2012

A study of anaerobic fermentation of biodiesel derived crude glycerol into butanol using Clostridium pasteurianum ATCC 6013

Keerthi Prasad Venkataramanan

A STUDY OF ANAEROBIC FERMENTATION OF BIODIESEL DERIVED CRUDE GLYCEROL INTO BUTANOL USING *Clostridium pasteurianum* ATCC 6013

by

KEERTHI PRASAD VENKATARAMANAN

A DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in
Biotechnology Science and Engineering
to
The School of Graduate Studies
of
The University of Alabama in Huntsville

HUNTSVILLE, ALABAMA
2012

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree from The University of Alabama in Huntsville, I agree that the Library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by my advisor or, in his/her absence, by the Chair of the Department or the Dean of the School of Graduate Studies. It is also understood that due recognition shall be given to me and to The University of Alabama in Huntsville in any scholarly use which may be made of any material in this dissertation.

V.K. 04/25/2012

(student signature) (date)

DISSERTATION APPROVAL FORM

Submitted by Keerthi Prasad Venkataramanan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering and accepted on behalf of the Faculty of the School of Graduate Studies by the dissertation committee.

We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this dissertation. We further certify that we have reviewed the dissertation manuscript and approve it in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering.

C. [signature] 4/4/12 Committee Chair
(Date)

Leland Carter 4-11-12
(Date)

[signature] 4-11-2012
(Date)

[signature] 4/15/12
(Date)

Katie Daci 4/18/2012
(Date)

[signature] 4/23/12 Program Coordinator
(Date)

[signature] 4/26/12 College Dean
(Date)

Rhonda Kay Deede 5/1/12 Graduate Dean
(Date)

ABSTRACT
The School of Graduate Studies
The University of Alabama in Huntsville

Degree Doctor of Philosophy Program Biotechnology Science and Engineering
Name of Candidate Keerthi Prasad Venkataramanan
Title A Study of Anaerobic Fermentation of Biodiesel-Derived Crude Glycerol into Butanol by *Clostridium pasteurianum* ATCC 6013

The rapidly expanding market for biodiesel is dramatically altering the cost and availability of glycerol. Biodiesel production processes generate about 10% (w/w) glycerol. Crude glycerol purification is economically unfeasible, and because of its composition, crude glycerol is considered a hazardous waste. This research investigated the value-added conversion of crude glycerol using *C. pasteurianum* into butanol. Butanol is of particular interest as a renewable biofuel. This work is significant in that it is the first report of butanol production from crude glycerol using this organism. The maximum butanol yield produced from crude glycerol was 0.30 g/g. These yields are substantially higher than the 0.15–0.20 g/g butanol yield typically achieved during the fermentation of glucose using *C. acetobutylicum.*

The effect of each impurity present in the biodiesel-derived crude glycerol on the growth and metabolism of glycerol by *C. pasteurianum* was analyzed. The crude glycerol contains methanol, salts (KCl/K_2SO_4), and free fatty acids. Salts and methanol were found to have no negative effects while, unsaturated fatty acids had a strong inhibitory effect on the utilization of glycerol by the bacteria. Hence, the removal of fatty acids from crude glycerol is an essential step towards the utilization of crude glycerol. Glucose-glycerol co-fermentation resulted in a shift in product distribution as 1,3-PDO formation was reduced by 90% and butanol formation was increased by 50%, as a result

of shift in electron flow. Acetate addition and biotin supplementation also resulted in an increase in butanol at the expense of 1,3-PDO.

C. pasteurianum was found to exert two different homeoviscous responses to counteract the butanol toxicity. Addition of exogenous butanol, when *C. pasteurianum* produced endogenous butanol, led to an increase in the ratio of saturated to unsaturated fatty acids (SFA/UFA), although, when *C. pasteurianum* did not produce endogenous butanol, led to a decrease in the ratio of SFA/UFA. Finally, *C. acetobutylicum* microarrays were optimized for analyzing *C. pasteurianum* transcriptome by lowering stringency during the post hybridization wash. Thus, *C. pasteurianum* is a versatile micro-organism that has the potential to be engineered as an industrial butanol producer using crude glycerol, a low cost feedstock.

Abstract Approval:

Committee Chair C. Ref 4/19/12

Program Coordinator 4/23/12

Graduate Dean Rhonda Kay Gaede 5/2/12

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Carmen Scholz, and co-advisors Dr. Katherine Taconi and Dr. Geoffrey Bothun, respectively, for giving me the opportunity in this research project and their guidance throughout all the stages of the work. I thank them for their mentorship, patience, unwavering support and providing an intellectually stimulating environment. I would like to thank Dr. Leland Cseke, for helping me during the course of microarray analysis and providing access to his laboratory space and instruments. I would like to thank Dr. Krishnan Chittur for his willingness to serve in my dissertation committee and for his valuable comments and suggestions during the microarray work.

I would like to thank Charger Products or BioTrain and the UAHuntsville Department of Chemical Engineering for providing an invaluable research, teaching and work experience. With Charger Products, I would like to thank Dr. Robert Zahorchak for being a good friend and a mentor. I would also like to thank Dr. Jonas Boateng for being a good friend and his valuable comments and suggestions during the course of my work. Also, my thanks are due to everyone at Educational Outreach, Hudson alpha Institute of Biotechnology and Open Biosystems, ThermoFisher Scientific for providing a wonderful work environment. I would also like to thank Dr. Joel Peek and Samantha Bond from Microarrays Inc. for granting access to their microarray scanner and providing valuable comments during my work.

I would like to thank Dr. Bernhard Vogler, Dr. Tracy Armstrong and Dr. Rodolphe Obeid for training me in NMR spectroscopy. I also thank Jennifer Perkins, Setosofia Agbenoto, Yance Syarif and Greg Bennett for their advice and help. My special thanks to

Yogi Kurniawan for his support and help in this project. I would also like to thank the current and past members of the Scholz lab for their support and help.

Finally, and most importantly, I would like to thank my wife, Dharini, my parents, my brother, my parents-in-law and friends for their unswerving support and encouragement during the course of this degree.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

CHAPTER 1

1. INTRODUCTION

1.1 Biofuel Industry

The biofuel industry is growing tremendously in an effort to reduce the dependency on petroleum-based fuels and address environmental concerns associated with their use (Pagliaro et al. 2007; Yazdani and Gonzalez 2007). One such sector is the biodiesel industry, which grew over the last decade, from 2 million gallons of biodiesel in the year 2000 to 315 million gallons in 2010 (Urbanchuk 2011). The production process of biodiesel employs transesterification of vegetable oils or animal fats, containing triacyl glycerols, with methanol in the presence of a strong base, such as potassium or sodium hydroxide, to form biodiesel and the co-product glycerol. For every 100 lbs of biodiesel produced, 10 lbs of glycerol is also produced (Yazdani and Gonzalez 2007). The glycerol stream contains a significant amount of impurities such as methanol, ash/salts (formed during the neutralization of the base with an acid), and residual fatty acids. Due to the presence of these impurities in the glycerol stream it is referred to as biodiesel-derived crude glycerol or simply crude glycerol.

The growth of the biodiesel industry has resulted in the accumulation of crude glycerol, as its application in comparison to pure glycerol is limited owing to the presence of impurities (Johnson and Taconi 2007). The price of crude glycerol has dropped significantly due to an increase in supply along with a simultaneous decrease in demand. This has resulted in the accumulation of crude glycerol leading to a glycerol glut (Johnson and Taconi 2007). Hence, the glycerol stream in the biodiesel plants has transformed from a co-product stream to a waste product. The purification costs of the glycerol stream are thrice the cost of the product and hence purification is not an economically feasible solution (Yazdani and Gonzalez 2007). The economic challenge of disposing the waste stream of crude glycerol has caused biodiesel companies such as Dow Chemicals and Procter & Gamble Chemicals to shut down their plants (Pagliaro et al. 2007). This clearly indicates that it is critical to find a solution to dispose or utilize crude glycerol from biodiesel plants to ensure the sustainability of the biodiesel industry.

1.2 Options for Crude glycerol

The most practical and feasible option for the crude glycerol is its conversion to value added products in the form of solvents and organic acids through bioconversion (Yazdani and Gonzalez 2007). Other options include disposal, which is hazardous due to the presence of methanol; purification, which is not economically favorable due to cost ineffectiveness; and co-firing the crude glycerol for thermal energy, which requires the removal of water and methanol, and also has environmental concerns. This leaves the bioconversion of crude glycerol by means of fermentation as a viable option. The highly reduced nature of glycerol, in contrast with sugars like glucose or xylose, results in the

production of twice the amount of reducing equivalents ($NADH^+$) compared to the catabolism of sugars such as glucose and xylose (Yazdani and Gonzalez 2007). This gives glycerol an advantage over sugars as a better substrate and, hence, anaerobic fermentation of glycerol is a promising approach.

Initially, the fermentation of glycerol was observed in pathogens such as *Citrobacter* and *Klebsiella* (Homann *et al.* 1990). Some species of algae, such as *Schizochytrium limacinum* (Yokochi et al. 1998), are capable of converting crude glycerol into omega-3 polyunsaturated fatty acids while *Yarrowia lipolytica* (Papanikolaou et al. 2002) has been shown to synthesize citric acid from crude glycerol. Species of *Clostridia* such as, *C. butyricum* (Raynaud et al. 2003; Gonzalez-Pajuelo, Andrade, and Vasconcelos 2004), and *C. pasteurianum* (Dabrock, Bahl, and Gottschalk 1992; Biebl 2001; Taconi, Venkataramanan, and Johnson 2009; Yazdani and Gonzalez 2007) can use glycerol as the sole carbon source for solvent production. However, the most studied solvent producing *Clostridia*, including *C. acetobutylicum* and *C. beijerinckii* do not have the natural ability to use glycerol as the sole carbon source (Johnson and Taconi 2007). Anaerobic utilization of glycerol has also been observed in *E. coli*, and after genetic modification, the utilization of crude glycerol has been improved (Atsumi et al. 2008; Dharmadi, Murarka, and Gonzalez 2006). *C. pasteurianum* is of particular interest due to its non-pathogenicity, unlike *Citrobacter* and *Klebsiella*, its ability to tolerate high concentrations of glycerol, and its ability to produce a wider range of products by using crude glycerol as the sole carbon source (Taconi, Venkataramanan, and Johnson 2009). The products include ethanol, 1,3-propanediol (1,3-PDO), butyrate and butanol (Dabrock and Gottschalk 1992; Taconi, Venkataramanan, and Johnson 2009). The metabolic pathway

of glycerol in *Clostridia* is described in Figure 1.1 (Johnson and Taconi 2007). The dashed lines (Figure 1.1) represent the electron flow while the boxed area represents the pathway for the production of butanol. The details of the enzymes regulating various steps are R1-glycerol dehydratase; R2-PDO dehydrogenase; O1-glycerol dehydrogenase; O2-dihydroxyacetone kinase; 1-lactate dehydrogenase; 2-pyruvate-ferredoxin oxidoreductase; 3-hydrogenase; 4-acetaldehyde dehydrogenase; 5-ethanol dehydrogenase; 6-thiolase; 7-butyryl-CoA dehydrogenase; 8-butyraldehyde dehydrogenase; and 9-butanol dehydrogenase, respectively.

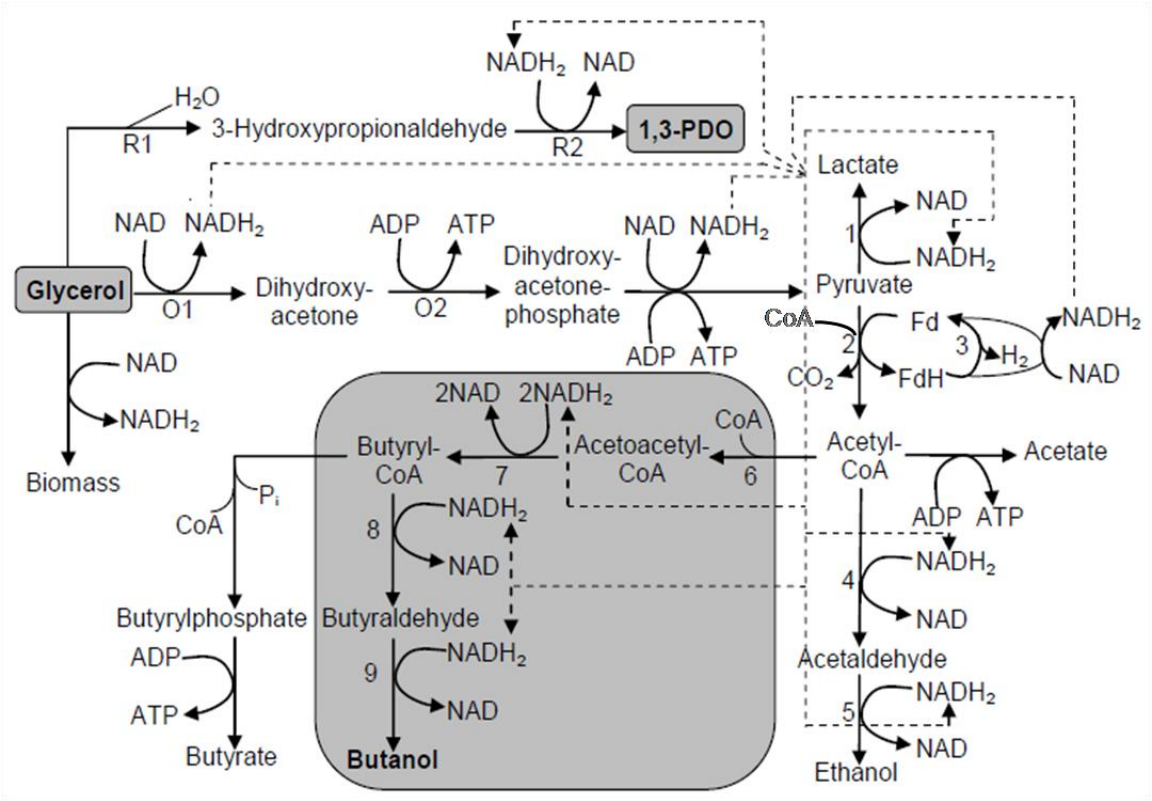


Figure 1.1 The Metabolic Pathway of Glycerol in *Clostridia*

1.3 *Clostridium pasteurianum* and Glycerol Metabolism

C. pasteurianum is one of the clostridial species able to consume glycerol as the carbon source for acid and solvent production. Glycerol is consumed by a dismutation process involving simultaneous oxidation and reduction (Johnson and Taconi 2007). The oxidation of glycerol is initiated by glycerol dehydrogenase resulting in the formation of dihydroxyacetone, which is later phosphorylated by a dihydroxyacetone kinase and is metabolized in the pathway to produce acetate, ethanol, butyrate and butanol (Luers et al. 1997). The reduction pathway is initiated by glycerol dehydratase to form an intermediate compound, 3-hydroxypropanaldehyde, which further gets reduced to 1,3-propanediol (PDO) (Luers et al. 1997; Macis, Daniel, and Gottschalk 1998). The final step in the production of PDO also results in the regeneration of the NAD^+. Thus *C. pasteurianum* shares most of it oxidative pathway with *C. acetobutylicum*, as the latter can produce acetone but does not have the ability to use glycerol as the sole carbon source (Taconi, Venkataramanan, and Johnson 2009). The product yields obtained from *C. pasteurianum*, particularly for butanol are as high as 0.4 g/g compared to 0.2 g/g obtained in *C. acetobutylicum* (Taconi, Venkataramanan, and Johnson 2009). This is the first study of *C. pasteurianum* as a potential butanol producer from biodiesel-derived crude glycerol.

