperature was 50°C and the refractive index detector temperature was 35°C. The flow rate was 0.45 mL/min using 5 mM H_2SO_4 as the mobile phase.

In reactor fermentations, the pH was monitored using a pH probe (Hamilton, Reno, USA). The redox potential was measured using a redox probe (Mettler-Toledo, Delaware, USA). Off-gas analysis was done using a BlueSens BlueVary (Herten, Germany) equipped with CO_2 and H_2 sensors (Sensor ID: CO_2 – 30783; H_2 – 31068). Biomass measurements were conducted by filtering 5 mL sample through a cellulose filter which had been previously dried and weighed. Following filtration, samples were rinsed with 10 mL H_2O before being dried until a constant weight was achieved. Biomass samples were taken in duplicate for every sample.

3.3) Results and Discussion

3.3.1) End-product differences between putative mutant and wild-type

A comparison of the growth and products formed between the putative mutants and the laboratory wild-type strain was conducted in batch fermentations. The desired behavior for the putative mutant was faster growth and a product profile that was more heavily weighted to the production of butanol.

Both of the putative mutants tested (E53J and E53D) displayed differences in the soluble products produced when grown in an identical batch fermentation to the laboratory wild type (Table 3.1). Of note, while the amount of butanol produced by the wild-type was higher on average compared to the putative mutants (9.133 g/L vs 8.780 g/L, respectively), the final amount of butyric acid produced differed significantly (0.224 g/L vs. 1.563 g/L). This shows a marked change in the metabolism, with increased flux of carbon towards the butanol/butyric acid pathway and less carbon being used for by-products. As described in Chapter 1 and elsewhere in literature, butyric acid can be converted to butanol, indicating that the overall butanol concentration could be further increased under the proper conditions (Sabra *et al.* 2016; Kao *et al.* 2013; Regestein *et al.* 2015). All other products were produced in similar amounts, with the exception of biomass being slightly higher in fermentations using the putative mutant.

Table 3.1: Comparison of the soluble products of wild-type and putative mutant strains of *C. pasteurianum*

	Wild-Type 1	Wild-Type 2	E53J	E53D
Acetic Acid (g/L)	0.394	0.413	0.541	0.542
1,3 PDO (g/L)	0.807	0.987	0.894	0.957
Ethanol (g/L)	0.503	0.396	0.37	0.31
Butyric Acid (g/L)	0.242	0.206	1.463	1.662
Butanol (g/L)	9.223	9.043	8.928	8.631
Time to fermentation completion (h)	18	16.5	15.5	13.5
Biomass (g/L)	2.81	2.84	3.05	2.96

3.3.2) Growth kinetics of putative mutant and wild-type

A comparison of the growth profiles for the two wild-type samples (WT 1 and 2) and the putative mutant strains (E53 J and D) is presented in Figure 3.1. From the offline data, the mutant strains seem to show a faster growth rate, based on the complete consumption of the glycerol present in the medium. The datasets were combined into two datasets, one for the wild-type samples and one for the putative mutant strains, and the solid line in the Figures is the best fit result using equations 3.1 – 3.3. As shown in the figure, the model fits the data very well, with an adjusted R^2 of 0.998 for the putative mutant dataset and 0.995 for the wild-type data. The parameters estimated by the model (with the 95% confidence bounds in brackets) are for the wild-type strain: K_S = 27.60 (-55.27, 110.71) and μ_{max} = 0.6749 (-0.7129, 2.063) and for the mutant strain: K_S = 11.1 (-4.51, 26.7) and μ_{max} = 0.3816 (0.115, 0.648). Based on this dataset, the mutant strains have the lower specific growth rate. However, it must be noted that batch fermentations are not suitable for estimations of Monod parameters. The parameters K_S and μ_{max} are highly correlated and are best estimated in specifically design experiments in continuous culture, in which the dilution rate is equal to the specific growth rate. By gradually increasing the dilution rate until washout of cells occurs, the maximum specific growth rate can be determined. The influence of the parameter K_S is only observed at substrate concentrations of similar and lower values than K_S, which is

typically <<1 g/L. Such low substrate concentrations only occur at the end of a batch fermentation, when the overall rates are high, due to large amounts of cell mass. Therefore, off-line data acquired every few hours, as in this data-set is typically not suitable to estimate K_S, and consequently, are not suitable to estimate μ_{max}. The large confidence bound of the values shown above is a direct result of the high correlation between the two parameters. As a result, the data presented here cannot be used for a direct comparison between the strains.

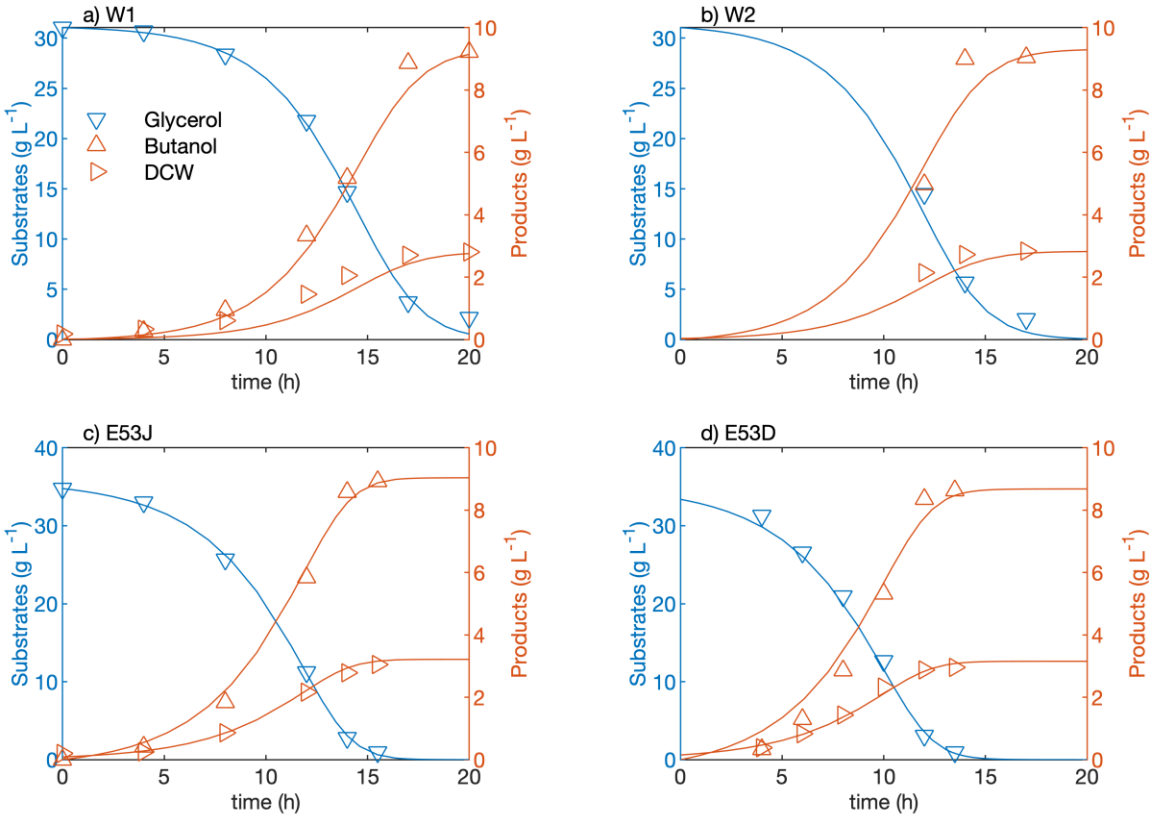

Figure 3.1: Glycerol consumption, butanol and cell biomass production over time of reactor-scale fermentations using two *C. pasteurianum* wild-type samples (top, graphs A and B) and two putative mutant samples (bottom, C and D). Solid lines are the best fit results for the consumption of glycerol and production of butanol and cell biomass.

The bioreactors used for the fermentations displayed in Figure 3.1 were also equipped with off-gas analyzers, allowing for further comparison of the gaseous products of the two strains. In addition to differences noted in the soluble products, differences in the CO_2 production rates were observed. The putative mutant strains (E53J1 an E53D1) displayed a 11.8% increase in the maximum CO_2 production rate over the wild-type strain (Original A and B) (Figure 3.2). As the formation of gasses is associated with butanol fermentation (see metabolic pathway in Figure 1.3), this increase in CO_2 production can be considered to reflect the metabolic rates of the organisms in the fermentations, and would reflect an increase in the glycerol consumption and butanol production rates (Munch *et al.* 2020b). Experiments in which a carbon balance could be performed in order to compare the efficiency of the two strains (yield of butanol per glycerol consumed) would also be beneficial as a determinant for which strain to use in further works.

As a result of the increased CO_2 production rates, a continuous fermentation strategy would hypothetically have higher overall productivity when using the putative mutant strain over the laboratory wild type. This, along with the behavior observed with the glycerol consumption and butanol formation rates, is further evidence that the putative mutant will have a faster growth rate and product production rate in continuous fermentation.

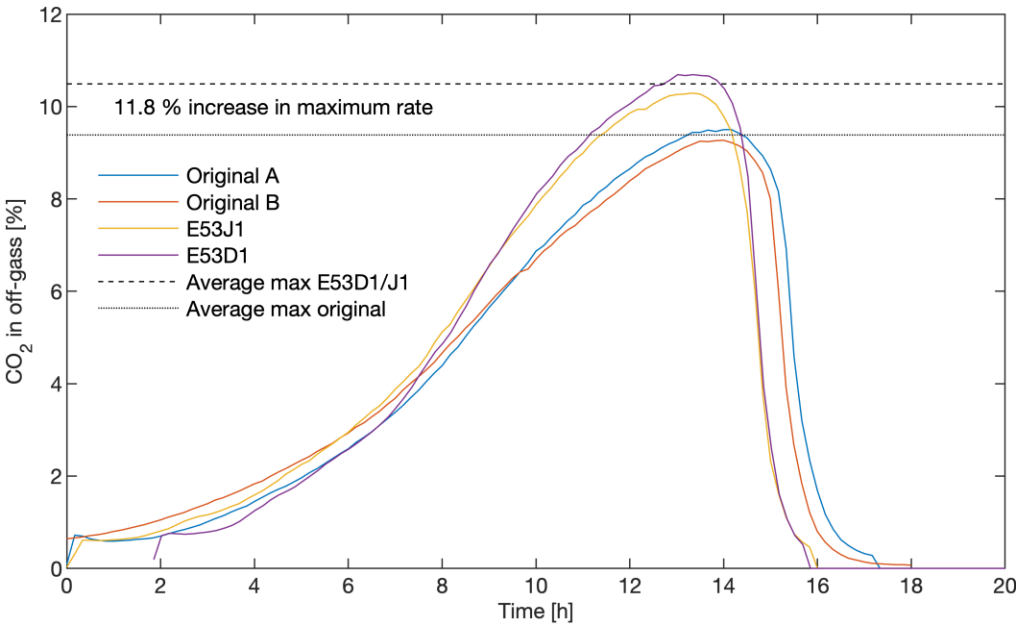

Figure 3.2: Comparison of the CO_2 production rates between the wild type *Clostridium pasteurianum* (Original A and B) and two different isolates of a putative mutant strain (E53J1 and E53D1).