1.4 Butanol Production in *Clostridia*

Butanol is a product of considerable interest due to its higher energy content compared to ethanol, as butanol (4C) has twice the amount of carbon atoms than ethanol (2C) (Huang et al. 2004). Butanol also has a better miscibility with petroleum products

and a lower vapor pressure. Butanol fermentation is of much importance as it can readily replace the process of butanol synthesis from the petroleum feedstock (Jones and Woods 1986). The butanol fermentation is carried out mostly by the species in the *Clostridium* genus. The fermentation of sugars in *Clostridium* generally results in the formation of the acetone, butanol and ethanol, and due to this product composition, the fermentation process is also known as the ABE (Acetone, Butanol and Ethanol) fermentation (Qureshi and Blaschek 2001; Ezeji, Qureshi, and Blaschek 2004; Ezeji, Qureshi, and Blaschek 2007; Ezeji, Qureshi, and Blaschek 2007). But the strains capable of metabolizing glycerol, such as *C. pasteurianum* and *C. butyricum* also produce 1,3-propanediol. *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, and *C. pasteurianum*, are the various strains that are able to synthesize butanol by fermentation. *C. acetobutylicum* can synthesize butanol when grown on sugars while *C. butyricum* and *C. pasteurianum* can ferment glycerol into butanol and *C. beijerinckii* can use both sugar and glycerol as the carbon source for butanol production.

C. acetobutylicum is the most studied clostridial strain for butanol production. The maximum butanol produced by *C. acetobutylicum* is 17.6 g/L and with genetic modification the butanol production was increased to 20 g/L (Tomas, Welker, and Papoutsakis 2003). There has been a considerable amount of genetic modification in the form of amplification of genes encoding enzymes in the butanol pathway and the genes encoding heat shock proteins have been reported to maximize butanol production (Narberhaus and Bahl 1992; Tomas, Welker, and Papoutsakis 2003; Papoutsakis 2008). Microarray analysis of *C. acetobutylicum* has revealed that the butanol production is coupled to many other metabolic pathways such as the acidogenesis (acid formation),

sporulation and the expression of stress response proteins (Tummala et al. 2003; Tomas et al. 2003; Tomas, Beamish, and Papoutsakis 2004; Alsaker, Paredes, and Papoutsakis 2005; Alsaker, Spitzer, and Papoutsakis 2004; Borden and Papoutsakis 2007; Borden, Paredes, and Papoutsakis 2005). These microarray results suggest the complexity of the regulation involved in butanol production by clostridia. In *Clostridia*, the metabolism of acid and solvent production takes place in two different phases, acidogenesis and solventogenesis (Jones and Woods 1986). The acidogenesis phase involves the production of acids in the form of acetic acid, butyric acid and lactic acid which result in a reduction in the pH. At this reduced pH, the solventogenesis gets initiated resulting in the production of solvents such as acetone, ethanol, butanol and 1,3-PDO.

1.5 Butanol Toxicity and Butanol Tolerance

Butanol is toxic to cells, as it accumulates in the cell membrane, affecting both the structural and functional integrity of the cell. The extent of solvent toxicity correlates to the $\log_{10}$ P value, where, P is the partition coefficient. Solvents with a $\log_{10}$ P value less than 4 are considered extremely toxic and butanol has a $\log_{10}$ P value of 0.8 and is considered to be one of the most toxic solvents (Sardessai and Bhosle, 2002). The mechanism of butanol toxicity is largely unknown, but has been predicted to affect the cell membrane by means of modifying its fluidity (Baer, Blaschek, and Smith 1987; Wang, Kashket, and Kashket 2005). It has also been suggested that the butanol affects the fluidity of the cells by means of altering the transmembrane ΔpH which is essentially the difference in the pH of the intracellular and the extracellular regions (Wang, Kashket, and Kashket 2005). It has been showed that the ΔpH was found to be larger in case of the

strains that were tolerant to butanol compared to wild type strain having lesser ability to tolerate butanol (Wang, Kashket, and Kashket 2005). Butanol also eliminates the ability of the cell to maintain an internal pH and inhibits membrane-bound ATPases and the uptake of glucose, which subsequently inhibits energy generation (Bowles and Ellefson 1985).

The butanol tolerance mechanism is initiated by the cells exposed to butanol by altering the composition of the lipids in the cell membrane (Baer, Blaschek, and Smith 1987). It has been observed in strains like *C. acetobutylicum* and *C. beijerinckii*, that an increase in butanol concentration in the media has altered the ratio of the saturated fatty acids to unsaturated fatty acids along with the changes in the fatty acid chain length (Baer, Blaschek, and Smith 1987; H. J Heipieper, Weber, et al. 1994; Liyanage, Young, and Kashket 2000). In butanol tolerant strains, the ratio of saturated to unsaturated fatty acids has been observed to increase, when they are exposed to butanol concentrations of 1.5% along with the increase in the number of longer fatty acids compared to shorter acyl chains (Baer, Blaschek, and Smith 1987).

It is widely accepted that most species of clostridia cannot tolerate more than 2 % (w/v) butanol (Fischer, Klein-Marcuschamer, and Stephanopoulos 2008; Knoshaug and Zhang 2009). In an attempt to develop a more butanol tolerant microorganism, efforts have focused on the metabolic engineering of *C. acetobutylicum*. *Escherichia coli* (Atsumi et al. 2008; Winkler, Rehmann, and Kao 2010; Lee et al.; Dharmadi, Murarka, and Gonzalez 2006) and *Saccharomyces cerevisiae* (Steen et al. 2008) are also considered good host candidates for metabolic engineering, since the genetic tools for altering these

organisms are more advanced than those for *Clostridia*. However, neither *E. coli* nor *S. cerevisiae* has been shown to have a higher butanol tolerance than *Clostridia*, as the butanol tolerance limit is 2 % (w/v).

1.5.1 *Clostridium pasteurianum* and Butanol production

Research has shown that *Clostridium pasteurianum* may offer several advantages over other organisms that have been considered for solvent production. A wild-type strain of *C. pasteurianum* has been shown to produce as much as 17 g/L butanol from pure glycerol (Biebl, 2001), which suggests that this organism has a natural ability to tolerate higher concentrations of butanol than other species of solventogenic C*lostridia*. Until now, it was unknown whether, *C. pasteurianum* is capable of producing butanol from crude glycerol, which contains significant amounts of methanol and salts, at yields comparable to those produced from purified glycerol. Also, *C. pasteurianum* had not been studied for butanol tolerance and there is no reported work on the lipid composition of *C. pasteurianum*. Investigating the effects of alcohols and solvents on lipid composition and the homeoviscous response by *C. pasteurianum* will help in understanding the butanol toxicity and tolerance, which will aid in engineering a high butanol tolerant and high butanol producing strains.

1.6 Objectives

Previous studies have shown that *C. pasteurianum* has the natural ability to utilize glycerol as the sole carbon source and make solvents in the form of butanol and 1,3-PDO. The goal of this study was to apply this inherent ability of *C. pasteurianum* to synthesize butanol from glycerol as a means to consume the excess crude glycerol from the biodiesel

industry, which threatens the sustainability of the industry. Also the maximum butanol tolerance level and the mechanism of butanol tolerance along with the homeoviscous changes in the lipid bilayer in *C. pasteurianum* have not yet been reported. The specific objectives of this study were to:

1. Compare the utilization of crude glycerol for butanol production to that of pure glycerol for the growth of the cells and product formation.
2. Study the effect of various parameters on growth and product formation such as effect of the addition of co-products in the butanol pathway; effect of the addition of a second carbon sources such as glucose, arabinose or xylose for co-metabolism with glycerol; effect of biotin addition and effect of the impurities in crude glycerol.
3. Identify the maximum butanol tolerance level for *C. pasteurianum* and study the mechanism of butanol tolerance by analyzing the lipid membrane.
4. Develop and optimize protocol for carrying out a microarray analysis using the probes of *C. acetobutylicum*. Identify the differentially expressed genes using microarray that are expressed during the metabolism of pure glycerol, crude glycerol and glucose.

1.7 Significance

This study is focused on developing a process that results in the utilization of biodiesel-derived crude glycerol to produce butanol using *Clostridium pasteurianum*. When successful, a metabolically engineered culture will produce more butanol utilizing crude glycerol, which means it must exhibit higher tolerance to butanol and the impurities

found in crude glycerol. This study will result in the quantification of the concentrations of butanol and impurities that partially and/or completely inhibit cell growth, substrate utilization, and product formation. This characterization has not previously been done for *C. pasteurianum* and is an important step toward understanding the robustness of the organism. Additionally, the impurities in crude glycerol, specifically methanol and salts, as well as butanol can have detrimental effects on the cell membrane. Therefore, the purpose of this study is also to specifically quantify the effects of increasing butanol, methanol, and salt concentration on the fluidity and stability of the lipid bilayer. It has been known for some time that butanol (and other alcohols) induces physical and chemical changes in the membrane structure. However, examining membrane structure using reconstituted and intact membranes obtained from samples collected under different cell culture conditions, which translates to differences in cellular growth and metabolism, has not been previously investigated. Linking changes in the structure of the membrane with changes in cellular metabolism and gene expression could be a powerful tool for predicting and validating how the cell alters its structure in response to stress, such as exposure to high concentrations of salts and alcohols. The development of a protocol to study gene expression, using probes from a related sequences species, will result in opening doors to study whole cell transcriptomic analysis at various conditions and will hence offer a big picture with respect to study of major metabolic changes during butanol production from glycerol.

CHAPTER 2

2. CRUDE GLYCEROL – A SOLE CARBON SOURCE

(This chapter has been published in *Environ. Prog Sustain Energy*, 28: 110-110 (2009) under the title "Growth and Solvent Production by *Clostridium pasteurianum* ATCC® 6013™ Utilizing Biodiesel-derived Crude-glycerol as the Sole Carbon Source")

2.1 Introduction

The goal of this research is to evaluate the potential for utilizing *C. pasteurianum* to produce butanol from crude glycerol via anaerobic fermentation. Cell growth and substrate utilization were compared using both crude glycerol obtained from a biodiesel production facility and purified glycerol. To date, this is the first reported effort of using fermentation to produce butanol from biodiesel derived crude glycerol using *C. pasteurianum*.

There are few reports of growth and product formation by other species of clostridia using crude glycerol as the sole substrate. In addition to *C. pasteurianum*, *Clostridium butyricum* can also metabolize glycerol. However, *C. butyricum* produces PDO, acetate, and butyrate and does not produce solvents. Early efforts to culture *C. butyricum* on crude glycerol were unable to observe growth of any strains obtained from culture collections. However, four new strains isolated from mud samples were able to grow on crude glycerol. Some of the isolates were able to metabolize crude glycerol equally as purified glycerol, while others could not (Petitdemange et al. 1995).

Subsequent efforts reported no inhibition of growth of *C. butyricum* at glycerol (both purified and crude) concentrations up to 20 g/L, and similar levels of growth inhibition by crude and purified glycerol at concentrations above 20 g/L, indicating that glycerol may cause growth inhibition, regardless of the source or presence of impurities (Gonzalez-Pajuelo, Andrade, and Vasconcelos 2004). Other authors reported equivalent growth characteristics for cultures of *C. butyricum* grown on both purified and crude glycerol, but noted a prolonged lag phase prior to substrate utilization in cultures grown on crude glycerol (Papanikolaou et al. 2000). Unfortunately, these efforts did not directly compare growth using glycerol as the substrate to growth using glucose.

2.2 Materials and Methods

Clostridium pasteurianum ATCC 6013™ was obtained from the American Type Culture Collection (Manassas, VA). To ensure anaerobic conditions, all cultures were prepared in a vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Freeze-dried cultures were reconstituted with 1 mL of media and heat-shocked at 70–80°C for 10 minutes. Of the 1 mL total volume, 800 µL was transferred to two culture flasks and 200 µL was reserved and stored as a stock culture. The heat-shocked culture was transferred to 75 mL culture vials containing 30–40 mL of nutrient media. Initial cultures were transferred directly to nutrient media containing K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, $CaCO_3$, yeast extract, and trace element solution, as described previously (Biebl 2001). All media components were purchased from either Fischer Scientific or Sigma-Aldrich. The carbon source was either purified (laboratory-grade) glycerol (Fisher Scientific) or biodiesel-derived crude

glycerol (Green River Biodiesel, Moundville, AL) at concentrations of 5 or 10 g/L (54.3, 108.6, and 271.4 mM). The crude glycerol was estimated to contain approximately 90–95% glycerol (the glycerol component contains 10-13% fatty acids, as determined later. See Chapter 4), 5–10% methanol and/or water, and 3–5% sodium sulfate, as specified by Green River Biodiesel. The initial pH of the media was approximately 7, and the media was not buffered. Cultures were incubated anaerobically at 37°C. Cell growth was determined using optical density (OD) at 610 nm. The pH of the fermentation broth was measured at regular intervals, and liquid samples were collected to determine substrate utilization and product formation. All experiments were performed in triplicate. Growth and substrate utilization observed by cultures transferred directly to glycerol media was very slow. Therefore, subsequent cultures were first grown on 8% w/v (80 g/L) glucose media. The cells were allowed to grow to an OD of at least 0.6 before being transferred to nutrient media containing 25 g/L pure glycerol. The $FeSO_4.7H_2O$ concentration of the nutrient media was also increased to 100 mg/L. After demonstrating sufficient glycerol utilization and solvent production, subcultures were transferred to nutrient media containing 25 g/L crude glycerol. Liquid samples were analyzed for glycerol, methanol (if crude glycerol was the substrate), 1,3-propanediol, acetate, butyrate, ethanol, and butanol using high pressure liquid chromatography. The HPLC system was equipped with a Bio-Rad Aminex HPX-87H column and a ProStar 355 refractive index detector (Varian, Walnut Creek, CA). The mobile phase was 5 mM H_2SO_4, pumped at a flow rate of 0.4 mL/min. Standards were prepared using HPLC grade chemicals purchased from either Fisher Scientific or Sigma-Aldrich. Cell growth and fermentation was performed in batch culture. Upon completion of the fermentation process (i.e., little to no substrate

detected in the fermentation broth), cells were centrifuged at 10,000 rpm for 10 min, and the cell pellets were resuspended in fresh medium.

2.3 Results and Discussion

2.3.1 Cell Growth

Clostridium pasteurianum ATCC® 6013™ was grown on both purified and biodiesel-derived crude glycerol at concentrations of 5 g/L (54.3 mM), 10 g/L (108.6 mM), and 25 g/L (271.4 mM). The original cultures were maintained for over 6 months, via transfers of cells to fresh media at increasing glycerol concentrations.