The profile of the byproducts formation (butyric acid, 1,3-PDO, ethanol, and acetic acid) re-enforces that the mutant strains have higher production rates when compared to the wild-type strains. The wild-type strains demonstrated the butyric acid re-assimilation discussed in Chapter 2, which was not observed as significantly in the putative mutants (Figure 3.3). However, even prior to the re-assimilation phase of the fermentation, the mutant samples had produced higher amounts of butyric acid while still producing similar other by-products. This could indicate a change in the redox homeostasis of the organism allowing for greater butanol/butyric acid production from glycerol.

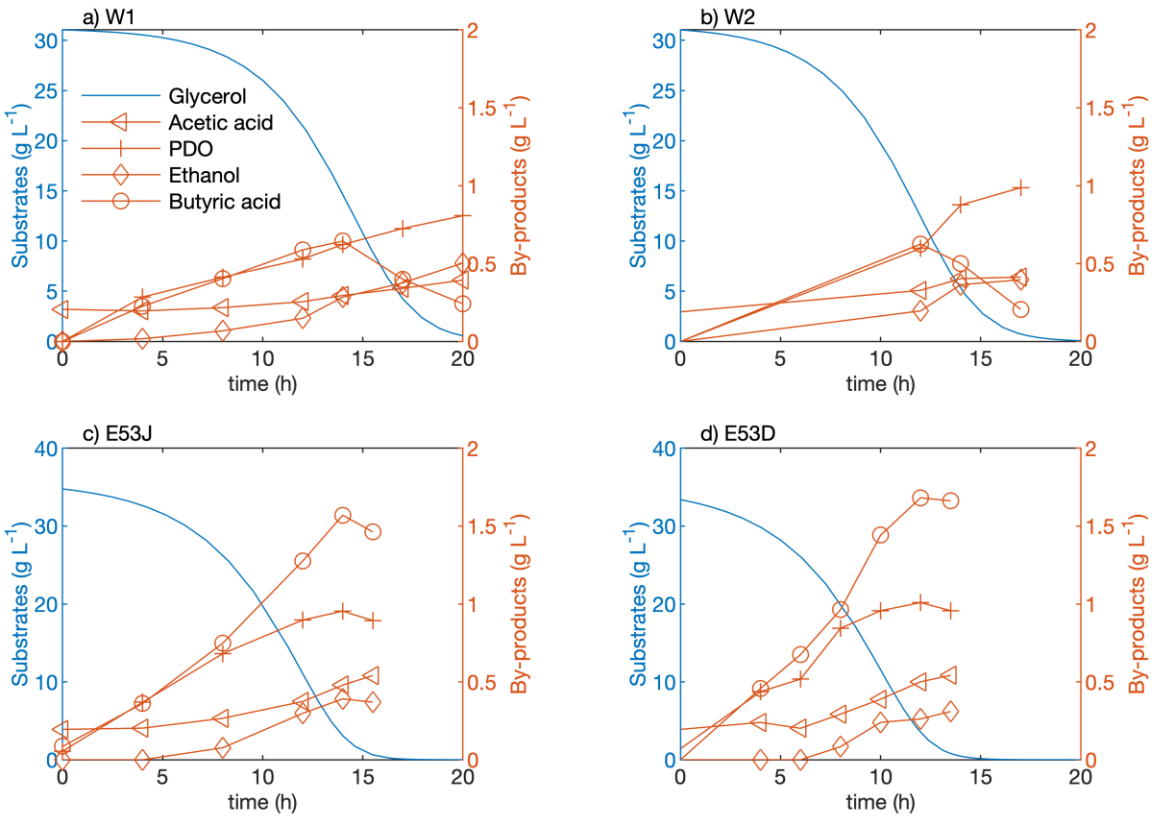

Figure 3.3: By-product formation for two wild-type *C. pasteurianum* fermentations and two fermentations using putative mutant samples. Solid blue line is the best fit for glycerol consumption using data displayed in Figure 3.1.

3.3.3) Whole genome sequence comparisons

The full gene sequences of the putative mutants were compared to sequences of the wild-type *C. pasteurianum* to locate the variants between the genomes to possibly explain the phenotypic differences observed. There were between 31 and 35 total variants detected in each of the four putative mutant samples, of which 29 were common amongst all samples (Figure 3.4). This non-uniform distribution of variants across samples could indicate that the microbial population is not homogenous, making determination of the genetic causes for the mutants altered growth rate and product profile difficult. The 29 common variants are listed and described in Table 3.2.

While the 29 common variants were found to not be located on genes directly related to the glycerol – butanol pathway (Figure 1.2), the mutations nonetheless had a positive effect on

the behavior of *C. pasteurianum* in the increased metabolic rates observed and increased carbon allocated to the butyric acid/butanol pathway. The lack of variants along the glycerol:butanol metabolic pathway indicate the phenotype results from changes in a different metabolic pathway, similar to results observed by Sandoval *et al.* upon deletion of the *SpooA* gene. In addition, the heterogeneity of the variants detected (each sample having variants not found in the other samples) indicate that the microbial culture in the fermentation is similarly heterogenous, with multiple phenotypes with increased growth rates and higher butanol production being displayed simultaneously in the fermentation. For future work, single colonies of the mutant will need to be isolated and cultivated to obtain homogenous cultures of mutants with advantageous phenotypes for further genetic analysis and study. This type of screening has been seen successes with other previous work with *C. pasteurianum* in identifying single phenotypes from a mixed population (Sandoval *et al*. 2015).

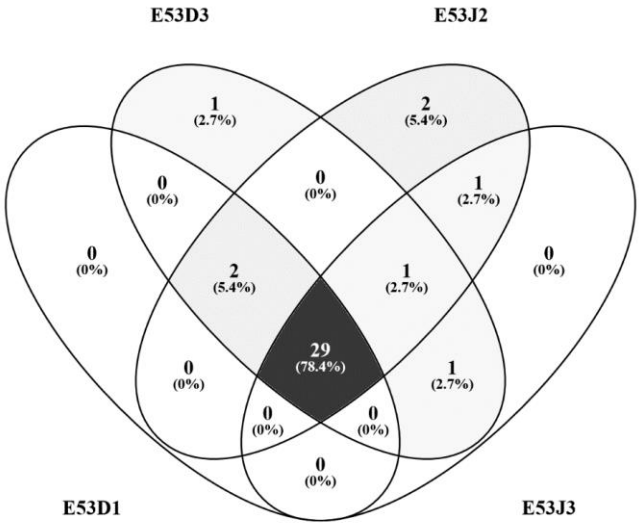

Figure 3.4: Overview of the unique and overlapping variants found in the found mutant samples

Table 3.2: Details of each gene, type of mutation, and possible product for each of the 29 common variants in all 4 sequenced mutant samples

Gene Symbol	Type of mutation (# of each type detected)	Product	Immediate Downstream Product (if promotor for protein)	Protein ID	Gene Coordinates	Variant ranges
rna-CLPA_RS00045	Promotor	23S ribosomal RNA	N/A	N/A	9688 - 11200	
gene-CLPA_RS02500	Nonsense (2) Missense (2)	Hypothetical protein	N/A	WP_034830280.1	555786 - 555980	555828 - 555930
gene-CLPA_RS03660	Promotor (13)	Hypothetical protein	tRNA nuclease WapA	WP_003446352.1	837986 - 838333	838054 - 838308
gene-CLPA_RS04330	Promotor (1)	flavodoxin family protein	N/A	WP_003447094.1	970941 - 971327	
gene-CLPA_RS20040	Promotor (6)	Hypothetical protein	SAM-dependent methyltransferase	WP_051803905.1	983131 - 983310	983141 - 983229
gene-CLPA_RS20055	Missense (4)	Hypothetical protein	N/A	WP_051803906.1	1249022 - 1249594	1249276 - 1249444

3.4) Conclusions and future recommendations

The oxidative treatment with $K_3FE(CN)_6$ of *C. pasteurianum* in a continuous fermentation resulted in 29 common mutations in 6 different genes for all sequenced samples. Each sample also possessed unique variants, indicating the cultures were a non-homogenous population of mutants displaying phenotypes beneficial to the growth rate and carbon selectivity for butanol. The batch fermentations conducted with the wild-type strain and the mutant strains showed, by virtue of the off-gas data, that the mutant strains are more likely to have higher growth rates. The product profile also shows an increase metabolic flux towards the butanol/butyric acid pathway with reduced by-products, possibly as a result of a change in redox homeostasis mechanisms in the mutant strains.

Future work to isolate individual phenotypes should begin with selective plating and isolation of single phenotypes in a colony. These colonies should be screened in a manner to determine which exhibit the higher metabolic rate, as described by the higher CO_2 production displayed in Figure 3.2, and the higher carbon selectivity for the glycerol-butanol metabolic pathway. Fermentations which display one or both of these features should be sequenced for identification of the mutations which result in these phenotypes.

In addition to this screening, further investigation into the genes in which variants were found in the E53D and E53J samples should be conducted. Using plasmid-based approaches to introduce these variants into wild-type *C. pasteurianum* will allow observation of changes in behavior and products as a result. As many of the variants are located in promoter regions, introduction of these mutations as a plasmid should result in the overexpression of genes, resulting in increased metabolic activity and butanol/butyric acid selectivity. Should no difference in behavior over the wild-type strain be observed, then these regions can be eliminated from future consideration and mutagenic work.

Chapter 4

Online measurement of CO_2 and total gas production in parallel anaerobic shake flask cultivations

Selected data presented in this chapter is part of a journal article authored by Garret Munch, Andreas Schulte, Marcel Mann, Robert Dinger, Lars Regestein, Lars Rehmann, and Jochen Büchs.

The information in this chapter has been slightly altered to meet formatting requirements. This work is substantially as it appears in Biochemical Engineering Journal, January 2020, Volume 153, (DOI: https://doi.org/10.1016/j.bej.2019.107418)

This chapter describes the work to develop a device which can simultaneously measure the off-gases production rates in several anaerobic fermentations in parallel shake flasks. This work builds on previous developments for monitoring the oxygen consumption and CO_2 production rates in aerobic shake flask fermentations (Anderlei *et al.* 2004). The device operates on a repeating pattern in which nitrogen is continuously flowing through the headspace of the flasks, maintaining an anaerobic atmosphere. At set intervals, the inlet and outlet valves close, sealing each flask completely, allowing for the changes in the headspace gas composition to be measured. The change to anaerobic fermentations required several modifications to the base design. The addition of a recirculation loop to continuously pull headspace gasses from the flask allowed for online analysis of the gasses as they passed over a nondispersive infrared CO_2 sensor without compromising the anaerobic atmosphere.

This device was first used to determine its ability to maintain conditions suitable for anaerobic fermentations outside of an anaerobic chamber. Following this success, the device was tested to determine how closely fermentations conducted matched similar fermentations conducted at the reactor scale. Next, fermentations with increasing concentrations of butanol in the initial medium were conducted to determine how the off-gas transfer rates would respond to increasing inhibitor present. Finally, a dual substrate (dextrose and glycerol) fermentation was conducted to assess the response to a complex fermentation, and how well the results in the shake flask match those at reactor scale. This device allowed for

fermentations to be screened using small volumes and multiple fermentations in parallel without a loss in accuracy.

The work described in this chapter satisfies the fourth objective of this thesis, with the successful development of an anaerobic screening device capable of obtaining off-gas transfer rates from fermentations conducted in shake flasks. The fermentations had similar product profiles and gas transfer rates as those observed at the reactor scale.

Abstract: Online measurements of off-gas streams are often crucial for studying bioconversion processes. However, for anaerobic processes, options for online off-gas analysis are typically restricted to lab-scale bioreactors or larger systems, while gas measurements at smaller scales typically do not discriminate between different gases. In this work, a method for online measurement of CO_2 and total gas production in anaerobic fermentations at the shake flask scale is described, extending capabilities of a previously reported device developed for aerobic processes to anaerobic bioprocesses. The novel design allows anaerobic fermentations to be performed in multiple parallel vessels, all of which collect online gas signals. The online gas signals are used to calculate the transfer rates, allowing near real-time visualization of the progress of eight fermentations operating in parallel. Conditions such as carbon source depletion, inhibition of growth, and exhaustion of a single carbon source in a dual-substrate fermentation can all be clearly distinguished. The combination of online signals and offline analysis allowed for carbon balances to be performed with high degrees of closure. The new design allows for higher throughput screening of anaerobic bioprocesses, an area lacking in small-scale options with off-gas analysis capabilities.

4.1) Introduction

The necessity of strictly oxygen-free conditions causes difficulties in the application of several online measurement techniques in anaerobic bioconversion processes (Z. Liu *et al.* 2011; Beutel and Henkel 2011; Leu, Libra, and Stenstrom 2010; Marques *et al.* 2016), especially on the small-scale level (Schäpper *et al.* 2009). However, the benefits of online signals for screening and process characterization are numerous (Kensy, Engelbrecht, and Büchs 2009; Clementschitsch *et al.* 2005; Buchenauer *et al.* 2009; Maskow *et al.* 2008;

Eliasson Lantz *et al.* 2010; Pohlscheidt *et al.* 2013). Small-scale anaerobic biotransformation, especially screening or optimization for operating conditions or biocatalysts are often conducted in simple serum bottles (Hyun, Young, and Kim 1998; Battersby and Wilson 1988; Yeh, Pennell, and Pavlostathis 1998; Sarchami, Johnson, and Rehmann 2016b; Regestein *et al.* 2015). Unfortunately, no commercial online measurement technique is known for anaerobic cultivations in serum bottles which incorporates active gas flow through the bottle. Using closed serum-bottles results in differences between the experimental conditions compared to stirred tank reactors, as the build-up of product gasses in the head space can alter the fermentations product profile (L Li, Wang, and Li 2019; Y. Liu and Wang 2017). Performing screening experiments directly in stirred tank reactor is normally not an option due to the low experimental throughput (Kensy, Engelbrecht, and Büchs 2009).

As a bridge between fermenter and shake flask scale, the Respiratory Activity Monitoring System (RAMOS) was developed for aerobic processes in 2001 and has been used extensively since (Anderlei and Büchs 2001; Anderlei *et al.* 2004; Herweg *et al.* 2018; Meissner *et al.* 2015; Wewetzer *et al.* 2015). This device allows for monitoring rates of oxygen consumption and carbon dioxide production of a microbial culture over time, known as the oxygen and carbon dioxide transfer rates (OTR and CTR), respectively. These measurements accurately reflect the metabolic activity of the fermentation over time, while the small scale allows for multiple conditions to be examined in parallel (Anderlei and Büchs 2001; Anderlei *et al.* 2004). Based on the off-gas measurements, effects such as oxygen limitation, pH inhibition, substrate limitation, and depletion of the carbon source can be identified (Anderlei and Büchs 2001). In addition, gas transfer rates are essential parameters for successful scale-up of several processes (Anderlei *et al.* 2004).

The predominant gasses being produced or consumed in anaerobic respiration and fermentation processes are CO_2, CO, H_2 and CH_4 (Weiland 2010; Thauer, Jungermann, and Decker 1977). Analogously to measuring the OTR, biological phenomena as discussed above should be identifiable during anerobic fermentations by measuring a given transfer rate of one or multiple gasses. Carbon dioxide is a commonly monitored parameter when working anaerobically, as it allows for carbon balance closure and indirect monitoring of the microbial metabolic activity (Saucedo-Castañeda *et al.* 1994; Boe *et al.* 2010). However,

most current technology for online measurement of CO_2 production requires fermentations at the reactor scale. Many existing flask-scale devices designed for use with anaerobic fermentation measure the total gasses produced and do not distinguish between the types of gasses. Others require direct sampling through a stopper using syringes. Some commercial products are able to measure specific types of gasses in shake flasks; however, they currently lack active sparging of the headspace, which could cause product inhibition via the buildup of product gasses or alter the product profile (Walker *et al.* 2009). Expanding the off-gas analysis capabilities for anaerobic fermentations at the shake flask scale would allow for collection of significant data. The ability to operate multiple flasks with differing medium, organisms, or inoculation conditions in parallel will also reduce the amount of time to screen different conditions.

There are many anaerobic bacteria currently under study for their application in a wide range of industries, particularly the production of alcohols (Saxena, Adhikari, and Goyal 2009). One organism of interest is *Clostridium pasteurianum*, a spore-forming gram-positive anaerobe capable of efficient conversion of glycerol to butanol (Biebl 2001; Erin Johnson and Rehmann 2016; Sarchami, Johnson, and Rehmann 2016b; Regestein *et al.* 2015). *C. pasteurianum* is a robust and rapidly growing organism which releases the gasses CO_2 and H_2 as part of the product pathway. It also displays well-known product inhibition due to the toxicity of butanol (Sabra *et al.* 2014; Xue *et al.* 2013; Dabrock, Bahl, and Gottschalk 1992).

This work describes the adaptation of the RAMOS device to support anaerobic fermentations, and the use of dynamic CO_2 (directly) and H_2 (indirectly) transfer rates to track metabolism and metabolic changes at the shake flask scale.

4.2) Materials and Methods

4.2.3) Description of anaerobic Respiratory Activity Monitoring System

The anaerobic Respiratory Activity MOnitoring System (anaRAMOS) was designed by modifying the existing RAMOS technology to support anaerobic respiration and fermentation processes. Detailed descriptions of the operation of the RAMOS device have been previously given (Anderlei and Büchs 2001; Anderlei *et al.* 2004), as well as applications in aerobic microbial cultivation (Meissner *et al.* 2015; Herweg *et al.* 2018).

For use with anaerobic fermentations, several modifications had to be made to the RAMOS device (Figure 4.1). High-purity nitrogen was sparged through the headspace of each flask at a rate of 10 mL/min to maintain an anaerobic atmosphere (corresponding to a sparging rate of approximately 2.4 vol/hr). A CO_2 sensor (sensor type MSH-P-CO_2, Dynamex, UK) was installed in a custom-designed stainless-steel sensor block. The sensor block was designed to house the CO_2 sensor in a manner that allowed for gasses to pass through while maintaining an air-tight seal to prevent oxygen contamination of the fermentation. A pressure sensor (Type 26PCA, Honeywell Inc.) was used to ensure that each individual flask and sensor unit was completely sealed when inlet and outlet valves were closed prior to beginning fermentations, and to measure the pressure changes during measurement phase during the fermentation (allowing to quantify a total gas transfer rate). A microfluidic piezo membrane pump (Bartels Mikrotechnik) was used to continuously move the headspace gas through a recirculation loop, from the headspace of the flask through the sensor block, over the sensor, and back into the flask, in a similar fashion as outlined by Takahashi and Aoyagi (2018) (Takahashi and Aoyagi 2018).