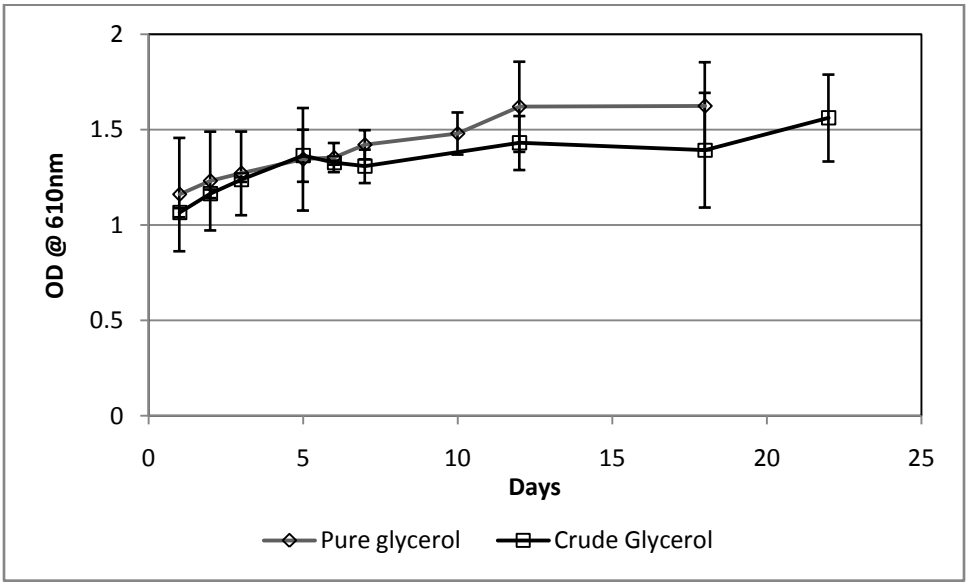


Figure 2.1 Comparison of Cell Growth [Initial Glycerol] = 5 g/L

Figure 2.1 compares the average optical density of cultures grown at an initial glycerol concentration of 5 g/L. As shown in the figure, growth on both purified and crude glycerol continued for up to 18 days on purified glycerol and 22 days on crude glycerol. While growth did appear slightly better on purified glycerol as compared to crude glycerol, the variation in the data indicates that the difference is not appreciable. Although cultures grown on 5 g/L glycerol continued to grow for an extended period of time, most of the growth occurred within the first 5–7 days, as indicated by the larger increase in OD during this time. Because growth is dependent on substrate concentration, the growth rate should decrease as the substrate is consumed. Most experiments grown on 5 g/L purified glycerol demonstrated nearly complete substrate utilization within 5–7 days. However, untransferred cultures required longer to consume the glycerol substrate, up to 18 days for pure glycerol. It was only after several transfers that the cells acclimated and the fermentation time decreased to 5–7 days. All cultures grown on crude glycerol required longer, up to 16 to 22 days, to metabolize all of the substrate. However, like the cultures grown on purified glycerol, most of the growth occurred within the first 5–7 days.

The cells grown on 5 g/L glycerol (pure and crude) were not precultured on glucose (or any other media). After the cells were reconstituted and heat-shocked, they were inoculated directly into the glycerol media. The original inoculum was recovered via centrifugation and transferred to subsequent experiments. Therefore, any growth should be attributed to glycerol utilization. Overall, the OD increased by a factor of almost 1.4. While this is not a large increase in cell density, cell growth did occur due to glycerol metabolism. Cell growth would have been limited by the low concentration of

substrate. Additionally, the more reduced state of the glycerol substrate may contribute to reduced growth, as will be discussed in more detail later in the chapter.

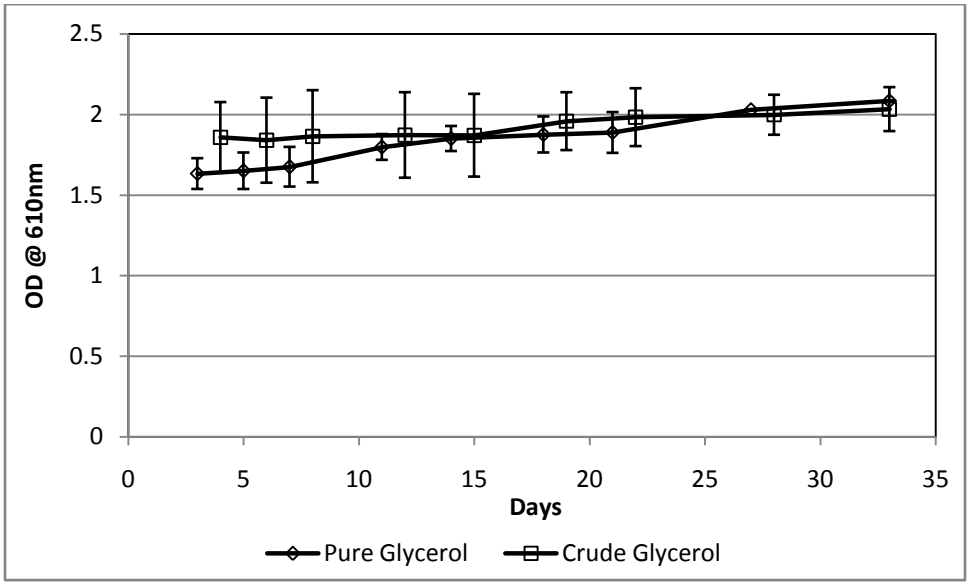


Figure 2.2 Comparison of Cell Growth [Initial Glycerol] = 10 g/L

Figure 2.2 compares cell growth using an initial glycerol concentration of 10 g/L. Cultures from the 5 g/L experiments were used to inoculate experiments containing 10 g/L glycerol. As at the lower concentration, there is no statistical difference in growth when comparing purified vs. crude glycerol. Growth continued for much longer, however, as the OD continued to increase for up to 33 days. The higher glycerol concentration also seemed to cause a lag in growth, particularly when crude glycerol was the substrate. As a result, cultures utilizing 10 g/L pure glycerol required substantially longer (27–33 days) to completely consume the substrate. Cultures grown on 10 g/L

crude glycerol were only able to metabolize 79–83% of the crude glycerol. However, the culture appeared to acclimate to glycerol utilization over time. In subsequent transfers, the amount of time required to metabolize the substrate decreased from 27 to 8 days. Overall, the OD only increased by a factor of about 1.2. Given that these cells had undergone multiple transfers, it is possible that the cells had entered the stationary phase at this point in experimentation.

Unfortunately, over time, the cultures used in the 5 and 10 g/L experiments degenerated and gradually lost all ability to produce solvents. Therefore, cultures used in the 25 g/L experiments were first pre-cultured on glucose media as described in Methods and Materials. The purpose of the pre-culturing was to grow the cells to an acceptable density prior to glycerol fermentation, and, ultimately, reduce the amount of time required to utilize the glycerol substrate. Figure 2.3 compares cell growth at an initial glycerol concentration of 25 g/L. Overall, growth was better at the higher concentration of glycerol. The initial culture increased in OD by a factor of 1.78 after growth on 25 g/L pure glycerol, and the overall OD doubled after one subsequent transfer. A portion of the initial culture was also transferred to 25 g/L crude glycerol, and although no growth occurred, the cells did appear to maintain the stationary phase. As indicated in Figure 2.3, the culture was able to completely consume the pure glycerol in 10 days. The amount of glycerol consumed ranged from 72 to 99% (avg. 92%). Cultures growing on 25 g/L crude glycerol required significantly longer, 14 to 24 days, to completely metabolize the substrate. However, the amount of time required to metabolize the crude glycerol decreased with subsequent transfers. The cultures metabolized 75–95% of the crude glycerol (avg. 86%). Figure 2.4 compares the maximum optical density observed

at each initial glycerol concentration. Differences in culture growth and pre-culture conditions among experiments contribute to the large standard deviations.

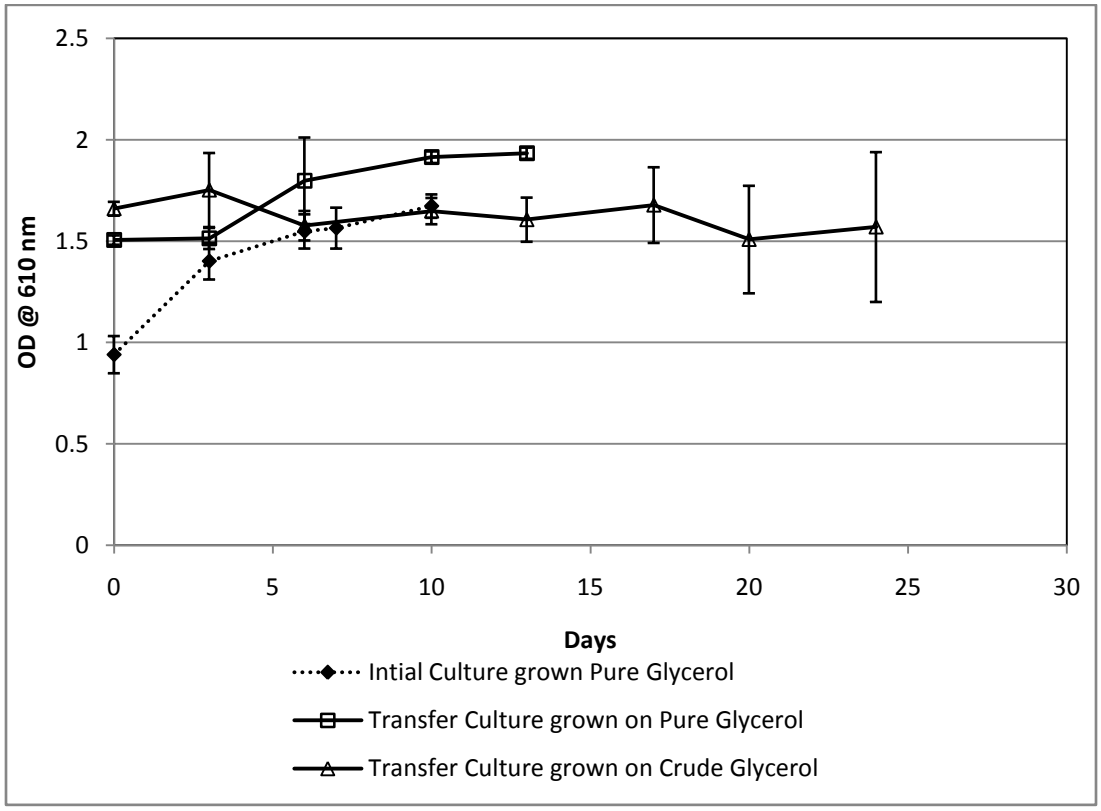


Figure 2.3 Comparison of Cell Growth (Initial concentration = 25 g/L)

Although overall growth of the culture was slow, results indicate that *C. pasteurianum* is capable of growth and cell maintenance on biodiesel-derived crude glycerol at rates comparable to those observed during growth on purified glycerol. *C. pasteurianum* is also capable of utilizing crude glycerol to produce solvents, particularly significant amounts of butanol. To the best of the authors' knowledge this is the first report of cell growth and solvent production by *C. pasteurianum* using crude glycerol as the sole carbon source. This data indicates that production of butanol via glycerol

fermentation is a promising alternative for biobutanol production that merits further investigation.

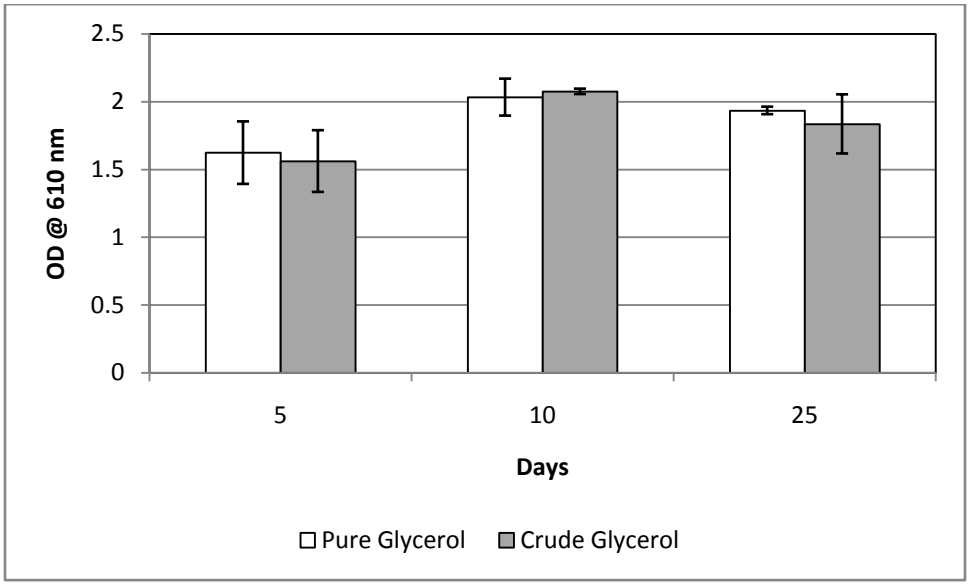


Figure 2.4 Maximum OD comparison for 5, 10 and 25 g/L pure and crude glycerol

Previous efforts evaluating growth of *C. pasteurianum* reported growth on purified glycerol in batch culture at concentrations ranging from 50 g/L up to 170 g/L (Dabrock, Bahl, and Gottschalk 1992; Biebl 2001). Our results indicate that *C. pasteurianum* can grow on biodiesel derived crude glycerol, at concentrations up to 25 g/L. It should be noted that cell growth in these experiments was slower and the overall optical density was lower than results in other published work. The difference in optical density is in part due to different procedures, as previous work measured OD at 578 nm (Dabrock, Bahl, and Gottschalk 1992). It is also possible that *C. pasteurianum* simply

does not grow as well when utilizing glycerol rather than other substrates such as glucose. Mitchell *et al.* compared growth of *C. pasteurianum* metabolizing glucose and mixtures of glucose and sorbitol (Mitchell et al. 1987). Sorbitol, like glycerol, is a more reduced substrate than glycerol. Cells were pre-cultured on either glucose or sorbitol. The growth of cells pre-cultured on glucose was studied using media containing glucose and sorbitol, while growth of cells pre-cultured on sorbitol was studied using media containing sorbitol only and media containing glucose and sorbitol. The published data indicates that both the growth rate (as indicated by changes in OD) and maximum OD achieved by sorbitol preculture was lower than that reported for the glucose pre-culture. The sorbitol culture did exhibit faster growth and a higher OD when metabolizing sorbitol and glucose as compared to sorbitol only. However, the pre-culture grown on glucose achieved a growth rate and maximum OD nearly twice that of the sorbitol pre-culture.

2.3.2 Product Formation

Cultures grown on both purified and crude glycerol were able to produce a range of products during fermentation. Table 2.1 shows the maximum product yields obtained by the cultures at utilizing 5, 10, and 25 g/L of both purified and crude glycerol. At an initial concentration of 5 g/L pure glycerol, all but three experiments metabolized essentially all of the glycerol initially present (n =12). All experiments produced PDO (n = 12). The maximum PDO yield observed was 0.41 g/g (g of PDO/g of glycerol), and the average yield was 0.20 g/g. In a few cases, PDO was the only product present at the end

of the fermentation process, and no solvents or acids were detected. Ethanol was produced in nearly all of the experiments (n = 9). Of the experiments that produced ethanol, the maximum and average ethanol yields were 0.36 and 0.17 g/g, respectively. Although butanol production typically occurs along with ethanol production (alcohologenesis), butanol production was observed in only five samples. The maximum butanol yield was 0.36 g/g, and the average was 0.12 g/g. Acetate was detected in eight experiments, while butyrate was detected in only three. Only trace amounts of acetate were produced, and the average acetate yield was 0.013 g/g. Butyrate production was slightly greater, with a maximum yield of 0.16 g/g and an average yield of 0.081 g/g. Although the yields were lower compared to pure glycerol, cultures grown on 5 g/L crude glycerol produced PDO, ethanol, butanol, acetate, and butyrate, as shown in Table 2.1. In experiments where all of the glycerol was utilized, the fraction of carbon recovered ranged from 0.13 to 1.09 and averaged 0.66. Cultures grown on 10 g/L purified glycerol produced considerably less products. Several transfers yielded no detectable products in the fermentation broth. Butanol and PDO were detected in two experiments. The average PDO yield was 0.084 g/g, and the average butanol yield was 0.084. The butyrate yield was 0.074 g/g. Only trace amounts of ethanol and acetate were detected. The fraction of carbon recovered was only 0.37, indicating that a significant amount may have been incorporated into the cells during growth. Cultures grown on 10 g/L crude glycerol were able to produce PDO (n = 3), ethanol (n = 2), and acetate (n = 2), but no butanol or butyric acid was detected. The average yields of PDO, ethanol, and acetate were 0.14, 0.20, and 0.11 g/g, respectively. The loss of solvent production was attributed to strain degeneration, which is a common occurrence in other species of clostridia.