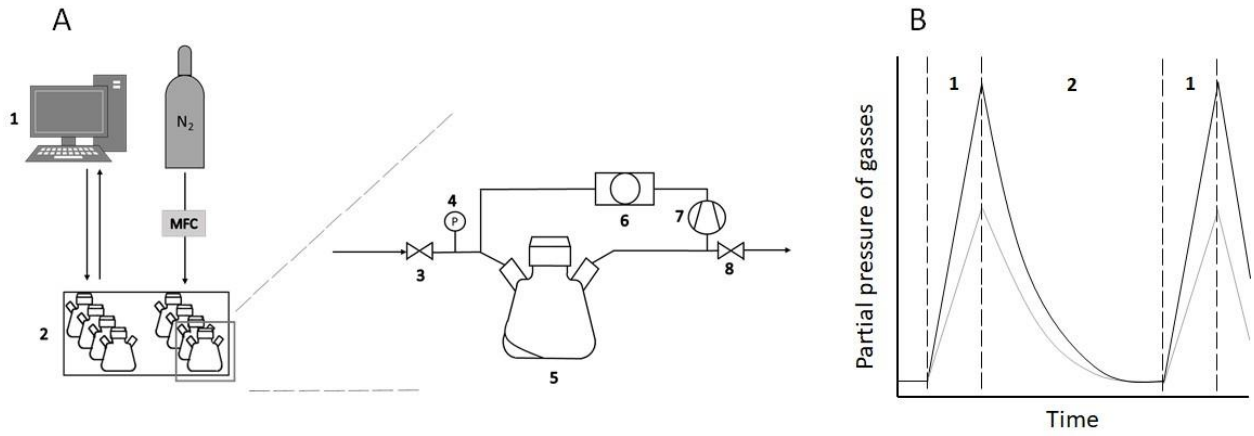

Figure 4.1: A) Schematic overview of the anaRAMOS system and details on single flask/sensor arrangement. 1: Computer controlling valves and logging CTR data. 2: Heating/shaking unit containing 8 anaerobic flasks in a parallel system with RAMOS circuitry board installed. 3: Gas inlet control valve. 4: Pressure sensor 5: anaRAMOS flask with culture. 6: CO_2 sensor connected to central circuitry. 7: Micropump. 8: Gas outlet control valve.
B) Illustrative graph demonstrating the principle of anaRAMOS operation via repeated phases. Phase 1: Measurement phase with no gas flow through flasks allowing fermentation gasses to accumulate in the headspace and be measured. Phase 2: Rinse phase with active sparging of nitrogen through the headspace. Black line represents the total pressure of gasses produced, measured via pressure sensor. Grey line represents the CO_2 measured directly via NDIR sensor.

The CTR was calculated using the CO_2 partial pressure in the headspace of the flask according to Equation 4.1:

$$\mathrm{CTR}\left(\frac{\mathrm{mmol}}{\mathrm{L}\cdot\mathrm{h}}\right) = \frac{n_{CO_2}}{V_l \cdot t} = \frac{\Delta p_{CO_2}}{\Delta t}\cdot\frac{V_g}{R\cdot T\cdot V_l} \qquad (4.1)$$

Where n_{CO_2} is the moles of CO_2 (mmol), V_l is the liquid filling volume of the anaRAMOS flask (L), t is time (h), Δp_{CO_2} is the partial pressure drop of CO_2 (bar) during the measuring phase, Δt is the duration of the measuring phase (h), V_g is the volume of the gas in the

anaRAMOS flask (L), *R* is the standard gas constant (8.314 J/mol/K), and *T* is temperature (K). The amount of CO_2 production can be obtained by integrating CTR over time. The total gas transfer rate (TGTR), a measurement of all the gasses produced during the measurement, is calculated according to Equation 4.2:

$$\mathrm{TGTR}\left(\frac{\mathrm{mmol}}{\mathrm{L}\cdot\mathrm{h}}\right) = \frac{n_{total\ gas}}{V_l \cdot t} = \frac{\Delta p_{total\ gas}}{\Delta t}\cdot\frac{V_g}{R\cdot T\cdot V_l} \tag{4.2}$$

While the remaining gas transfer rate (RGTR) is calculated by subtracting the CTR from the TGTR.

$$\mathrm{RGTR} = \mathrm{TGTR} - \mathrm{CTR} \tag{4.3}$$

Due to the established metabolism of *C. pasteurianum* when grown on glycerol, the RGTR can be assumed to be a result of hydrogen gas production, and as such will be referred to as the Hydrogen Transfer Rate (HTR) [32,38].

The total amount of CO_2 and H_2 produced at a time t can then be estimated by numerically integrating the measured CTR/HTR data.

$$\mathrm{CO}_{2,est}\ (mmol/L) = \int_0^t CTR\ dt$$
$$\mathrm{H}_{2,est}\ (mmol/L) = \int_0^t HTR\ dt; \tag{4.4}$$

4.2.2) Organism and Medium

Clostridium pasteurianum DSM 525 was purchased from the DSMZ (Braunschweig, Germany) and revived following instructions. All cryogenic stocks and pre-cultures were prepared using Reinforced Clostridium Medium (RCM) containing the following (in g/L): peptone, 10 (Roth); beef extract, 10 (BD); yeast extract, 3 (Roth); dextrose, 5; NaCl, 5; soluble starch, 1 (Merck); sodium acetate, 1; pH adjusted to 6.8. For fermentations, a modified medium described by Biebl (Biebl 2001) was used. It contained (unless stated otherwise) in g/L: glycerol, 20; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 5; $MgSO_4{\cdot}7H_2O$,

0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4$, 0.05; yeast extract, 1.0 (Roth). Citric acid buffer was added in a concentration of 0.05 M to maintain pH in the range of 5 – 5.8, and 0.25 g/L L-cysteine hydrochloride was added to reduce the medium. 2 mL of trace element solution was added per liter of medium, the trace element solution containing (in g/L): $FeCl_2 \cdot 4H_2O$, 1.5, dissolved in 25% HCl solution; $CoCl_2 \cdot 6H_2O$, 0.19; $MnCl_2 \cdot 4H_2O$, 0.1; $ZnCl_2$, 0.07; H_3BO_3, 0.062; $Na_2MoO_4 \cdot 2H_2O$, 0.036; $NiCl_2 \cdot 6H_2O$, 0.024; $CuCl_2 \cdot 2H_2O$, 0.017. All cultures were grown in flasks with a 250 mL nominal filling volume, and cultivated on a shaker with a shaking diameter of 50 mm. Cultures were grown in a 2-step preculturing method: 1 mL of frozen glycerol stock culture was added to 9 mL RCM and incubated undisturbed in an anaerobic chamber for 16 – 18 hours. 3 mL of this culture was added to 27 mL of fresh RCM and incubated in the anaRAMOS at 35°C and 100 rpm until the CTR reached a minimum level of 10 mmol CO_2/L/h (approx. 5 hours). Once this point has been reached, the flask was returned to the anaerobic chamber where a small aliquot was removed to determine the OD of the preculture. For all experiments, the main cultures were inoculated with a volume resulting in a starting OD (600 nm) of 0.1, and the flasks had a working volume of 30 mL.

4.2.3) Experimental Growth

Three different experimental conditions were examined to demonstrate the efficacy of the anaRAMOS for tracking changes in anaerobic fermentations using the CTR and HTR, as well as replicate yields and behaviors previously reported at the fermenter scale. All experiments used the flasks, shaker, preculturing steps, and medium as stated above, with temperature set at 35°C, shaking at 100 rpm, and a filling volume of 30 mL. For every experiment, each condition was performed in duplicate, and experiments were repeated at least twice to demonstrate reproducibility of results. Values are given as the average of the duplicate flasks and repeated experiments. The first set of experiments imitated conditions reported by Johnson and Rehmann (2016), and was conducted with varying amounts of glycerol (5 g/L, 10 g/L, and 20 g/L). The second investigated differences in growth rate due to inhibition of the starting culture. For this, varying amounts of butanol (0, 2.5, 5, and 7.5 g/L butanol) were added to the starting culture, all of which contained 20 g/L glycerol. The third used mixed carbon sources (dextrose and glycerol) in a dual-substrate fermentation strategy. The concentration of glycerol was kept constant at 20 g/L for all fermentations, while 0, 1, 2.5, and 5 g/L of dextrose was added to the starting fermentation medium.

4.2.4) Analytical Methods

Liquid samples were collected from the flask in 5 mL aliquots, placed into pre-weighed 15 mL falcon tubes and centrifuged at 4000 rpm (18000 x g) for 10 minutes. Supernatant was kept for subsequent analysis, while the cell pellet was washed with 5 mL distilled water, centrifuged again at 4000 rpm for 10 minutes, the water discarded, and the pellet placed in an 80^{o}C oven until a constant weight was achieved. Supernatant was stored at -20^{o}C until it could be analyzed by High Pressure Liquid Chromatography (HPLC) using a Dionex HPLC system with an organic acid resin column (300 x 8 mm, CS-Chromatography) at 60^{o}C, with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.8 mL/min. Carbon balances were performed including the measured amounts of butanol, butyric acid, ethanol, acetate, 1,3-PDO, CO_2, and biomass. A previously reported molecular formula to account for the carbon in biomass, $C_4H_7O_2N$ (101.1 g/mol) was used (Erin Johnson and Rehmann 2016).

4.3 Results and Discussion

4.3.1) Gas transfer rates of shake flask cultivations with *C. pasteurianum*

The following experiments were conducted to demonstrate the ability of the anaRAMOS to accurately reflect the condition of an anaerobic fermentation via monitoring the CTR and HTR, and to replicate product profiles of fermentations reported in literature at the bench scale. *C. pasteurianum* was selected to demonstrate the anaRAMOS capabilities as only CO_2 and H_2 are formed via the main glycerol metabolic pathway (E. Johnson *et al.* 2016; Biebl 2001). Fermentation conditions were kept as close to those reported by Biebl (2001) and Johnson and Rehmann (2016) as possible, to allow for limited comparison between fermentations performed in the anaRAMOS and those at the fermenter scale (Biebl 2001; Erin Johnson and Rehmann 2016). Figure 4.2 shows an example of the online data (CTR) the anaRAMOS can collect in a single experiment (single flask). To match the CTR with offline data (glycerol, butanol, etc.), replica flasks were sacrificed at discrete time points for analysis of the liquid phase. The figure clearly show that online and offline data are in agreement. The CTR increases with the rate of substrate consumption and identified the point of substrate depletion. Subsequent fermentations therefore use online data only to enable parallel experiments to visualize the capability of the anaRamos to conveniently study effects of substrate concentration, product inhibition and dual-substrate consumption. Different initial

amounts of glycerol were used to visualize the depletion of the carbon source in the medium at various time points using the gas transfer rates, as well as to visualize the effect of a higher culture density and activity on the gas transfer rates using the anaRAMOS.

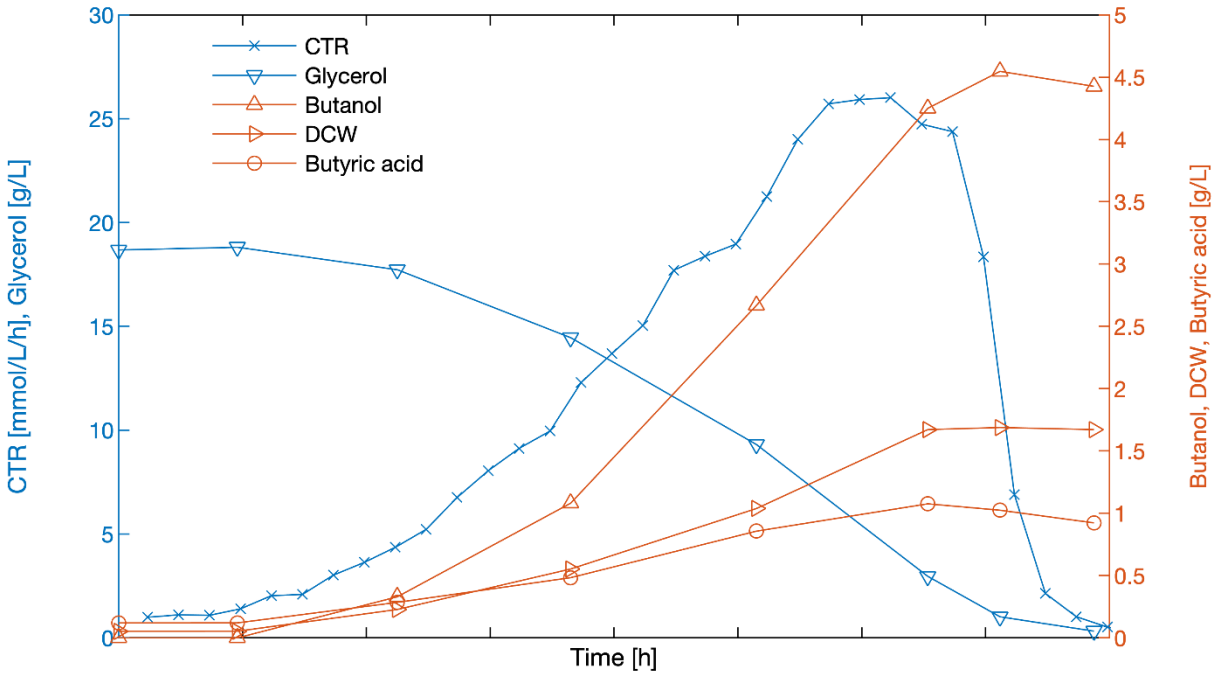

Figure 4.2: Example data from single run of the anaRAMOS demonstrating combination of online and offline signals obtained at the shake flask scale. Fermentation volume was 30 mL, which initially contained 20 g/L glycerol and was incubated at 35ºC at 100 RPM.

By examining the CTR and HTR, changes with respect to fermentation time and glycerol exhaustion are clearly visible (Figure 4.3, top). A sharp drop indicates the depletion of glycerol in the medium, and as a result, a cessation of metabolic activity and the end of the fermentation. As the amount of initial glycerol was increased, both the duration of the fermentation as well as the maximum CTR and HTR increased (11.6, 20.5, and 35.6 mmol/L/h and 12.7, 23.8, and 36.8 mmol/L/h, respectively). Both gas transfer rates increased at similar rates, matching previous reports and the understanding of the glycerol metabolic pathway (Groeger *et al.* 2017; Erin Johnson and Rehmann 2016; Biebl 2001). The HTR was slightly higher than the CTR for all three conditions, however the HTR and CTR followed

the same patterns and had very similar values throughout the course of the fermentation. This behavior indicated that in this case the HTR was closely linked to the CTR, as expected based on the reported glycerol metabolic pathway (Sarchami *et al.* 2016). By numerically integrating the CTR curve, the amount of CO_2 produced per volume of medium can be estimated (Figure 4.3, bottom). As expected, the estimated amounts of CO_2 emitted was higher for samples containing higher initial glycerol concentrations. The samples containing 5, 10, and 20 g/L initial glycerol emitted 35.9, 73.2, and 169.7 mmol/L, respectively, which correspond to a 2.0x CO_2 increase between 5 and 10 g/L glycerol sample, and a 2.3x increase between 10 and 20 g/L glycerol. This could indicate a change in the metabolism as the fermentation continues (initial effects of toxicity of accumulating butanol), though the total amount of H_2 increased in a similar fashion. Another possible explanation is changes in the fermentation pH's, as the final pH's for the 5, 10 and 20 g/L glycerol samples were 5.49, 5.27, and 5.05, respectively. Changes in pH have been shown to have an effect on the amount of CO_2 produced during a similar fermentation at the reactor scale (Erin Johnson and Rehmann 2016).

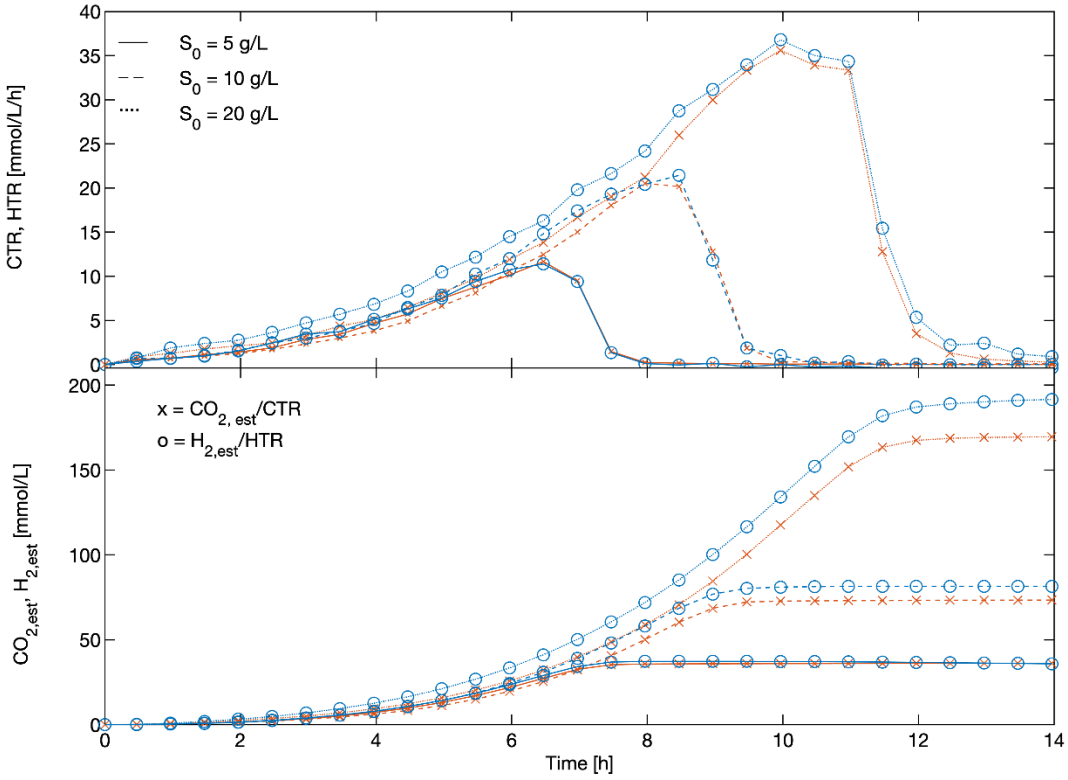

Figure 4.3: Top: CTR and HTR profiles of fermentation with *C. pasteurianum* in medium containing 5, 10, and 20 g/L glycerol as the carbon source.

Bottom: Total CO_2 and H_2 produced over the course of the fermentations. Fermentation volume was 30 mL, which initially contained 5, 10, or 20 g/L glycerol and incubated at 35ºC at 100 RPM.

Of particular interest are the products of the fermentation. *C. pasteurianum* is a well-documented consumer of glycerol and producer of butanol (Ahn, Sang, and Um 2011; Venkataramanan *et al.* 2012; Gallazzi *et al.* 2015; Regestein *et al.* 2015). Offline sampling shows that consumption of glycerol matches the gas production and the overall time of

fermentation was comparable to that found at the reactor scale (Erin Johnson and Rehmann 2016). However, a direct comparison to results with active pH control is not possible, as all shake flask experiments were pH buffered systems. The product profiles and carbon balance closures at the end of the fermentations shown in Figure 3 are summarized in Table 4.1. Of note, samples containing only 5 g/L initial glycerol saw a nearly equal amount of butanol and

butyric acid produced (0.65 and 0.72 g/L, respectively), which follows patterns previously reported for *C. pasteurianum* when grown on low amounts of carbon source (Gallardo, Alves, and Rodrigues 2014; Biebl 2001).

Table 4.1: Products and carbon balances of fermentations conducted in the anaRAMOS with differing initial glycerol concentrations. Values shown correspond to end-point of fermentations displayed in Figure 4.3.

Initial Glycerol Concentration (g/L)	Butanol (g/L)	1,3 PDO (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Biomass (g/L)	CO_2 (mmoles/L)	Carbon Balance Closure (%)
5	0.65	0.62	0.47	0.73	0.65	35.9	92.8
10	1.58	0.96	0.51	1.15	1.33	73.2	94.7
20	3.75	1.46	0.58	1.60	1.98	169.7	94.9

4.3.2) Visualization of inhibition using the gas transfer rates

The second set of experiments were conducted to test the ability of the anaRAMOS to discern differences in growth rates due to inhibiting effects. Butanol was selected as the inhibitor due to its relevance as a fermentation end-product and its known inhibitory effect on the growth of *C. pasteurianum*. Butanol was added in increasing concentrations (0, 2.5, 5, and 7.5 g/L butanol) to the culture medium immediately prior to inoculation. The effects of the inhibition are clearly reflected by the CTR and HTR (Figure 4.4, top).