Table 2.1 Maximum product yield, g/g glycerol consumed (mol/mol glycerol consumed)

Products / Variants	**PDO**	**Ethanol**	**Butanol**	**Acetate**	**Butyrate**
5 g/L Pure glycerol	0.41 (0.50)	0.36 (0.72)	0.36 (0.45)	0.045 (0.07)	0.16 (0.17)
5 g/L Crude glycerol	0.018 (0.02)	0.23 (0.46)	0.03 (0.04)	0.025 (0.04)	0.07 (0.07)
10 g/L Pure glycerol	0.074 (0.09)	0.01 (0.02)	0.12 (0.15)	0.02 (0.03)	0.074 (0.08)
10 g/L Crude glycerol	0.275 (0.33)	0.225 (0.45)	Nd	0.16 (0.245)	Nd
25 g/L Pure glycerol	0.39 (0.47)	0.025 (0.05)	0.31 (0.385)	0.094 (0.14)	0.23 (0.24)
25 g/L Crude glycerol	0.064 (0.078)	0.065 (0.13)	0.30 (0.37)	0.037 (0.057)	0.054 (0.056)

Cultures grown on 25 g/L glycerol demonstrated much more consistent product formation, and by far, PDO and butanol were the dominant products. All experiments utilizing purified glycerol (n = 6) produced PDO, acetate, ethanol, butyrate, and butanol. The PDO yield ranged from 0.12 to 0.42 g/g, and the average was 0.33 g/g. Acetate and butyrate, which are the precursors to ethanol and butanol, were produced at yields ranging from 0.03 to 0.09 g/g for acetate and 0.03 to 0.23 g/g for butyrate. All cultures grown on 25 g/L pure glycerol produced ethanol and butanol. The ethanol yield ranged from 0.01 to 0.025 g/g, with an average yield of 0.016 g/g. Butanol yields ranged from 0.18 to 0.31 g/g, and the average was 0.24 g/g. The overall carbon recovery ranged from

0.65 to 1.1 and averaged 0.92, indicating that most of the substrate was converted to soluble products detectable in the liquid phase.

All cultures grown on 25 g/L crude glycerol (n = 6) produced PDO, acetate, ethanol, butyrate, and butanol. However, there were distinct differences in the product distribution. Cultures grown on crude glycerol produced much less PDO, as the highest yield observed was 0.064 g/g, and the average yield was 0.038 g/g. The yields of acetate and butyrate were also slightly lower than those observed on pure glycerol, ranging from 0.013 to 0.036 g/g for acetate and 0.01–0.05 g/g for butyrate. The ethanol yield ranged from 0.02 to 0.06 g/g and averaged 0.026 g/g. By far, butanol was the dominant product, as the butanol yield ranged from 0.19 to 0.30 g/g and the average was 0.22 g/g. The carbon recovery was also much lower in experiments utilizing crude glycerol, as only 0.4–0.55 (avg. 0.45) mole fraction of the carbon was recovered in the liquid products. It is possible that these cultures produced carbon dioxide; however, this was not captured or measured.

Because the media was not buffered, the pH of the fermentation broth decreased over time. At an initial glycerol concentration of 5 g/L, the final pH was 6.22 ± 0.23. As the initial glycerol concentration increased, the final pH decreased, as would be expected. In batch fermentation, the higher initial substrate concentration results in higher product concentrations. At initial glycerol concentrations of 10 and 25 g/L, the average final pH was 5.78 ± 0.23 and 5.16 ± 0.23, respectively. There was no apparent difference in the final pH when comparing experiments that used purified glycerol to those using crude glycerol. Figures 2.5a and 2.5b show the fermentation profiles as a function of time with either purified or crude glycerol as the substrate. As shown in the figures, *C.*

pasteurianum exhibits biphasic fermentation, first producing acetic and butyric acids, which causes a decrease in pH. The accumulation of acids then triggers solvent production, in this case, primarily butanol. The data in Figure 2.5a show that PDO production primarily occurs in the early stages of fermentation, prior to the pH decrease due to acid accumulation. The trends in the data shown in Figure 2.5b reflect the lag in substrate utilization and product formation that was observed in the cultures utilizing crude glycerol. This lag was likely caused by the impurities in the crude glycerol, including methanol and salts.

Previous studies evaluating glycerol utilization by *C. pasteurianum* also reported incomplete glycerol utilization. Dabrock *et al.* reported that batch cultures demonstrated glycerol utilization up to 170 g/L (Dabrock, Bahl, and Gottschalk 1992). However, batch cultures were only able to completely metabolize about 28 g/L glycerol (300 mM), and at higher concentrations, significant amounts of glycerol remained in the fermentation broth. Biebl reported that cultures could not completely metabolize the glycerol at concentrations above about 50 g/L (543 mM). Biebl also reported that the carbon recovery decreased to 75–85% at glycerol concentrations of 50 g/L and above, while up to 95% of the carbon was recovered at a concentration of 30 g/L.

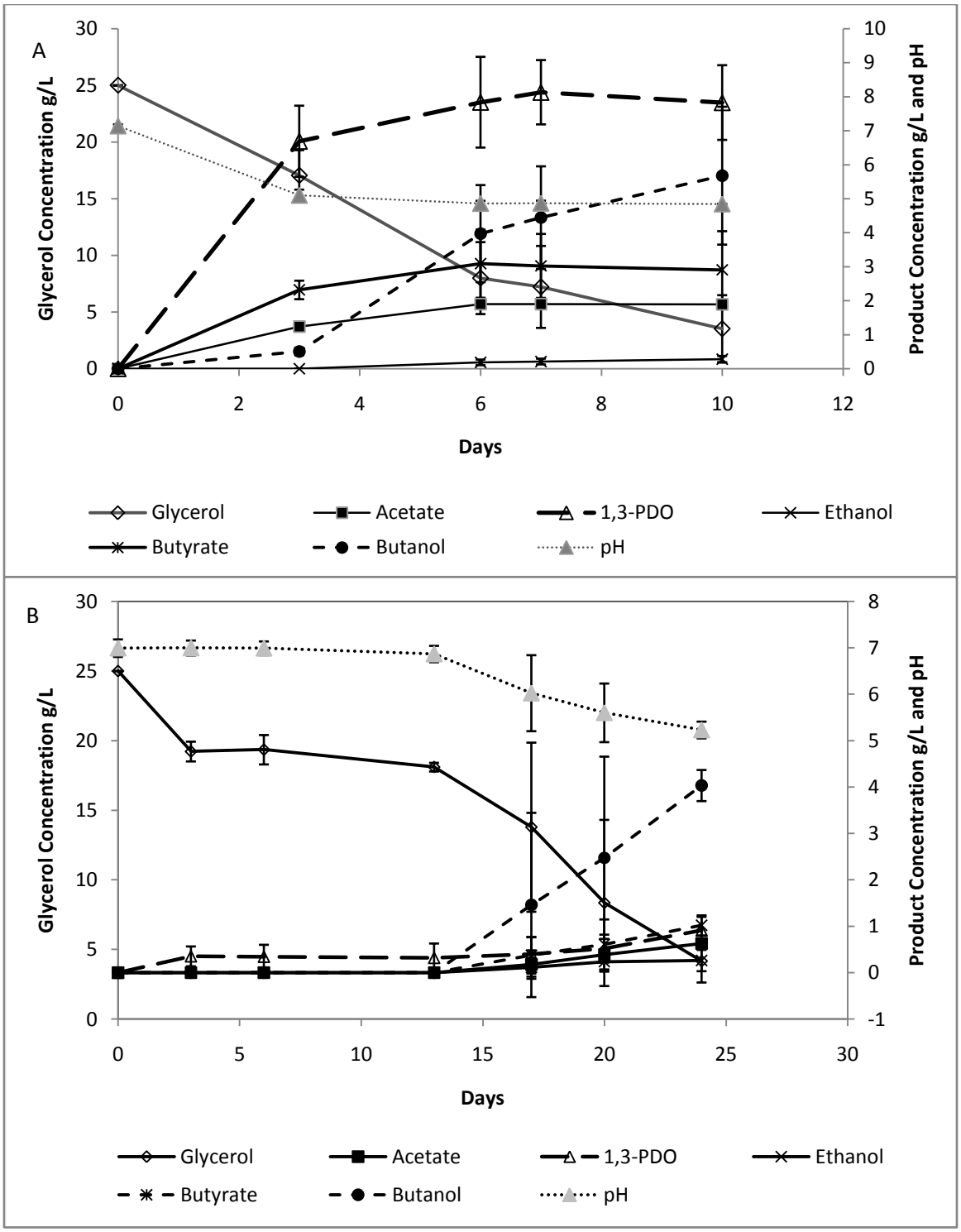


Figure 2.5 Fermentation profile for *C. pasteurianum* (A) 25 g/L pure glycerol. (B) 25 g/L crude glycerol

Table 2.2 Maximum and average product yields, g/g glycerol consumed (mol/mol glycerol consumed) using *C. pasteurianum*

	PDO	Ethanol	Butanol	Acetate	Butyrate	Lactate
Dabrock *et al*	**0.28 (0.34)**	**0.15 (0.3)**	**0.24 (0.3)**	**0.015 (0.023)**	**0.00285 (0.003)**	**0.245 (0.25)**
	0.17 ±0.07	0.053 ± 0.065	0.17 ± 0.066	0.01 ± 0.004	0.003 ±0.0002	0.10 ± 0.10
Biebl	**0.22 (0.27)**	**0.13 (0.26)**	**0.31 (0.39)**	**0.027 (0.041)**	**0.125 (0.13)**	**0.03 (0.031)**
	0.12 ± 0.06	0.031 ± 0.037	0.20 ± 0.055	0.016 ± 0.009	0.038 ± 0.04	0.011 ± 0.013
This Study	**0.42 (0.51)**	**0.36 (0.72)**	**0.36 (0.45)**	**0.16 (0.245)**	**0.23 (0.24)**	Nd
	0.17 ± 0.14	0.095 ± 0.11	0.18 ± 0.10	0.057 ± 0.036	0.066 ± 0.057	

The slow rates of growth and substrate utilization observed in this work may be partially due to a low initial cell concentration as well as the low substrate concentration. It should be acknowledged that significant growth and substrate utilization should occur within 36–48 h, rather than over 10–20 days, as observed in some of these experiments. However, over time, cultures did demonstrate faster rates of growth and substrate utilization, indicating that cultures can acclimate to glycerol utilization. Improved glycerol utilization was also observed when cells were pre-cultured on glucose media, likely due to a higher cell density.

Table 2.2 compares the maximum and average product yields reported in previous studies to those obtained in this work. Dabrock *et al.* reported significant production of

PDO, ethanol, butanol, and lactate during glycerol fermentation, per mole of glycerol, experiments produced as much as 0.28 g/g (0.34 mol/mol) of lactate, and the average lactate yield was 0.17 g/g (0.21 mol/mol). These authors also reported much lower yields of ethanol and PDO compared to other efforts. It should be noted that all yield data was generated from experiments initially containing 40 g/L glycerol (435 mM), and only 77–90% of the substrate was converted to products. Additionally, yield data was reported for continuous cultures grown under either phosphate or iron limitation. Dabrock *et al.* also concluded that iron limitation apperated to inhibit the formation of butanol and ethanol and speculated that the alcohol dehydrogenase enzymes responsible for butanol and ethanol formation are iron dependent, as they are in *C. acetobutylicum*.

Biebl also reported significant production of PDO, ethanol, and butanol. However, experiments produced only trace amount of lactate, if any at all. Biebl compared product yield in batch culture as a function of both initial glycerol concentration and pH. Cultures were not grown under any nutrient limitation. Experiments comparing product yield at varying glycerol concentrations were performed using concentrations of 29.5, 54.2, 83.7, 114.6, and a fed batch culture that was provided 66.5 g/L over 25 h. The pH was held constant at 6.0. At concentrations above 54.2 g/L, 55–75% of the glycerol remained in the fermentation broth. Experiments evaluating pH were performed at constant pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 at an initial glycerol concentration of 50 g/L. Glycerol concentration only seemed to affect the ethanol yield, which increased as the initial glycerol concentration increased. The highest ethanol yield of 0.13 g/g (0.265 mol/mol) was obtained when the pH was increased to 7.5. Other product yields varied, seemingly independent of the initial glycerol

concentration. The highest butanol yield observed was 0.31 g/g (0.39 mol/mol), at an initial pH of 4.5. However, this was attributed to fluctuations the culture conditions or slight differences in the pre-cultures rather than some function of pH, as butanol yield at higher pH values showed no trend as a function of pH. Biebl [20] also reported that batch and chemostat cultures favored butanol and ethanol production, while pH auxostat cultures that provide an excess of all nutrients favor PDO production.

The distribution of products observed in this work is considerably different from what has previously been reported. In most cases, this work produced an average PDO yield comparable to those obtained by others. However, the maximum PDO yield obtained in this work was 0.41 g/g, much higher than the maximum PDO yield reported by Dabrock *et al*. or Biebl. In the case of the cultures growing on 25 g/L crude glycerol, however, the PDO yield was much lower, averaging only 0.038 g/g. The reason for this low yield is not certain, and more experimentation using higher concentrations of crude glycerol is necessary to determine if it was caused by impurities in the crude glycerol or differences in the culture.

The maximum (0.36 g/g) ethanol yield observed in this work is much higher than previously reported. However, the average ethanol yield of 0.095 g/g is lower than that reported by others. The maximum and average acetate yields were also higher in these experiments compared to others. The discrepancies in ethanol production may have been caused by differences in the pH of the fermentation broth at each of the different glycerol concentrations. Biebl previously reported that ethanol production appeared to be pH dependent, as ethanol production increased significantly at pH values of 6–7.5 compared with pH values of 4.5–5.5 (the pH was held constant at each value). In this work, the

highest ethanol yield of 0.36 g/g was achieved in experiments containing 5 g/L glycerol. Although these experiments were not buffered, the pH of the 5 g/L experiments decreased only slightly, to an average of 6.22, due to the small amount of products formed. In contrast, the final pH of the 25 g/L experiments was on average 5.16, which is in the pH range at which Biebl reported significantly less ethanol production. The fact that these experiments consistently produced very little ethanol when the pH was below 6.0 seems to support the conclusion that ethanol production by *C. pasteurianum* is pH-dependent.

The overall average butanol yield of 0.18 g/g obtained in this work is comparable to that reported by Dabrock *et al.* and Biebl. Additionally, this work has produced the highest overall yield of butanol–0.36 g/g. Experiments consistently produced 0.19–0.31 g/g butanol when 25 g/L glycerol was provided as the substrate, regardless of whether the glycerol was purified or crude. In experiments comparing 25 g/L glycerol, there was no significant difference in the butanol yield produced by cultures metabolizing purified glycerol as compared to those metabolizing crude glycerol. To date, the highest butanol yield obtained from crude glycerol is 0.3 g/g, and the average butanol yield from crude glycerol is 0.22 g/g.

The experiments in this study were performed in media that was not buffered, and as a result, the pH of the fermentation broth decreased as products were formed. In comparison, previous work evaluating glycerol fermentation by *C. pasteurianum* was performed in media that was buffered at a constant pH value. As indicated in Figures 2.5a and 2.5b, in unbuffered media, *C. pasteurianum* produces acetate and butyrate prior to the onset of solventogenesis. To the best of the author's knowledge, this is the first confirmation that *C. pasteurianum* does exhibit biphasic glycerol fermentation, similar to

the biphasic fermentation exhibited by *C. acetobutylicum* during solvent production from glucose. The acetate and butyrate yields observed in this work are slightly higher that those reported by Dabrock *et al.* and Biebl. No quantifiable amounts of lactate were detected via HPLC analysis, although trace amounts may have been present.