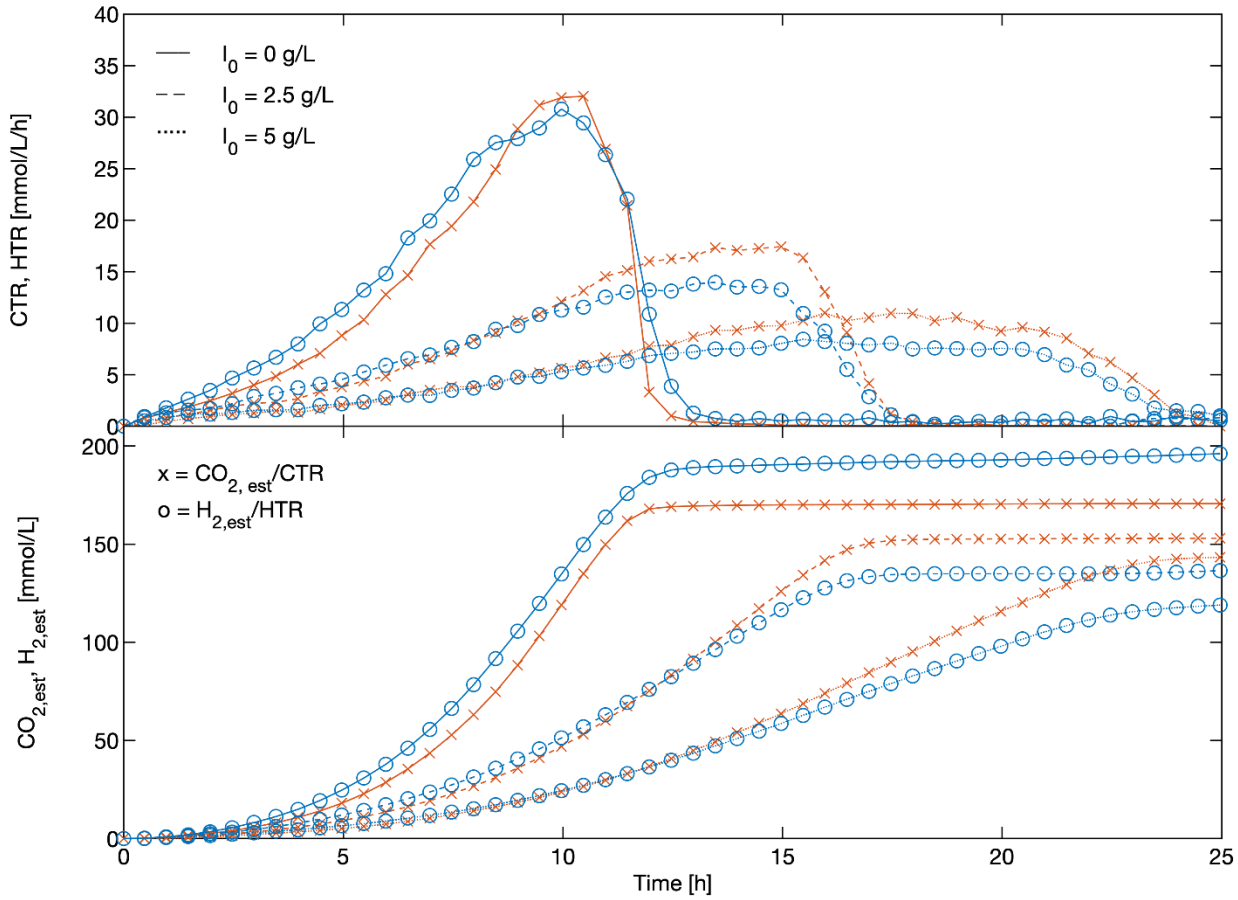

Figure 4.4: Top: CTR and HTR of inhibited *C. pasteurianum* fermentation due to increasing concentrations of butanol added (I_0) to medium at time of inoculation.

Bottom: Total CO_2 and H_2 emitted over the course of the fermentations. Fermentation volume was 30 mL, which initially contained 20 g/L glycerol and 0, 2.5, or 5 g/L butanol, and incubated at 35°C at 100 RPM.

The addition of butanol had an immediate and pronounced effect on the metabolism of *C. pasteurianum*. The addition of 2.5 g/L butanol reduced the maximum CTR from 32.0 mmol/L/h with no butanol added to 17.3 mmol/L/h, and extended the fermentation time from 13 to 18.5 hours. The HTR was decreased from 30.8 to 13.9 mmol/L/h, indicating that the inhibitory effect of the added butanol had a stronger effect on the H_2 formation. Increasing the amount of butanol further to 5 g/L reduced the maximum CTR to 11.0 mmol/L/h, the

HTR to 8.4 mmol/L/h, and the fermentation time was extended to over 24 hours. During the solventogenic pathway CO_2 and H_2 are formed at equimolar ratio during the pyruvate to acetyl-CoA step (Biebl 2001). The additional CO_2 must therefore be formed differently, likely connected to required maintenance energy in order to address the solvent stress. In this particular case, the online signal can therefore be used to identify conditions under which carbon is deviated from the desired fermentation product. When 7.5 g/L of butanol was added, complete inhibition of the cultures was observed, and no glycerol was consumed (data not shown). It should be noted that while many previously reported studies show *C. pasteurianum* fermentations remaining active in the presence of butanol at concentrations higher than 7.5 g/L (Moon *et al.* 2011b; Gallazzi *et al.* 2015; Ahn, Sang, and Um 2011; Biebl 2001; Erin Johnson and Rehmann 2016), an important distinction is the time of exposure. In this current setup, *C. pasteurianum* was exposed to high butanol concentrations while at low cell density, and being transferred from rich butanol free medium to a minimal medium with high solvent concentrations. The data clearly demonstrates the effectiveness of monitoring inhibition of fermentations via the CTR and HTR, and the ability to monitor multiple gassed gives further insight into the biochemical process.

The total CO_2 produced (Figure 4, bottom) by the fermentations decreased as the amount of butanol in the fermentation medium increased, indicating a change in the metabolism of the cells due to the inhibition. Samples grown with no butanol added produced 169.8 mmol/L, similar to what was previously demonstrated. However, flasks containing 2.5 g/L butanol produced only an estimated 151.6 mmol/L, despite a longer fermentation time. Flasks containing 5 g/L butanol produced even less CO_2, an estimated 143.2 mmol/L in total.

HPLC analysis showed the glycerol to be completely consumed at the time of the drop in the gas transfer rates for all samples, with the exception of the sample initially containing 7.5 g/L butanol, which showed no change in the glycerol concentration (data not shown). The carbon balances for 2.5 g/L initial butanol closes at ~ 92%, similar to the data shown in Table 1. The data becomes unreliable at 5 g/L initial butanol, likely due to increased evaporative loss of butanol at elevated concentration.

4.3.3) Using CTR and HTR to monitor dual-substrate consumption

Finally, a dual-substrate fermentation was performed to demonstrate the ability of the anaRAMOS to reflect changes in the uptake of different substrates, and demonstrate that changes in metabolism can be reflected by the change in the rates of the gasses produced. Dextrose and glycerol were used as the carbon sources for *C. pasteurianum*. Previous work by Sabra *et al.* has shown that *C. pasteurianum* is capable of consuming dextrose and glycerol simultaneously (Sabra *et al.* 2014). When grown with dextrose as the sole substrate, *C. pasteurianum* produces increased concentrations of butyric acid and less butanol compared to when grown solely on glycerol (Dabrock, Bahl, and Gottschalk 1992). As well, when *C. pasteurianum* is grown in the presence of extracellular butyric acid, a reversible metabolic pathway utilizes the butyric acid to increase the butanol production rate (Regestein *et al.* 2015; Gallardo, Alves, and Rodrigues 2014). For this reason, a fermentation using both dextrose and glycerol as carbon sources with *C. pasteurianum* has been of previous interest at the reactor scale (Sabra *et al.* 2014). Similar fermentation conditions were selected for the anaRAMOS to determine the effects of the dual substrate on the CTR and HTR. Specifically, it is of interest whether the gas transfer rates can indicate the point of depletion of dextrose, and if the product profile is similar to those found at the reactor scale in previously reported studies.

The gas transfer rates indicate clearly the point of dextrose depletion in the medium by a sharp decrease and subsequent increase as the cells begin metabolizing the remaining glycerol (Figure 4.5). For the lowest dextrose concentration (1 g/L added dextrose) the decrease in the transfer rates occurs at 4.5 hours post inoculation with a small but noticeable decrease in the CTR from 7.6 to 7.1 mmol/L/h, and a drop in the HTR from 9.3 to 9.0 mmol/L/h. Following this slight decrease, the transfer rates resumes increasing until glycerol is exhausted and the gas transfer rates reach 0 mmol/L/h, signaling the end of the fermentation. The HTR does not reach as high a peak as the CTR prior to the end of the fermentation, only reaching 25.2 mmol/L/h, compared to a peak of 31.9 mmol/L/h when no dextrose was added. Initially adding 2.5 g/L dextrose resulted in a later decrease in the CTR at 6 hours post inoculation, as well as a more pronounced decrease in the CTR from 16.7 to 10.0 mmol/L/h and the HTR decreased from 17.5 to 10.9 mmol/L/h. The HTR once again did not demonstrate as pronounced an increase as the CTR following the depletion of the

dextrose in the medium (Figure 4.5). These trends continued when adding 5 g/L dextrose, causing the gas transfer rates to decrease at 7.5 hours post inoculation, though this decrease occurred over a period of 1.5 hours before beginning to rise again, and was extremely pronounced for both gasses. The CTR decreased from 21.9 to 8.5 mmol/L/h, while the HTR decreased from 19.2 to 9.6 mmol/L/h. As well, in the 5 g/L dextrose sample the CTR did not increase at the same rate as with lower dextrose samples, the CTR plateaued with a peak at 16.5 mmol/L/h. The lower CTR peak and plateau is similar to those observed in fermentations in the presence of inhibitory conditions. This inhibition was most likely due to a decrease in the pH. Samples taken at the time of the CTR drop showed that the pH in the 5 g/L dextrose sample had dropped to values between 4.9 and 5.0, despite the presence of buffer in the medium. Previous research has shown increasingly delayed growth and activity for *C. pasteurianum* as pH values fall further below a value of 5.9, which aligns with the results demonstrated here (Erin Johnson and Rehmann 2016). Interestingly, the HTR remained plateaued in the range of 9.6 mmol/L/h until the end of the fermentation, indicating a marked shift in the metabolism when compared to samples containing lower initial dextrose concentrations. Previous reports have indicated that an increase in butyric acid production alters the redox balance for *C. pasteurianum*, requiring less hydrogen to be produced as a means of balancing the reducing equivalents, which could explain the decrease in the HTR observed (Groeger *et al.* 2017; Sabra *et al.* 2016, 2014). This disparity in the CTR and HTR at certain time periods during the fermentation allows for identification of times when the metabolic balance has been altered from an established baseline.

Offline analysis was conducted both at the point of the sudden decrease in the gas transfer rates, indicating dextrose depletion, and the second CTR decrease indicating the end of the fermentation (Table 2). HPLC analysis demonstrated that the fermentation had proceeded as expected according to previously reported results for dual-substrate growth with *C. pasteurianum* (Sabra *et al.* 2014). Specifically, that *C. pasteurianum* was able to simultaneously consume both the dextrose and the glycerol present in the medium. The product profile had changed when compared to fermentations with solely glycerol. The initial growth, where dextrose was present, resulted in higher butyric acid and biomass production over butanol, especially when compared to fermentations containing solely glycerol. However, when 5 g/L of dextrose was added to the initial medium, the concentration of

butyric acid detected at the end of the fermentation was lower than in samples taken at the depletion of dextrose in the medium. Similar behavior has been previously reported, showing that *C. pasteurianum* grown on both dextrose and glycerol will produce initially high concentrations of butyric acid, and subsequently use that butyric acid to aid the formation of butanol (Sabra *et al.* 2014). However, these previous reports were conducted at the reactor scale, while the results presented in this study use a filling volume of only 30 mL in a shake flask.

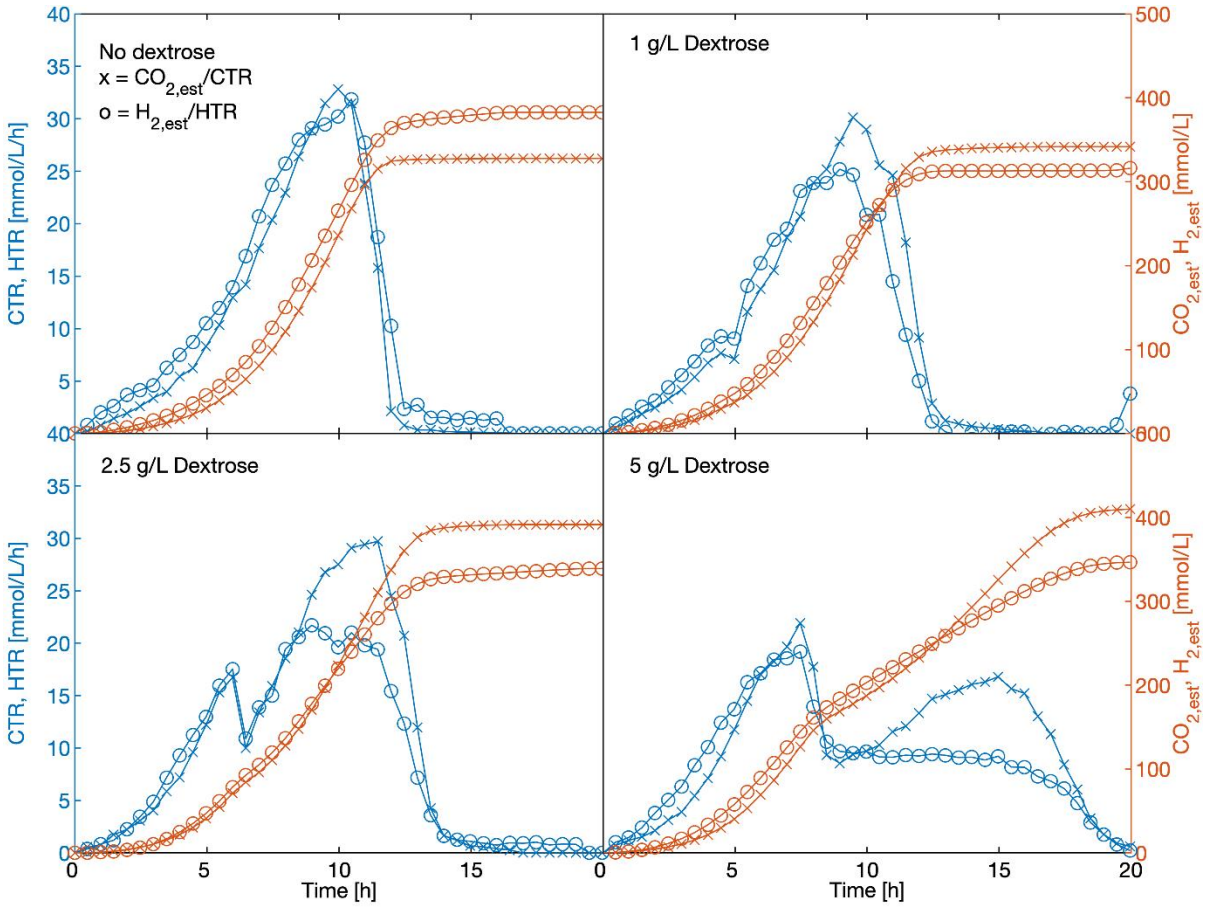

Figure 4.5: CTR, HTR, CO_2 production, and H_2 production over the course of *C. pasteurianum* fermentations containing 20 g/L glycerol and varying amounts of dextrose in the starting medium. Sharp decrease in CTR and HTR indicate time of dextrose depletion in medium. Lines marked with an x are the CTR and CO_2, lines marked with open circles are

the HTR and H_2. Fermentation volume was 30 mL, which initially contained 20 g/L glycerol and 0, 1, 2.5, or 5 g/L dextrose, and incubated at 35ºC at 100 RPM.

The total CO_2 produced displayed interesting behavior patterns as the dextrose concentration was increased. The reference fermentation containing no additional dextrose produced 164.9 mmol CO_2/L, again matching previous experiments. The addition of 1 g/L dextrose resulted in a moderate increase, up to 171.6 mmol CO_2/L, which does not greatly differ from samples with no additional dextrose. However, addition of 2.5 g/L dextrose saw an increase of the total CO_2 to 195.3 mmol/L, a more pronounced increase over previous samples, while the addition of 5 g/L dextrose resulted in 205.5 mmol/L. The additional carbon from dextrose increased the overall amount of CO_2 produced, however the increase in CO_2 produced does not appear directly correlated with the amount of dextrose added. In a complex fermentation using multiple substrates, a large number of biological factors could result in the observed changes in products. However, online monitoring of the gas transfer rates allows for reliable monitoring of the fermentation, assessing degrees of inhibition, and identifying specific time ranges in which the metabolism of the cells has changed to allow for targeted sampling, such as when a carbon source has been depleted.

Table 4.2: Comparison of products between dual-substrate fermentations containing dextrose and glycerol. Samples were taken either when the CTR indicated depletion of dextrose, or when CTR indicated completion of the fermentation.

	Analysis when dextrose depleted			Analysis when fermentation complete			
	1 g/L dextrose added	2.5 g/L dextrose added	5 g/L dextrose added	No added dextrose	1 g/L dextrose added	2.5 g/L dextrose added	5 g/L dextrose added
Ethanol (g/L)	0.23	0.32	0.41	0.61	0.48	0.65	0.63
1,3 PDO (g/L)	0.17	0.39	0.39	1.3	1.5	1.4	1.6
Butyric acid (g/L)	0.74	1.3	2.5	1.4	1.6	1.6	1.6
Butanol (g/L)	0.12	0.39	0.6	3.5	3.9	4.5	5.6
Biomass (g/L)	1.3	2.8	3.1	2.0	3.1	3.2	3.3
CO_2 (mmols/L)	27.6	51.5	81.3	164.9	171.6	195.3	205.5

Glycerol remaining (g/L)	18.8	16.4	15.6	0	0	0	0
Carbon balance closure	90.8%	90.4%	93.7%	93.8%	87.0%	90.3%	90.8%

4.3.4) Visualization of changing metabolism by plotting CTR vs. HTR

The disparity in the CTR and HTR at certain time ranges during the fermentation allows for identification of times when the metabolic balance has been altered. As mentioned, a fermentation using *C. pasteurianum* consuming glycerol will produce both CO_2 and H_2 in roughly equal volumes and rates (H Biebl 2001; Sabra *et al.* 2016). However, changing conditions can alter this balance in a manner that can be visualized using the CTR and HTR. Plotting the CTR vs. the HTR highlights the times in the fermentation which are different from the standard fermentation containing only glycerol (Figure 4.6).