Typically, the lag phase prior to solvent production is 7–15 hours following the onset of fermentation. Although pH is recognized as one of the most important factors affecting the outcome of the ABE fermentation, there is no firm consensus as to what specific pH or undissociated acid concentration is optimum for solvent production. Depending on the strain, the optimum pH for solvent production varies from 4.3–5.5, in order to achieve the necessary concentrations of undissociated acids (Jones and Woods 1986). The concentration of undissociated butyric acid is also important, as concentrations of 1.6–1.9 g/L are required to induce solvent production (Monot, Engasser, and Petitdemange 1984). Because it appears that *C. pasteurianum* does produce ethanol in a pH-dependent manner, the effect of pH on butanol production should also be further investigated, specifically to determine the pH and undissociated acid concentration associated with the onset of butanol production.

One characteristic that is consistent when comparing this work to others is the wide variation in product yields, as indicated by the large standard deviations reported in Table 2.2. The variability in product formation has previously been attributed to slight differences in culture conditions and/or the pre-culture. The instability and degeneration of cultures of *C. acetobutylicum* (which results in the loss of the ability to produce solvents) has been a considerable challenge in studying solvent formation via glucose fermentation, and solvent production is believed to be closely linked to the regulation of

spore formation at the genetic level. In addition to *C. acetobutylicum*, *C. beijerinckii* is also capable of producing butanol from glucose and other sugars. In fact, the highest butanol yields have been achieved using a stable, hyper-butanol producing mutant of *C. beijerinckii*. Stability and butanol production were improved via directed chemical mutagenesis using n-methyl-N-nitro-N-nitrosoguanidine (Annous and Blaschek 1991; T. C. Ezeji, Qureshi, and Blaschek 2007). Similar directed mutagenesis techniques could be applied to *C. pasteurianum* to develop mutants that exhibit better stability or tolerance to impurities such as methanol. Until a stable culture is developed, however, it will be difficult to determine the extent to which parameters, such as pH, substrate concentration, impurities, etc., affect solvent production.

The preliminary data reported in this work and by others represents some of the highest reported yields of butanol production via fermentation. Previous efforts reported up to 0.31 g butanol per g glycerol consumed (Biebl 2001), while this work produced a maximum of 0.36 g butanol per g glycerol consumed and 0.30 g of butanol per g of crude glycerol consumed. To date, the highest butanol yield achieved during glucose fermentation is 0.31 g butanol per g glucose. This yield was achieved using *C. beijerinckii* in a fed-batch system that employed H_2/CO_2 gas stripping to continuously remove products (T. C. Ezeji, Qureshi, and Blaschek 2004). Typical total solvent yields using *C. beijerinckii* range from 0.3 to 0.4 g/g glucose, with an ABE ratio of 3:6:1. Butanol yields from *C. acetobutylicum* are typically 0.15–0.20 g/g glucose. In order for biofuels such as butanol to be economical, however, lignocellulosic feedstocks must be used in place of corn. As a result of the pretreatment processes, the resulting mixture contains a variety of C-5 and C-6 sugars as well as impurities that inhibit cell growth and

product formation (T. Ezeji, Qureshi, and Blaschek 2007). Therefore, if butanol is produced from lignocellulosic feedstocks, the yield would likely be much lower than the maximum, unless significant improvements are made in feedstock pretreatment and/or metabolic engineering of clostridia.

Comparatively, the yield of butanol from glycerol (0.3–0.36 g/g) using *C. pasteurianum* is significantly higher than that typically achieved by either *C. beijerinckii* or *C. acetobutylicum*. Although it remains unclear what long-term impact the impurities in crude glycerol have on solvent formation, this work was able to achieve an average butanol yield of 0.22 g per g crude glycerol. The maximum butanol yield obtained using crude glycerol was 0.30 g/g, which is comparable to the yields obtained when *C. acetobutylicum* metabolizes glucose. Additionally, *C. pasteurianum* does not produce acetone, which should simplify the separation and purification of butanol. Unlike *C. acetobutylicum* and *C. beijerinckii*, *C. pasteurianum* also produces significant amounts of ethanol. Although ethanol is a less than ideal fuel, it could be used along with butanol for blending with petroleum fuels. Finally, the carbon recovery observed during glycerol fermentation by *C. pasteurianum* ranges from 75 to 90%, compared to a recovery of less than 60% during sugar fermentation. As a result, less carbon will be lost via the production of CO_2.

2.3.3 Methanol

Because crude glycerol contains impurities including salts and methanol, the methanol concentration in samples containing crude glycerol varied substantially. The average methanol concentration detected in samples of fermentation broth was 1.78 ±

2.98 g/L (n = 24). Methanol concentrations ranged from 0.38 to 13.1 g/L, and no methanol was detected in six samples. There was no direct correlation observed between the methanol concentration and glycerol concentration (i.e., samples with highest concentrations of crude glycerol did not have the highest concentration of methanol). The variability in methanol concentration can likely be attributed to the HPLC column. Although it is capable of detecting methanol, it is not optimized for this compound, particularly when glycerol is also present. In fact, HPLC analysis of samples of methanol in water compared to methanol and glycerol in water showed that the methanol peak varies unpredictably in size when glycerol is present. Samples of 50 g/L glycerol in water were independently analyzed via head-space gas chromatography at Green River Biodiesel and found to contain 0.3 wt % methanol. Therefore, despite the large variation in samples, the methanol concentration detected via HPLC analysis is within the expected range based on the amount of methanol initially present in the crude glycerol.

2.4 Conclusion

Although the market value for biodiesel-derived crude glycerol has varied in recent months, the typical value is estimated between $0.05 and $0.15 per pound. If a butanol fermentation process were integrated into a biodiesel production facility, there would essentially be no cost associated with purchasing the feedstock. Comparatively, feedstock costs for sugar-based fermentation processes are usually 60–80% of total production costs and represent one of the most important factors affecting the cost of butanol production (Qureshi and Blaschek 2001). Authors of other research investigating

the production of ethanol via anaerobic fermentation of glycerol using *E. coli* have estimated that anaerobic fermentation of glycerol could reduce total production costs by up to 40% as compared to conventional ethanol fermentation processes (Yazdani and Gonzalez 2007).

Although the potential to produce butanol from biodiesel derived crude glycerol is promising, much work is needed to fully understand and optimize the process. As mentioned previously, culture stability is critical to the process, and can possibly be achieved through development of mutant strains. It is also important to identify and quantify the environmental parameters, such as pH, nutrient and trace metal concentration, etc., that have a significant impact on solvent formation and product distribution. Because the genome of *C. pasteurianum* has not been sequenced, options for genetic engineering are somewhat limited. Both *C. acetobutylicum* and *C. beijerinckii* have been sequenced, revealing some significant differences between the two organisms. Given the relatively low homology between *C. pasteurianum* and other clostridia, particularly *C. acetobutylicum* (Cummins and Johnson 1971), it is unclear how genetic information from the other two species might apply. Most experts agree, however, that genetic engineering will play an important role in the future of the biofuels industry (Papoutsakis 2008; Fischer, Klein-Marcuschamer, and Stephanopoulos 2008). Given the advantages to using *C. pasteurianum* to produce butanol from glycerol, it would be advantageous to begin determining the extent to which the tools developed for the metabolic engineering of *C. acetobutylicum* and *C. beijerinckii* (Papoutsakis 2008) might be applied to *C. pasteurianum*. For example, the sequences of the genes responsible for solvent formation in *C. acetobutylicum* and *C. beijerinckii* could be used to determine if

these same genes are present and active in *C. pasteurianum*. Techniques developed to overexpress, knockout, or knockdown genes in *C. acetobutylicum* (Fischer, Klein-Marcuschamer, and Stephanopoulos 2008) could be applied to *C. pasteurianum* to reduce PDO production or increase solvent production at the genetic level. Alternatively, the genes responsible for glycerol metabolism and/or alcohologenesis in *C. pasteurianum* could be expressed in *C. acetobutylicum*. Identifying that *C. pasteurianum* has the ability to produce butanol via the fermentation of crude glycerol opens up a wealth of potential research opportunities that could potentially improve the viability of biobutanol production and contribute to the development of a diverse portfolio of biomass-based processes that can generate comparable alternatives for petroleum products.

CHAPTER 3

3. PARAMETERS AFFECTING GLYCEROL FERMENTATION

3.1 Introduction

C. pasteurianum was shown to utilize biodiesel-derived crude glycerol as the sole carbon source (Chapter 2) and its ability to produce butanol at a yield comparable to the one obtained from pure glycerol. Though, the bacteria were able to consume glycerol and produce butanol, it is important to analyze various methodologies to enhance glycerol utilization and improve product yield. It has been shown that the product distribution during fermentation can be altered by modifying the growth conditions, particularly, media composition and pH. The approaches to modifying the media have been performed by incorporating additional carbon source such as glucose. The addition of glucose to glycerol utilization has been studied with *C. butyricum* (Abbad-Andaloussi et al. 1998; Malaoui and Marczak 2001; Vasconcelos, Girbal, and Soucaille 1994). In *C. butyricum*, the presence of glucose during glycerol fermentation has resulted in an increase in the amount of 1,3-PDO produced (Abbad-Andaloussi et al. 1998; Malaoui and Marczak 2001; Vasconcelos, Girbal, and Soucaille 1994). It was envisioned that the distribution of products formed during fermentation can be modified by supplementing the media with another carbon source such as glucose, arabinose and xylose (Jin et al.

2011). L-arabinose and D-xylose are of interest as they are the constituent hexose and pentose of the hemicelluloses, a constituent polymer of lignocelluloses. Hence, examining the utilization of arabinose and xylose will result in the use of sugars from renewable sources for the co-fermentation with glycerol. The addition of byproducts of the butanol pathway, acetate and butyrate, have also been explored on *C. butyricum* (Chen and Blaschek 1999; Colin et al. 2001). It has been found that the addition of acetate resulted in the reduction of 1,3-PDO production, while the addition of butyrate increased the production of 1,3-PDO (Chen and Blaschek 1999; Colin et al. 2001).

Apart from the above mentioned factors, iron concentration in the media and the supplementation of vitamins such as biotin and p-aminobenzoic acid has been shown to affect the product distribution, substrate utilization and solvent productivity in *C. acetobutylicum* (Soni, Soucaille, and Goma 1987).

The augmentation of solvent production and enhancement of substrate utilization through modification of media by supplementing with solvent pathway byproducts, secondary carbon source, and vitamins have been envisioned for *C. pasteurianum* towards the utilization of crude glycerol for butanol production. The experimental details and their results are discussed in detail in this chapter.

3.2 Materials and Methods

Clostridium pasteurianum ATCC™ 6013 was obtained from American Type Culture Collection (Manassas, VA). Freeze dried pure cultures of *C. pasteurianum* were revived using nutrient-rich reinforced clostridial growth media (RCM) and glycerol stock cultures were prepared for future use. The composition of RCM is tabulated in Table 3.1.

Table 3.1 Composition of RCM

Components	Concentration (g/L)
Peptone	10.0
Beef extract	10.0
Yeast extract	3.0
Dextrose	5.0
NaCl	5.0
Soluble Starch	1.0
Cysteine HCl	0.5
Sodium acetate	3.0
Agar	0.5

Pre-cultures for glycerol utilization experiments were grown in RCM. The growth was monitored using optical density ($\lambda = 600$ nm) and the cultures were used as inoculum once they reached the mid-exponential phase of growth. The growth experiments were conducted in 75 mL cell cultures flasks containing 40 mL of media and 5 mL of inoculum. Biebl medium (Biebl 2001) was used for glycerol utilization as described earlier in chapter 2 (Taconi, Venkataramanan, and Johnson 2009). All experiments were carried out in batch cultures and in triplicates. The cultures were monitored for growth, substrate utilization and product formation as mentioned earlier in Chapter 2.

The co-fermentation of sugars was studied using hexose (dextrose) and pentose (D-xylose and L-arabinose) with 20 g/L of pure glycerol at a concentration of 10 g/L of

the sugars, maintaining a 2:1 ratio of glycerol and sugars. Cells grown on glycerol or sugar alone were used as controls. Acetate and butyrate, the acid co-product in butanol pathways were studied for their impact on butanol production by supplementing the media with sodium acetate and sodium butyrate respectively. Acetate and butyrate were studied at concentrations of 2 g/L with an initial glycerol concentration of 25 g/L of pure glycerol. Biotin was supplemented in the media at 0.8 mg/L with an initial concentration of 25 g/L of pure glycerol. A stock solution of 400 mg/L of biotin was made in sterile, autoclaved deionised water and an appropriate amount was added to the media, after the media were sterilized by autolclaving. All these experiments were conducted using pure glycerol as it was difficult to have successful experiments with crude glycerol, which had a reproducibility of less than 10 %. The results from these experiments have the potential to be transferred to crude glycerol, provided crude glycerol is purified by removing the growth inhibiting and growth impairing impurities.

3.3 Results and Discussion

3.3.1 Co-fermentation of glycerol with sugars

The co-fermentation of glycerol was studied with glucose, arabinose and xylose. Controls for the experiments were cultures grown on each carbon as the sole carbon source. The cultures on the control experiments were grown until little or no carbon source was left behind, as analyzed using HPLC.

Figure 3.1 to Figure 3.4 compare the optical densities (measured at 600 nm) and pH profiles of the cultures grown at various conditions. The optical densities of the

control as well as the cultures grown under co-fermentation followed the S-shaped (sigmoidal) growth curve typically observed in bacteria. Figure 3.1 compares the metabolism during glycerol-glucose co-fermentation. Figure 3.1A compares the growth profile of cultures in glucose, glycerol and glycerol & glucose. The growth curves of cultures grown in all three conditions are similar. A comparison using the maximum specific growth rate, μ_{max}, calculated during the maximum growth rate in the exponential log phase, shows that the cultures in co-metabolism of glycerol and glucose grew at a faster rate than the two controls. The μ_{max} for cultures on glycerol were calculated as 1.562 ± 0.118 day^{-1}, while the cultures on glucose and glycerol-glucose had a μ_{max} of 1.130 ± 0.055 day^{-1} and 1.355 ± 0.115 day^{-1}, respectively. Though the glycerol-glucose cultures had a μ_{max} slightly lower than the cultures grown only on glycerol and glucose, respectively, the three cultures were able to grow to the same maximum cell density. The overlapping curves of the three cultures also indicate the absence of a diauxic growth, usually observed in bacteria grown on two carbon sources. During diauxic growth, the bacteria utilize one carbon source first and reach a lag phase, which is followed by another exponential growth phase resulting from the utilization of the second carbon source. The absence of diauxic growth also indicates that glycerol and glucose were simultaneously consumed for growth and metabolism by *C. pasteurianum*.

Similarly, comparison of the pH profile (Figure 3.1B) of the cultures supports the growth data. The cultures grown on glucose reached a highly acidic pH of 4.5, which can be explained by the production of acids (Table 3.1). The pH profile of the cells on glycerol-glucose co-fermentation closely aligns with the pH profile of the cells on

glycerol only. The initial difference in the glycerol-glucose cultures can be described due to the simultaneous consumption of both the carbon sources.

Figure 3.2 compares the growth and pH profiles of *C. pasteurianum* cells on the co-metabolism of glycerol and L-arabinose along with the controls. The growth profiles clearly indicate that the glycerol control had a superior growth rate in comparison to the cultures on dual carbon sources and the control on L-arabinose. The μ_{max} of the cultures on glycerol and L-arabinose mixture were found to be 1.005 ± 0.020 day^{-1}, and the cultures on arabinose had a μ_{max} of 0.933 ± 0.054 day^{-1}.

The comparison of the pH profiles (Figure 3.2B) indicates that the controls grown on L-arabinose had the least change to their pH due to the very low production of acids (Table 3.1), while the cultures on dual carbon source (glycerol and L-arabinose) had the maximum drop in the pH to 5.0 (Figure 3.2B). The analysis of growth and pH profile does not show any diauxic growth during co-fermentation of glycerol and L-arabinose.

Figure 3.3 compares the growth and metabolism of *C. pasteurianum* during D-xylose addition to glycerol with its respective controls. The growth of cells in D-xylose or in the combination of glycerol and D-xylose, was very slow and had a very low growth rate. The cultures on D-xylose had a μ_{max} of 0.794 ± 0.390 day^{-1} while the addition of D-xylose to glycerol had the lowest μ_{max} of 0.632 ± 0.050 day^{-1}. The pH profile of all the cultures (Figure 3.3B) have a similar profile. Unlike the co-fermentation of glycerol with glucose and L-arabinose respectively, the co-fermentation of glycerol with xylose resulted in the utilization of glycerol only while more than 90 % of D-xylose was left unconsumed in the media.

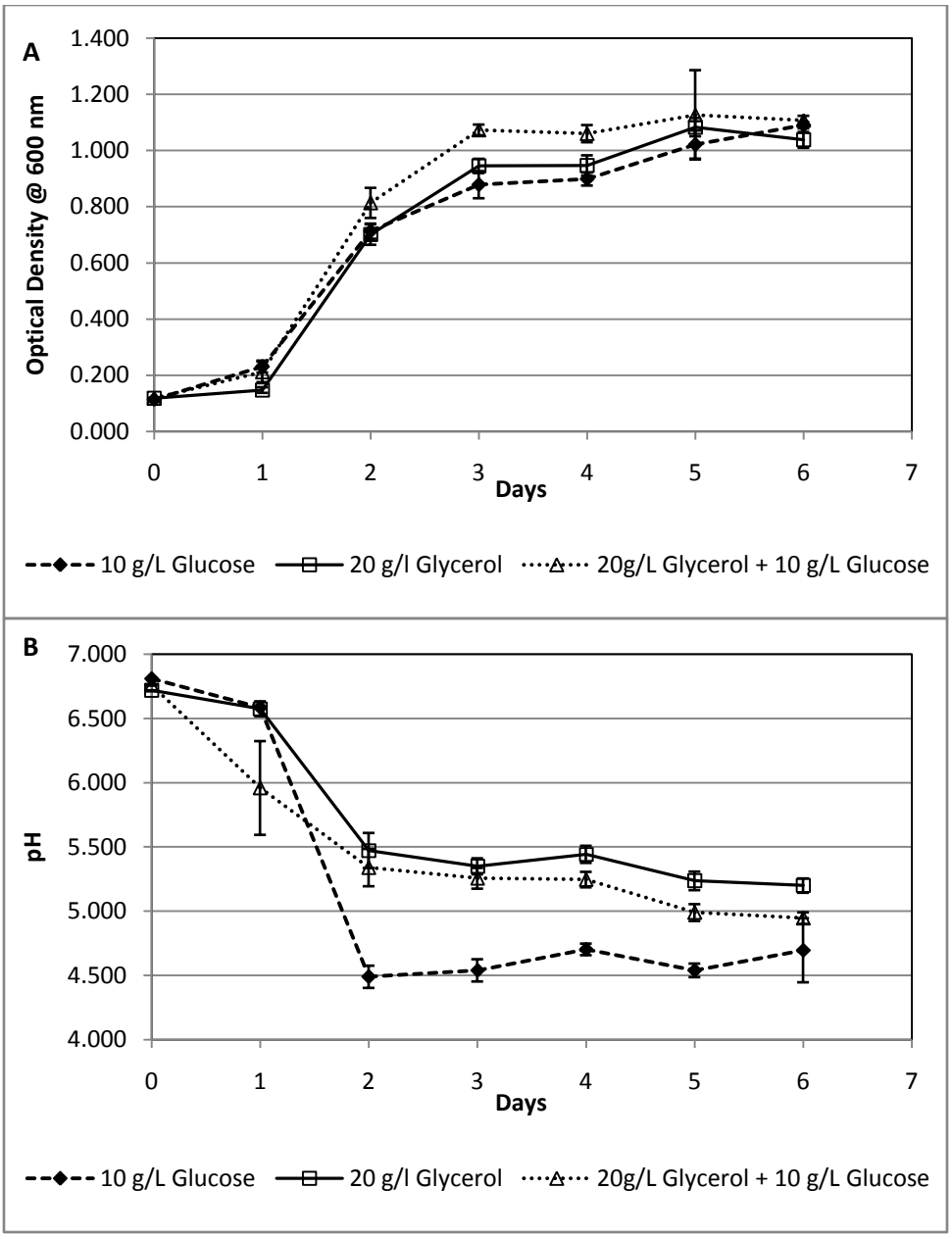


Figure 3.1 Comparison of metabolism during glycerol-glucose co-fermentation. (A) Growth profile. (B) pH Profile.

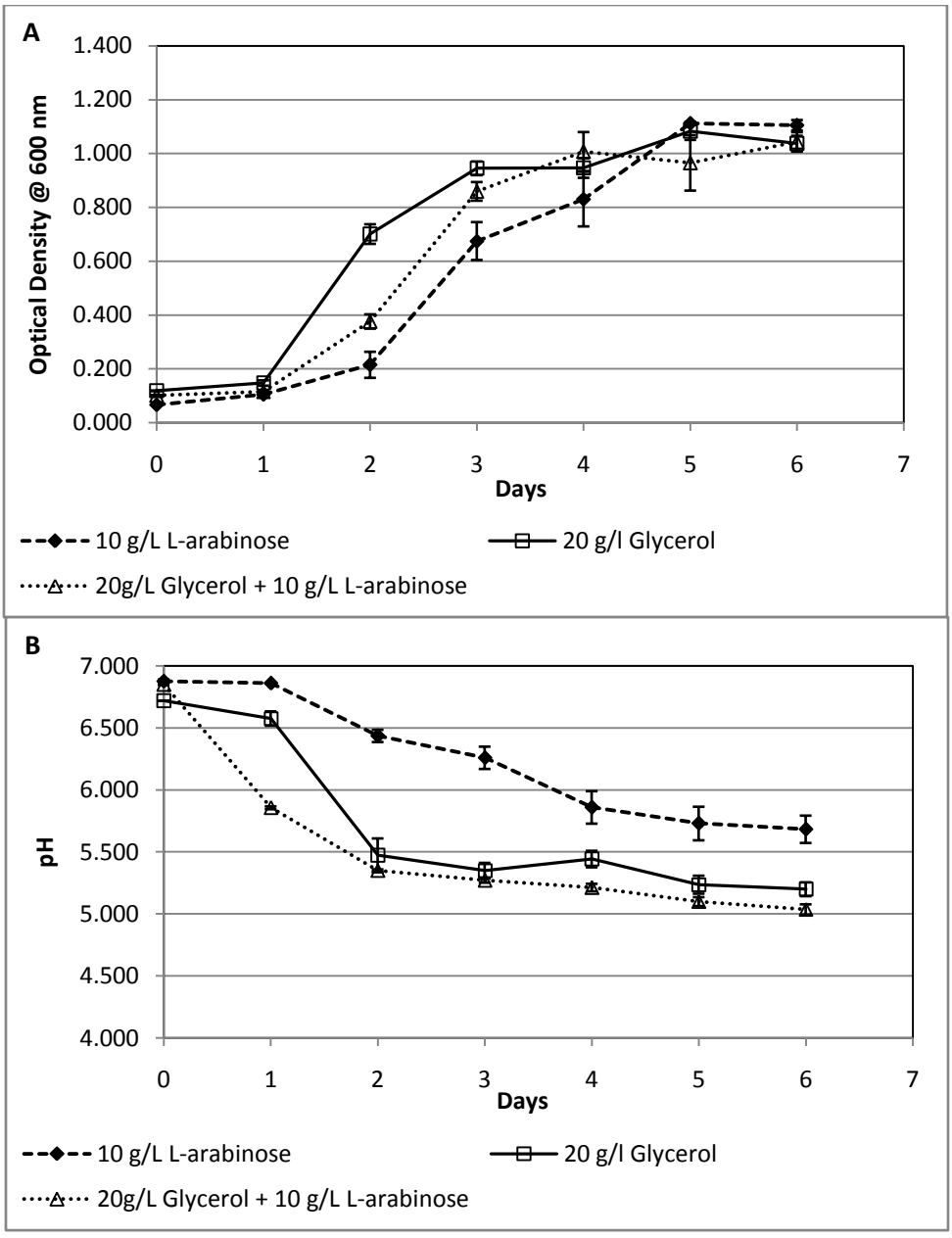


Figure 3.2 Comparison of metabolism during glycerol & L-arabinose co-fermentation. (A) Growth profile. (B) pH Profile.

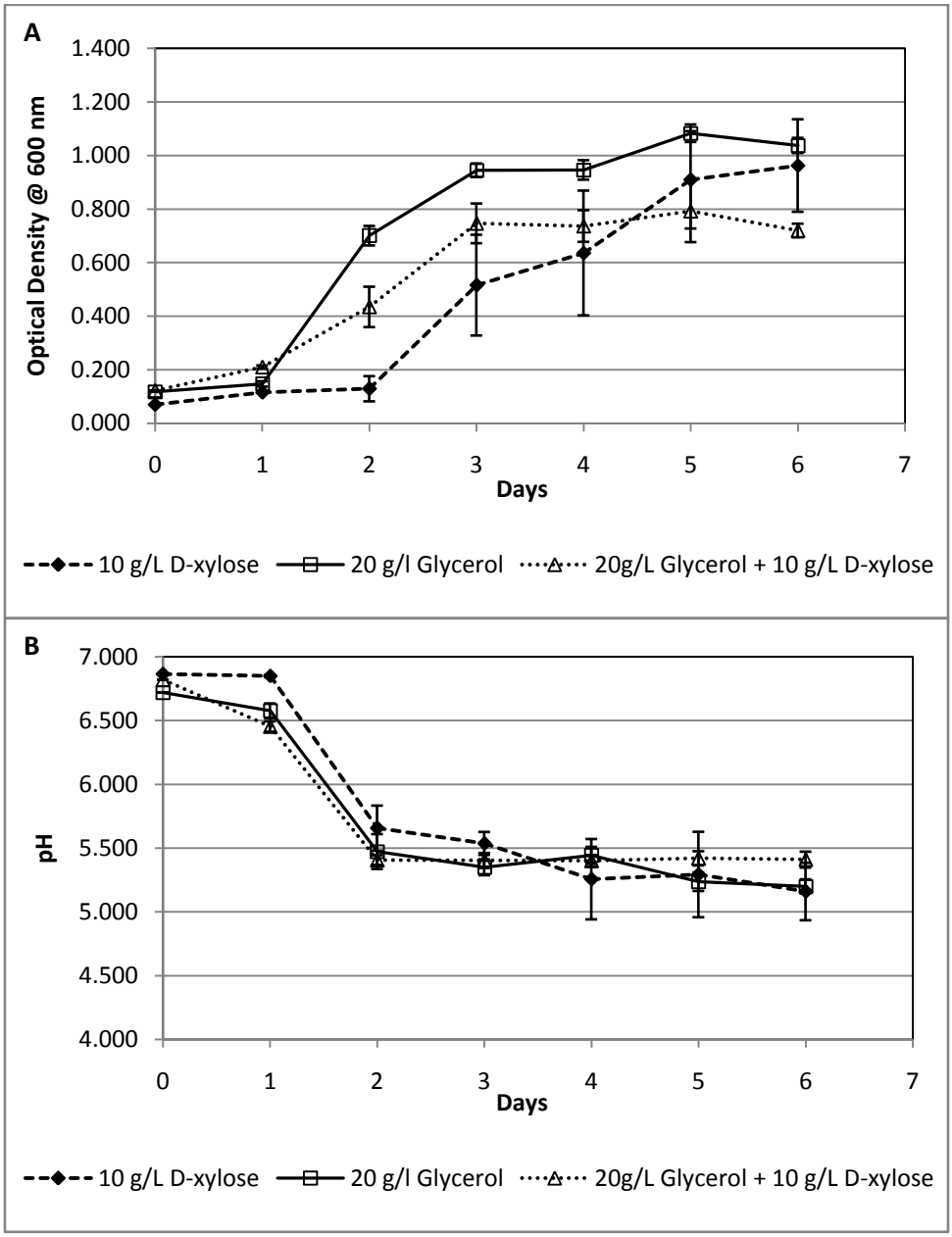


Figure 3.3 Comparison of metabolism during glycerol & D-xylose co-fermentation. (A) Growth profile. (B) pH Profile.

Table 3.2 Control Experiments - Product yields g/g (mol/mol).

	Acetate	1,3-PDO	Ethanol	Butyrate	Butanol
10 g/L Glucose	0.21 ± 0.01 (0.64 ±0.02)	0.02 ± 0.0 (0.04 ± 0.0)	0.02 ± 0.0 (0.07 ± 0.0)	0.41 ± 0.01 (0.83 ±0.02)	0.02 ± 0.0 (0.04 ± 0.00)
10 g/L L-arabinose	0.06 ± 0.01 (0.15 ± 0.02)	0.28 ± 0.07 (0.55 ± 0.02)	0.17 ± 0.03 (0.57 ± 0.11)	0.01 ± 0.0 (0.02 ± 0.0)	0.005 ± 0.008 (0.01 ± 0.02)
10 g/L D-xylose	0.25 ± 0.08 (0.62 ± 0.20)	0.22 ± 0.04 (0.43 ± 0.08)	0.22 ± 0.04 (0.72 ± 0.14)	0.02 ± 0.01 (0.04 ± 0.01)	0.07 ± 0.05 (0.13 ± 0.11)

Table 3.3 Co-fermentation of Glycerol and Sugars: Product yields g/g (mol/mol).

	Acetate	1,3-PDO	Ethanol	Butyrate	Butanol
20 g/L Glycerol	0.04 ± 0.001 (0.06 ±0.002)	0.20 ± 0.01 (0.24 ± 0.01)	0.02 ± 0.002 (0.05 ±0.005)	0.05 ± 0.003 (0.05 ±0.003)	0.22 ± 0.02 (0.27 ± 0.02)
20 g/L Glycerol + 10 g/L Glucose	0.07 ± 0.003 (0.11 ±0.004)	0.07 ± 0.008 (0.09 ±0.01)	0.03 ± 0.001 (0.06 ±0.001)	0.06 ± 0.005 (0.06 ±0.006)	0.46 ± 0.022 (0.57 ±0.027)
20 g/L Glycerol + 10 g/L L-arabinose	0.05 ± 0.002 (0.07 ±0.004)	0.33 ± 0.03 (0.40 ±0.04)	0.10 ± 0.003 (0.20 ± 0.01)	0.07 ± 0.01 (0.07 ± 0.01)	0.21 ± 0.01 (0.26 ± 0.01)
20 g/L Glycerol + 10 g/L D-xylose	0.03 ± 0.01 (0.05 ± 0.01)	0.11 ± 0.02 (0.13 ± 0.02)	0.06 ± 0.01 (0.13 ± 0.03)	0.03 ± 0.01 (0.03 ± 0.01)	0.19 ± 0.04 (0.24 ± 0.06)

Table 3.1 summarizes the product yields of the control experiments containing 10 g/L (55.5 mM/L) glucose, 10 g/L (66.6 mM/L) L-arabinose and 10 g/L (66.6 mM/L) D-xylose. The cells grown on glucose produced the acids, acetate and butyrate, as the major products, constituting more than 90 % (mol/mol) of the products formed. Butyrate was the major product under glucose utilization with a 41 % (g/g) yield. Ethanol, 1,3-PDO and butanol were formed in trace amounts (Table 3.1) and this shows that the growth of *C. pasteurianum* in glucose results in acidogenesis with little or no solventogenesis.