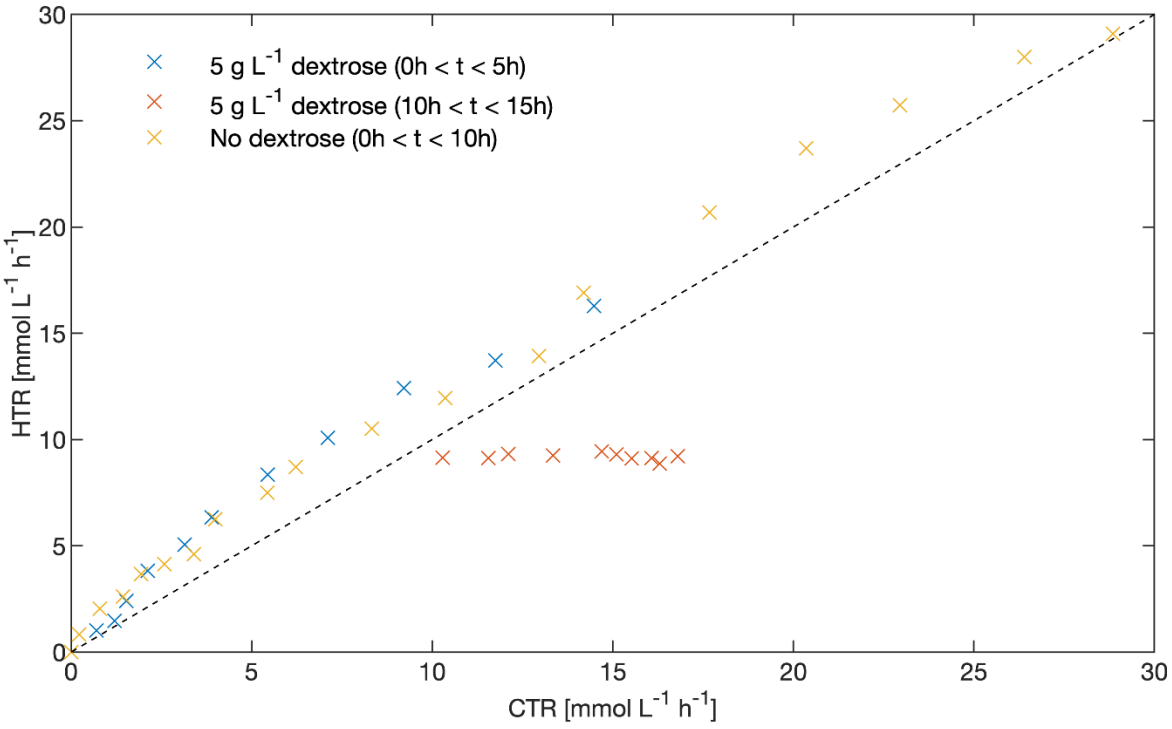

Figure 4.6: CTR vs. HTR of *C. pasteurianum* fermentations containing only glycerol as the carbon source (dark squares) or a mix of glycerol and dextrose. The 0 g/L dextrose sample shows the ratio of CTR/HTR remaining consistent through the course of the fermentation. The 5 g/L dextrose initially follows a similar trend (0 to 5 hours), however following the depletion of dextrose in the medium (10 to 15 hours) the CTR/HTR trend differs greatly.

A fermentation containing only 20 g/L glycerol follows a consistent pattern throughout the fermentation, in this case with slightly higher HTR than CTR, however the two increase in a near linear manner. However, when 5 g/L dextrose is added to the initial fermentation medium, there becomes two distinct patterns identifiable. At the onset of the fermentation (0 to 5 hours following inoculation) the CTR/HTR values are similar to those from fermentations without additional dextrose. However, following the depletion of the dextrose in the medium, the CTR/HTR values begin to differ as the CTR rose and the HTR plateaued. This could be attributed to previously reported behavior from *C. pasteurianum*in which butyric acid production resulted in lowered H_2 production (Sabra *et al.* 2016). By maintaining an online monitoring of the CTR/HTR ratio values, it will be possible to have another tool in identifying conditions that deviate from an established norm ratio in fermentations, possibly identifying times of interest for further investigation, or to indicate the depletion or utilization of cofactors and components in the medium.

4.4) Conclusions

This work demonstrates the capabilities of the RAMOS device to be successfully adapted to allow the cultivation of anaerobic organisms. The adapted device termed anaRAMOS allows for online off-gas analysis of fermentations at the shake flask scale, allowing for multiple fermentation conditions to be analyzed in parallel. Monitoring the evolved gasses allows for the calculation of the CTR and the HTR (in this particular case), which are an accurate representation of the metabolic activity of the fermentation. The anaRAMOS device allows for smaller fermentation volumes to be used, and more conditions to be screened in parallel, while emulating larger reactor scales. Future improvements to the device can extend capabilities to directly monitor other off-gasses than CO_2, such as H_2, as well as monitor fermentations with other anaerobic organisms.

Chapter 5

Conclusions, Contributions, and Recommendations

This chapter outlines the main conclusions from this thesis, the contributions to the field, and outlines some recommendations for future work.

5.1) Conclusions

The experimental results outlined in this thesis demonstrate the efficacy of using butyric acid, regardless of source, to increase butanol yield from glycerol fermentations with *C. pasteurianum*. In addition, a population of mutants generated previously by E. Johnson via random mutagenesis has been sequenced and compared to wild-type strains to locate specific mutations. Finally, a novel screening device capable of online monitoring off-gas production in shake flasks during anaerobic fermentations was developed.

The addition of butyric acid at the onset of the fermentation resulted in inhibition of the fermentation and had no discernable effect on the butanol yield. However, delayed addition, specifically once the fermentation has become well established, saw significant uptake of the butyric acid and a yield increase from 0.449 mol carbon butanol / mol carbon substrate to 0.519 mol carbon butanol / mol carbon substrate. The source of the butyric acid had a small effect, with xylose-derived butyric acid (process fluid from xylose fermentation by *C. tyrobutyricum*) having a lesser effect when compared to chemically pure.

Using molasses as a potential source of inexpensive sugar substrates as part of a dual-substrate strategy allowed *C. pasteurianum* to produce butyric acid early in the fermentation and subsequently re-assimilate the acid during later glycerol metabolism. Optimization of the initial molasses to glycerol ratio resulted in 1.9 g/L butyric acid being produced and 1.8 g/L butyric acid being re-assimilated, with a ratio of 0.459 mol carbon butanol / mol carbon substrate being achieved. Scaling up to reactor scale with the addition of pH control increased this ratio further to 0.480 mol carbon butanol / mol carbon substrates, while utilizing a substrate which did not previously lead to butanol production from *C. pasteurianum*.

The DNA isolated from the putative mutants showed several areas with mutations, signifying that the putative mutant is indeed a novel mutant strain. The locations of the mutations were

not among the glycerol – butanol metabolic pathway as may have been expected, indicating that the mutations may be having a secondary effect on the cells which is beneficial to the glycerol metabolism.

A screening device which allowed for off-gas analysis of multiple parallel shake flask fermentations was developed and employed to analyze glycerol fermentations by *C. pasteurianum*. The anaRAMOS was able to accurately replicate fermentations conducted at the reactor scale in shake flasks while still collecting valuable off-gas data, allowing for carbon balances to be conducted. The gas transfer rates obtained by the anaRAMOS reflected the presence of inhibitory compounds in the medium, as well as a complex dual-substrate fermentation using both dextrose and glycerol.

5.2) Contributions

The work conducted in this thesis will contribute to the field of fermentation science and biobutanol production in three primary ways. The first is the highly effective use of molasses sugars in a dual substrate fermentation with *C. pasteurianum*. While previous work has shown the ability to produce large amounts of butanol with a similar strategy, the work outlined here was able to design a strategy which resulted in a substantially higher yield of butanol from the carbon sources by optimizing the ratio of glycerol to molasses. This type of strategy could allow for more efficient processes to be developed in which molasses and glycerol are used simultaneously and minimize the amount of unutilized substrate. The second contribution was the characterization in batch fermentation and DNA analysis of mutant strains of *C. pasteurianum* generated by E. Johnson that displayed high productivity and superior product profiles. This work will help to develop a highly productive process using one of the isolates, or help guide the development of designed mutant strains with increased productivity. Finally, the design of the anaRAMOS will help bridge the gap between shake flask and reactor experiments and allow for more data collection while reducing material cost and time for experiments. The design could be used in both laboratory and industrial settings to expediate the screening and scale-up process.

5.3) Future Recommendations

The results of this work allow for recommendations for future work to be made to build upon the work done. This section details the recommendations.

Chapter 2 demonstrated the possible efficacy of delayed butyric acid addition to glycerol fermentations by *C. pasteurianum*, regardless of the source of butyric acid or the presence of residual fermentation by-products. Further optimization of this addition to identify the optimal time for addition based on a measurable metric, such as the optical density of the culture, would increase the reproducibility of this process. Further studies can use this strategy to add butyric acid in a slow, continuous manner to fed-batch or chemostat fermentations. This work can further explore the possibility of using butyric acid in process fluid from xylose fermentations using *C. tyrobutyricum* as a low-cost source. In addition, this work could explore using biodiesel-derived waste glycerol as the main glycerol source to further reduce substrate costs. Finally, Chapter 2 identified an optimum ratio of glycerol to molasses to allow high amounts of butyric acid re-assimilation, thus increasing the yield of butanol in batch fermentations. This principal can also be applied to a chemostat fermentation, in which both molasses and glycerol are continuously added to the fermentation. This work will require additional optimization to ensure sufficient resident time for butyric acid production and re-assimilation. Waste carbon sources, from molasses production and biodiesel-derived waste glycerol, can be explored as low-cost substrates.

In Chapter 3, the DNA of a putative mutant strain of *C. pasteurianum*, previously generated by E. Johnson, was sequenced and compared to the wild-type laboratory stock and published reference genomes. Several clusters of mutations were identified, the locations of which were not homogenous amongst the samples sequenced, indicating the population was equally non-homogenous and contained several variants. Future work should be conducted, beginning with plating and isolating single colonies of the mutant population. Subsequent screening for enhanced butanol production with an established mono-culture will allow for more accurate isolation and sequencing of highly productive mutants. Once the highly productive mutants have been sequenced, mutations in regions of interest can be replicated in wild-type *C. pasteurianum* using DNA-editing techniques. This will allow identification of the mutations resulting in increased butanol yield, and their effect on the overall metabolism of the organism.

Finally, in Chapter 4, a screening device was developed to monitor the off-gasses of several parallel anaerobic fermentations in shake-flasks. The off-gas analysis capabilities can be expanded by the addition of additional sensors to the sensor blocks, allowing for gas transfer rates of gasses other than CO_2 and H_2 to be monitored. The phase length and nitrogen sparging rates can be modified to allow slower growing organisms to be cultivated in the anaRAMOS. Additional flasks undergoing identical phase cycles, but without the sensor data, can be incorporated to the anaRAMOS. This will allow offline sampling of the fermentation without the requirement of sacrificing a flask collecting off-gas data. Conversely, updating the anaRAMOS data processing to accommodate changes in the volume of the flask due to sampling could be explored as a possible method to allow sampling without compromising the integrity of the off-gas data.

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