Similarly, the control cultures grown on L-arabinose, produced 1,3-PDO as the major product with a conversion of 28% (g/g) and a maximum yield of 36.5 % (g/g). Ethanol and 1,3-PDO constituted nearly 86 % (mol/mol) of the products yielded on arabinose. Growth on D-xylose resulted in the production of acetate, 1,3-PDO and ethanol as the major products with trace amounts of butyrate and butanol being formed. The control experiments with hexose and pentoses as the sole carbon source show unique array of products, based on the substrate used.

As discussed earlier in chapter 2 and as shown in Table 3.2, the fermentation of glycerol results in the production 1,3-PDO and butanol as the major products, during the biphasic production of acids and solvents. The control experiment with glycerol yielded 1,3-PDO and butanol at 20 % (g/g) and 22 % (g/g), respectively. The ratio of butanol/1,3-PDO is close to one, 1.1 g/g (1.125 mol/mol). The objective of this experiment is focused on determining the effect of various sugars to alter this ratio, when used as co-substrates.

3.3.1.1 Effect of Glucose on Glycerol Fermentation

The addition of glucose as a co-substrate at 10 g/L (55.5 mM/L) to 20 g/L (217.2 mM/L) of glycerol, resulted in an increased production of butanol along with a simultaneous decrease in the formation of 1,3-PDO. The butanol production doubled from 0.22 g/g to 0.46 g/g with the addition of glucose at half the concentration of glycerol. At the same time, the 1,3-PDO yields dropped significantly, from 0.20 g/g to 0.07 g/g, a 65% reduction. The simultaneous reduction in the yield of 1,3-PDO and increase in the yield of butanol, also results in altering the ratio of butanol/1,3-PDO to 6.57 g/g (6.33 mol/mol). This 6 fold increase in the ratio of butanol/1,3-PDO, during the addition of glucose for co-fermentation with glycerol, indicates a simultaneous consumption of the two carbon sources. The cultures grown on glucose produced only acids (acetate and butyrate) and no butanol, while cultures on glycerol produced trace amount of acids, and, butanol and 1,3-PDO as the major products. This drastic change in the distribution of products indicates a simultaneous utilization of glucose and glycerol during co-fermentation.

The co-fermentation of glucose with glycerol using *C. butyricum* resulted in an increase in 1,3-PDO production (Abbad-Andaloussi et al. 1998; Malaoui and Marczak 2001; Vasconcelos, Girbal, and Soucaille 1994). Abbad-Andaloussi *et al.* showed an increase of 1,3-PDO from 57 % (mol/mol) to 92 % (mol/mol) in the presence of glucose as the co-substrate during glycerol fermentation (Abbad-Andaloussi et al. 1998). *C. butyricum* has the ability to produce 1,3-PDO, butyrate, acetate and ethanol from glycerol and does not posses an inherent ability to produce butanol from glycerol (Saint-Amans et al. 2001).

In *C. acetobutylicum*, it has been shown that the glycerol-glucose co-fermentation brings a shift in product pattern from an acid based production on glucose to an alcohol based production in glucose-glycerol co-fermentation (Vasconcelos, Girbal, and Soucaille 1994). In *C. acetobutylicum*, the shift in product during glucose-glycerol co-fermentation has been correlated to the increase in the level of NADH and ATP, along with an increase in activity of NAD-dependent alcohol dehydrogenases leading to an alcohol based fermentation (Vasconcelos, Girbal, and Soucaille 1994).

The decrease in 1,3-PDO production in *C. pasteurianum*, during glucose supplementation of glycerol fermentation, can be explained by the presence of a possible repression of the 1,3-PDO pathway either by the presence of an alternate reduction pathway from glucose and/or catabolite repression of the glycerol dehydratase enzyme catalyzing the conversion of glycerol into 1,3-PDO through 3-hydroxypropionaldehyde. During glycerol-glucose co-metabolism, a repression in the 1,3-PDO pathway along with an increase in the activity of the enzymes in the butanol pathway (NAD-dependent butanol dehydrogenase) and an increase in the level of NAD and ATP (due to the presence of glucose and compensating for the repression in the 1,3-PDO pathway) , can explain the shift in product pattern towards higher butanol formation.

The ability of *C. pasteurianum* to divert glycerol to produce more butanol by shutting down 1,3-PDO formation, during co-fermentation with glucose, clearly suggests the presence of an alternate reducing pathway to compensate for the regeneration of NAD. The availability of NAD is a key requirement to run the oxidative branch of glycerol catabolism leading to the formation of butanol (Figure 1.1). Also, from the control experiments (glucose as the sole carbon source and glycerol as the sole carbon

source) and the experiments involving co-fermentation of glycerol and glucose, it is evident that the butanol production results only from the fermentation of glycerol.

Comparison of the overall product yield (total concentration of products formed/ concentration of glycerol) with respect to glycerol, shows that overall product yield increases from 0.53 ± 0.025 g/g (0.68 ± 0.032 mol/mol) to 0.69 ± 0.022 g/g (0.89 ± 0.028). This 30 % increase in the overall product yield for glycerol during co-fermentation with glucose indicates that the supplementation of glucose not only provided an alternate reducing pathway, but also served as an energy source for cell multiplication and growth, while more of glycerol was used for solventogenesis.

3.3.1.2 Effect of Pentoses on Glycerol Fermentation

The effect of pentoses on co-metabolism with glycerol was studied using L-arabinose and D-xylose, the major products from the hydrolysis of hemicelluloses. The effect of pentoses was studied by maintaining the initial ratio of glycerol/pentose to 2:1. Control experiments were carried out with individual carbon sources at the same concentration as used in co-fermentation.

From the production data in Table 3.2, addition of L-arabinose to glycerol, does not affect the yield of butanol. The yield (calculated with respect to glycerol) of 1,3-PDO increased by 65 % to 0.33 g/g and hence the ration of butanol/1,3-PDO shifted from 1 to 0.64. The yield of ethanol also increased 5 fold from 0.02 g/g to 0.10 g/g. The solvent production by *C. pasteurianum* on L-arabinose predominantly resulted in the formation of 1,3-PDO and ethanol (85 % of overall products) and traces of acetate, butyrate and

butanol. The addition of L-arabinose did not result in an increase in the production of one compound at the expense of the other as observed in the co-fermentation of glycerol with glucose. The product formation during the co-fermentation of glycerol and L-arabinose resulted in the production of products that were observed in both the control experiments containing glycerol and L-arabinose as the sole carbon source. These results indicate that there was no product based suppression of 1,3-PDO production, which was produced from both carbon sources during co-fermentation. The addition of L-arabinose during glycerol fermentation did not alter the carbon flow of glycerol and both carbon sources were consumed in a manner independent of each other.

The addition of D-xylose to glycerol fermentation by *C. pasteurianum* was studied at the same concentration of 2:1. Unlike the addition of glucose and L-arabinose for co-fermentation with glycerol, which were completely consumed, addition of D-xylose resulted only in a utilization of only 25 ± 16 % of D-xylose. D-xylose was, however, effectively consumed as a sole carbon source and resulted in the production of acetate, 1,3-PDO and ethanol (88 % of the overall products) with 0.78 g/g of product formation. Addition of xylose in *Klebsiella pneumonia,* during glycerol fermentation, resulted in an increase in the production of 1,3-PDO (Jin et al. 2011). In *C. pasteurianum*, the addition of xylose as a co-substrate during glycerol fermentation did not result in an increase in the formation of any of the products. Also the addition of xylose lowered the growth rate of the cells (Figure 3.3).

3.3.2 Effect of Co-product and Biotin Addition

The effect of co-product addition was studied for the addition of acetate and butyrate in the form of sodium salts at 2 g/L, while the effect of biotin supplementation was studied at 0.8 mg/L. Figure 3.4 compares the growth and pH profile of the cultures on acetate and butyrate addition and biotin supplementation. Table 3.3 compares the product yields when co-products were added. The metabolism of the cells in terms of growth and pH was not drastically different due to the addition of compounds such as butyrate and biotin, with respect to the control (Figure 3.4). Acetate addition resulted in a faster drop in pH and resulted in a small increase in the maximum optical density. Butyrate addition resulted in a slightly lower maximum optical density.

The product distribution during the addition of acetate resulted in a 23 % increase in butanol formation (Table 3.4) with a corresponding decrease in 1,3-PDO production (33 %). This shift of the butanol/1,3-PDO ratio due to the addition of acetate, shows the effect of acetate addition in moderating the carbon flow between the oxidation and the reduction pathway. In contrast, the addition of butyrate did not increase butanol production neither did butyrate affect an increase 1,3-PDO formation. On the other hand, the supplementation of biotin in the media altered the butanol/1,3-PDO ratio by both increasing butanol formation by 25 % and decreasing 1,3-PDO by 50 %.

The effect of acetate and butyrate addition on glycerol fermentation has been reported on *C. butyricum* (Chen and Blaschek 1999; Colin et al. 2001) and *C. beijerinckii* (Chen and Blaschek 1999). Colin *et al.* reported a decrease in 1,3-PDO formation during the supplementation of acetate in the media and an increase in butyrate was also observed by Chen *et al.* (Chen and Blaschek 1999; Colin et al. 2001). It should be noted that *C.*

butyricum does not produce any butanol from glycerol, hence the decrease in 1,3-PDO resulted in a corresponding increase in butyrate (Chen and Blaschek 1999; Colin et al. 2001). Butanol is the major product in *C. pasteurianum* and hence addition of acetate resulted in an increase in butanol. Increase in butanol production on acetate addition was reported in *C. beijerinckii (*Chen and Blaschek 1999). The effect of acetate on increasing butanol production was correlated to the increase in the coenzyme-A transferase (Chen and Blaschek, 1999), an enzyme that plays a key role in the butanol pathway. Colin *et al.* furthermore reported that the addition of acetate increased biomass, which was also observed in this study as the addition of acetate resulted in a noticeable increase in the optical density value (Figure 3.4A). The effect of acetate addition resulted in a faster progress in metabolism as the pH drop was predominant, compared to the control.

Butyrate addition in *C. butyricum* was reported to have increased 1,3-PDO production with a corresponding decrease in butyrate production (Chen and Blaschek 1999; Colin et al. 2001). Butyrate supplementation in the media was reported to increase butanol production in *C. acetobutylicum* (Yusof et al. 2010). Yusof et al. studied the effect of butyrate addition at a concentration of 2 g/L and found that it resulted in an increase of butanol production by 1.6 folds. No such effect of butyrate was observed in this study. Addition of butyrate lowered the maximum optical density by 20 % (Figure 3.4). The reduction in biomass during butyrate addition was also reported in *C. butyricum* (Colins *et al.* 2001).

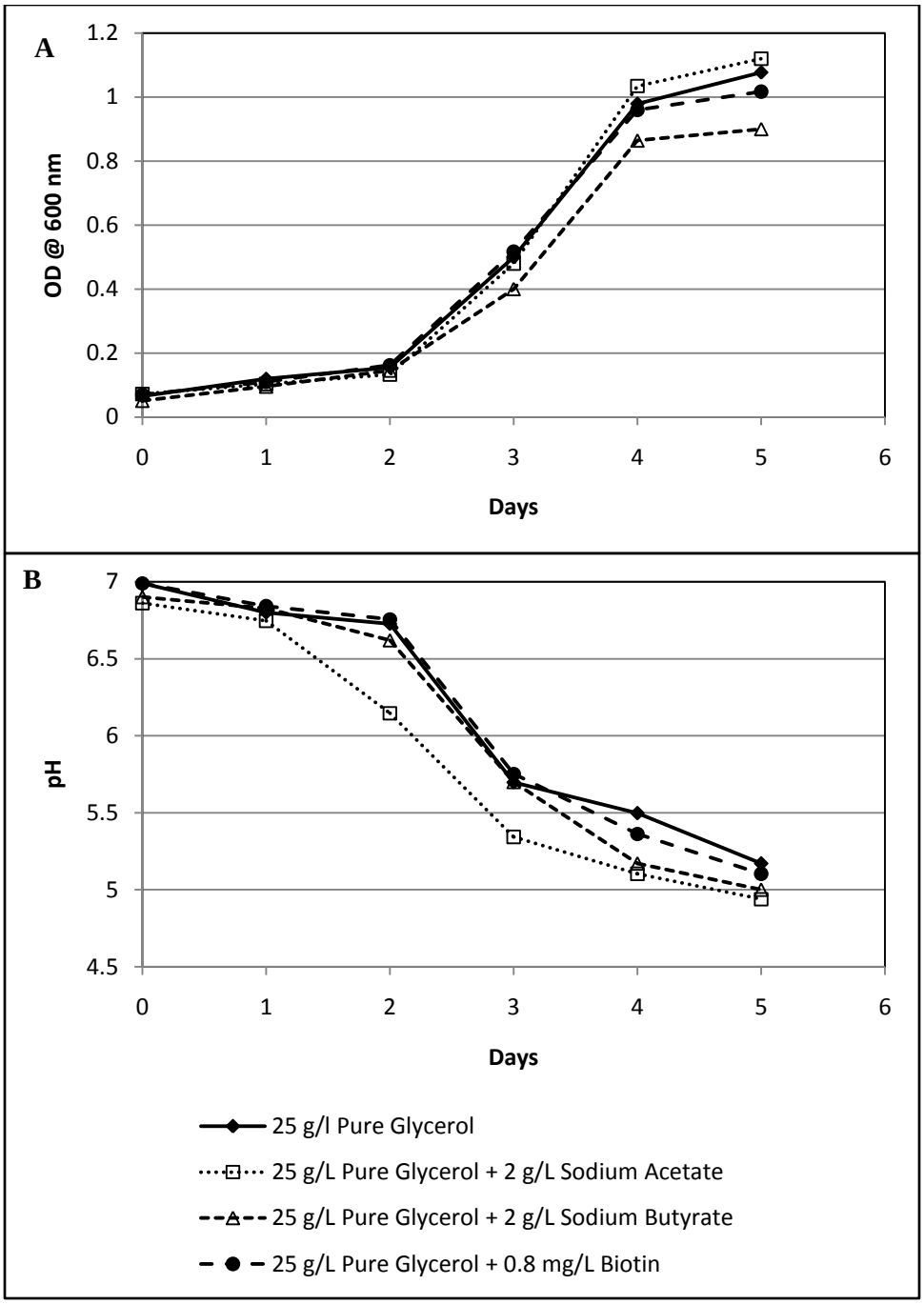


Figure 3.4 Effect of addition of acetate, butyrate and biotin on glycerol fermentation (A) Growth profile (B) pH profile

Table 3.4 Comparison of Product Yield in g/g (mol/mol)

	Acetate	1,3-PDO	Ethanol	Butyrate	Butanol
25 g/L Pure Glycerol	0.03 ± 0.002 (0.04±0.004)	0.21 ± 0.01 (0.25 ±0.02)	0.07 ± 0.01 (0.15 ± 0.01)	0.02 ± 0.01 (0.02 ± 0.01)	0.22 ± 0.02 (0.28 ± 0.02)
25 g/L Pure glycerol + 2 g/L Sodium acetate	0.05 ± 0.01 (0.08 ±0.009)	0.14 ± 0.01 (0.17 ±0.01)	0.05 ± 0.01 (0.11 ±0.02)	0.03 ± 0.01 (0.03 ±0.01)	0.27 ± 0.004 (0.33 ±0.005)
25 g/L Pure glycerol + 2 g/L Sodium butyrate	0.05 ± 0.001 (0.08 ±0.001)	0.22 ± 0.005 (0.26 ± 0.01)	0.01 ± 0.001 (0.02 ±0.001)	0.12 ± 0.001 (0.13±0.001)	0.21 ± 0.01 (0.27 ± 0.01)
25 g/L Pure glycerol + 0.8 mg/L Biotin	0.03 ± 0.003 (0.04 ±0.005)	0.10 ±0.01 (0.12 ±0.02)	0.02 ± 0.003 (0.04 ±0.01)	0.02 ± 0.01 (0.02 ±0.01)	0.26 ± 0.01 (0.32 ± 0.01)

The effect of biotin supplementation was reported to increase solvent production and biomass in *C. acetobutylicum* (Soni, Soucaille, and Goma 1987). Similarly, the biotin supplementation in the media has resulted in an 18% increase in butanol formation with a corresponding decrease of 1,3-PDO formation by more than 50 %.

3.4 Conclusion

The effect of various compounds, from co-fermentation of sugars, addition of organic acids and supplementation of a vitamin, were studied on glycerol fermentation by *C. pasteurianum.* This study shows that the carbon flow from glycerol into various products can be regulated by using appropriate additions to the media. Co-fermentation of glycerol with glucose resulted in the maximum increase of butanol formation by 110 %. Addition of acetate, in the form of sodium acetate at 2 g/L, and biotin (0.8 mg/L), also resulted in an increase in production of butanol (~25 %). Co-fermentation of glycerol with L-arabinose and D-xylose, respectively, did not have a positive impact on butanol yield. Similar results were obtained from addition of butyrate. Co-fermentation of glycerol and glucose is an interesting finding in this study, as it not only increases butanol production, but, it also mediates the increase in butanol through carbon shift from 1,3-PDO to butanol. Glucose, acetate and biotin can be used to maximize butanol production from glycerol, provided their addition to the media does not increase cost on feedstocks. The use of glucose from lignocellulosic compounds may help in justifying the use of glucose in glycerol fermentation by *C. pasteurianum*, but that aspect still remains to be largely unexplored.

CHAPTER 4

4. IMPACT OF IMPURITIES IN CRUDE GLYCEROL

(This chapter has been published in *Applied Microbiology and Biotechnology:* Volume 93, Issue 3 (2012), Page 1325-1335 under the title "Impact of impurities in biodiesel-derived crude glycerol on the fermentation by *Clostridium pasteurianum* ATCC 6013")

4.1 Introduction

Though, *C. pasteurianum* has been shown to grow in the presence of crude glycerol as the sole carbon source with an average yield of 0.22 g/g of butanol which was comparable to the yield (0.24 g/g) obtained on pure glycerol (Taconi, Venkataramanan, and Johnson 2009), the amount of time taken for the complete utilization of glycerol was much slower. The batch fermentation time in crude glycerol ranged from 14-24 days as to 10 days in pure glycerol at the same glycerol concentrations of 25 g/L. This delay in the utilization of crude glycerol is solely attributed to its nature and composition. The impurities in crude glycerol clearly distinguish crude glycerol from pure glycerol and hence in this work, our objective is to determine the inhibitory effect of each of the individual impurities (methanol, salts and free fatty acids). The findings of this work will result in determining the role of the impurities as an inhibitor of the transport and utilization of glycerol by *C. pasteurianum*. The results of this study will have an important significance as it can lead to the development of methods to eliminate

particular impurities from crude glycerol, to enhance its utilization and value-added conversion in an efficient and effective process.

4.2 Materials and Methods:

4.2.1 Materials:

Biodiesel-derived crude glycerol was obtained from Green River Biodiesel, Moundville, AL. All chemicals were purchased from either Fisher Scientific or Sigma-Aldrich. The fatty acids were obtained from TCI America. Deuterated dimethylsulfoxide (d_6-DMSO) and D_2O were obtained from Cambridge Isotope Laboratory**.**

4.2.2 Bacterial Strain, Media and Fermentation:

Clostridium pasteurianum ATCC™ 6013 was obtained from American Type Culture Collection (Manassas, VA). Freeze dried pure cultures of *C. pasteurianum* were revived using nutrient-rich reinforced clostridial growth medium (RCM) and glycerol stock cultures were prepared for future use. RCM is composed of peptone (10 g/L), beef extract (10 g/L), yeast extract (3 g/L), dextrose (5 g/L), NaCl (5 g/L), soluble starch (1 g/L) cysteine HCl (0.5 g/L), sodium acetate (3 g/L) and agar (0.5 g/L). Pre-cultures for glycerol utilization experiments were grown in RCM. The growth was monitored using optical density (λ = 600 nm) and the cultures were used as inoculum once they reached the mid-exponential phase of growth. The growth experiments were conducted in 75 mL cell cultures flasks containing 40 mL of media and 5 mL of inoculum. Biebl medium (Biebl 2001) was used for glycerol utilization as described earlier (Taconi, Venkataramanan, and Johnson 2009). The medium was composed of K_2HPO_4 (3.4 g/L),

KH_2PO_4 (1.3 g/L), $(NH_4)_2SO_4$ (2.0 g/L), $CaCO_3$ (2.0 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2.2H_2O$ (0.02 g/L), $FeSO_4.7H_2O$ (100 mg/L), yeast extract (1 g/L) and 2 mL of trace element solution SL7 per liter of the medium. The trace element solution, SL7, consisted of 25% HCl (10 ml of 25% HCl for 1 liter), $FeCl_2 \cdot 4H_2O$ (1.5 g/L) $CoCl_2 \cdot 6H_2O$ (190.0 mg/L), $MnCl_2 \cdot 4H_2O$ (100.0 mg/L) $ZnCl_2$ (70.0 mg/L), H_3BO_3 (62.0 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (36.0 mg/L), $NiCl_2 \cdot 6H_2O$ (24.0 mg/L) and $CuCl_2 \cdot 2H_2O$ (17.0 mg/L). Unless otherwise specified purified glycerol or biodiesel derived crude glycerol was used as the sole carbon source. All experiments were conducted in an anaerobic chamber (95% N_2 and 5% H_2) at a temperature of 37ºC. The 5% hydrogen in the chamber was used to maintain anaerobic conditions, as the hydrogen reacted with palladium catalyst (supplied by the chamber manufacturer) to remove trace amounts of oxygen. The pH of the samples was measured at regular intervals and the samples were analyzed for glycerol, acetic acid, 1,3-propanediol, ethanol, butyric acid and butanol by HPLC equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column using 5mM sulfuric acid as the mobile phase. All experiments proceeded until glycerol was completely utilized. The fermentation period was determined based on the time taken for complete utilization of glycerol. The dry cell weight was measured by centrifuging 1 mL of the media containing the cells at 10000 rpm for 10 minutes and the pellets were washed with deionized water and dried in a preweighed glass test tube in an oven at 100°C for 24 hours.

4.2.3 Effect of Impurities:

The effect of each impurity on the growth behavior was studied individually by adding varying amounts to the fermentation (defined media) at 25 g/L pure glycerol. Methanol was added at 2.5 g/L and 5.0 g/L. Potassium chloride (KCl) and potassium sulfate (K_2SO_4) were added at 2.5, 5.0, 7.5 and 10% (wt of salt/wt of initial substrate concentration) of the initial glycerol concentration. The fatty acids were added using sodium salts of stearic acid, oleic acid and linoleic acid at 5, 10 and 15% (wt of fatty acid/wt of initial substrate concentration). Media containing 25 g/L of pure glycerol were used as the control. The effects of fatty acids were also studied by partially purifying the crude glycerol by precipitating the fatty acids using concentrated hydrochloric acid. The crude glycerol was diluted in deionized water in the ratio of 1:3 and hydrochloric acid was added to lower the pH to between 2 and 3. At this pH the fatty acids precipitated and were removed by centrifugation at 5000 rpm for 15 minutes.

4.2.4 GC-MS Analysis of Fatty acids

The free fatty acids were extracted three times with 1 ml portions of 1:1 (v/v) hexane/diethyl ether. The organic phases were combined and evaporated to dryness using rotoevaporator at room temperature. Dry fatty acid mixture were kept under argon in a desiccator at -80°C and taken out for derivatization. Fatty acid methyl ester (FAME) procedures were adapted from Supelco using BF_3-Methanol 10 % w/w. FAME derivatives were analyzed by a Shimadzu GC-MS QP 2010 system using a SHR5xLB silica capillary column (30m× 0.25mm ID , composed of 100% Dimethyl poly siloxane). Operating conditions were as follows: operating in electron impact mode at 80 eV; ultra

high pure helium (99.999%) was used as carrier gas at a constant flow of 1.7ml / min with a nominal pressure of 120 kPa, injector temperature of 240 °C. MS were recorded in electron ionization (EI) mode, with energy of 80 eV. Mass spectra were taken at 80 eV; a scan interval of 0.5 seconds and fragments from 50 to 550 Da. Ion-source temperatures was 250 °C with 3.00 min solvent cut time. The compounds were analyzed with GC-MS solution and identified by comparison with the data in the NIST libraries.

4.2.5 NMR Spectroscopy:

All ^{1}H-NMR spectra were recorded on a Varian Unity Inova 500 (500 MHz) spectrometer equipped with a 5 mm triple resonance inverse detectable probe. DMSO-d_6 and D_2O were used as solvents and contained trimethylsilane (TMS) as internal reference.

4.3 Results:

4.3.1 Effect of Methanol and Salts:

According to the specifications of the manufacturer (MSDS), methanol is present in crude glycerol at 12-18%. Hence 2.5 g/L and 5.0 g/L methanol, corresponding to 10% and 20% respectively, were added to the fermentation of 25 g/L pure glycerol. The addition of methanol to media did not hinder growth or metabolism. Figure 4.1 summarizes the growth pattern obtained from cultures grown in presence of methanol while Table 1 summarizes the product yield obtained from the same experiments. The fermentation products constituted acetate, 1,3-PDO, ethanol, butyrate and butanol. All products, with the exception of 1,3-PDO, were produced in identical amounts. The yield

of 1,3-PDO dropped from 0.19 g/g to 0.13 g/g and 0.12 g/g (g of product produced/ g of substrate utilized) in presence of 2.5 g/L and 5 g/L methanol, respectively. The addition of methanol did not lower the yield of butanol (Table 4.1), which remained at 0.24 g/g.

Table 4.1 Effect of methanol on glycerol fermentation in terms of product yield (g of product produced/g of substrate utilized)

Products / Variants	Acetate (g/g)	1,3-PDO (g/g)	Ethanol (g/g)	Butyrate (g/g)	Butanol (g/g)	Biomass (mg/mL)
Control	**0.01 ± 0.000**	**0.19 ± 0.011**	**0.05 ± 0.003**	**0.0 ± 0.000**	**0.24 ± 0.019**	**3.41 ± 0.218**
2.5 g/L Methanol	**0.01 ± 0.000**	**0.13 ± 0.002**	**0.05 ± 0.007**	**0.00 ± 0.003**	**0.24 ± 0.003**	**3.35 ± 0.206**
5 g/L Methanol	**0.01 ± 0.000**	**0.12 ± 0.014**	**0.06 ± 0.005**	**0.00 ± 0.003**	**0.23 ± 0.016**	**3.43 ± 0.186**

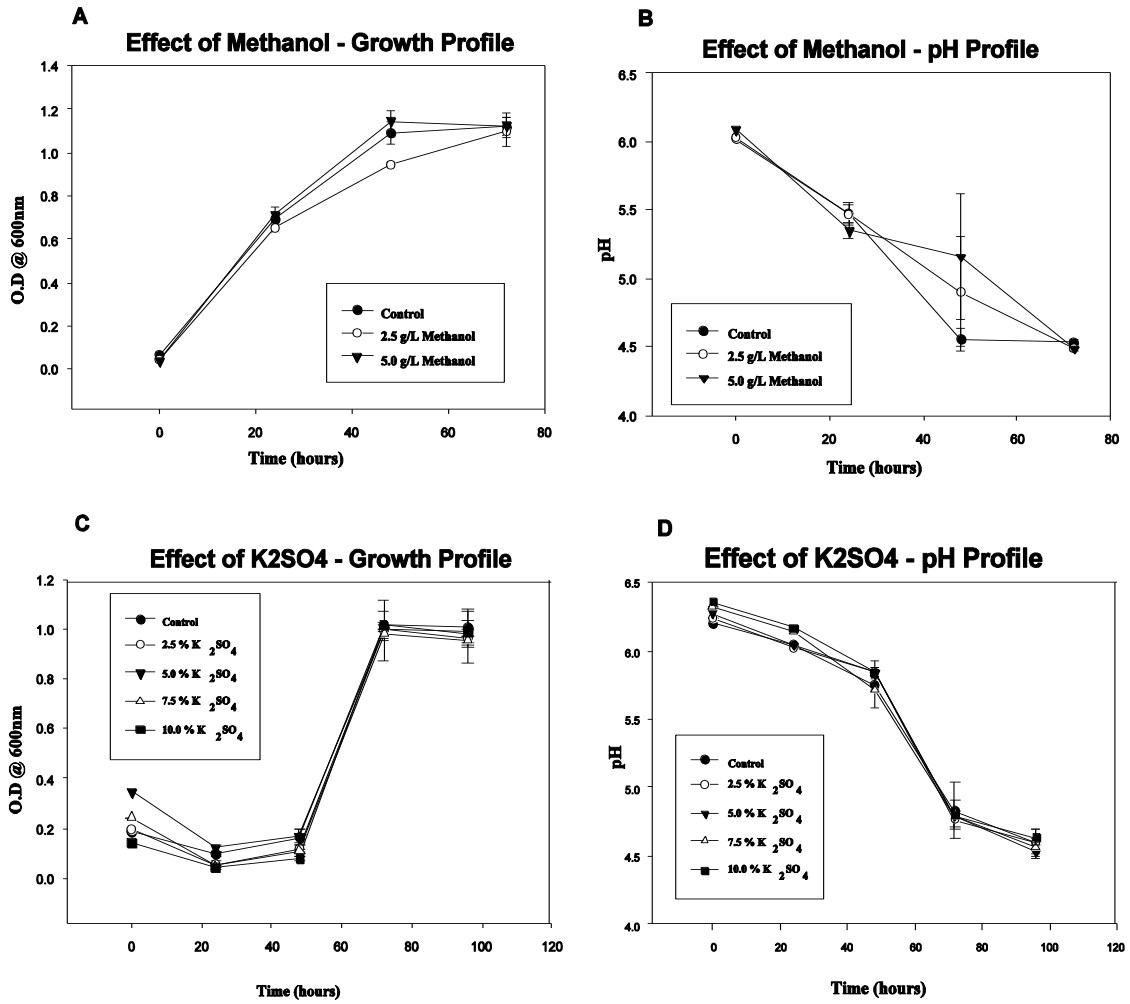


Figure 4.1 Effect of Methanol and Salts on growth and metabolism. The effect of methanol was studied at concentrations of 2.5 g/L and 5 g/L. (A) Growth (B) pH profile. The effect of the addition of K_2SO_4 at concentrations of 2.5, 5.0, 7.5 and 10.0 % . (C) Growth and (D) pH profile. The growth and pH profile for the addition of KCl (Appendix A, Figure A1) resembled the data obtained from the addition of K_2SO_4 shown in (C) and (D), respectively.

The effect of the salts was studied by adding potassium chloride and potassium sulfate at concentrations of 2.5, 5.0, 7.5 and 10 % (wt of the salt/wt of the initial substrate). These concentrations were selected based on the salt concentrations reported in the MSDS of the crude glycerol (3-10 %). Fermentation of pure glycerol without any addition of these salts served as a control. Growth and product formation data for the effect of K_2SO_4 are shown in Figure 4.1 and Table 4.2. The growth and pH profile of KCl addition were similar to the results obtained from the addition of K_2SO_4 (Figure 4.1). The product yield of the desired product, butanol, was not affected by the addition of these salts, but the production of 1,3-PDO was observed to be negatively affected. The yield of 1,3-PDO was lowered from 0.17 g/g to 0.12 g/g during the addition of 10 % (wt of salt/wt of the substrate) K_2SO_4 and to 0.11 g/g for all the KCl concentrations added (Table 4.2).

The addition of methanol to the media did not affect the yield of butanol and also there was no effect observed on the growth of the cells in presence of 2.5 g/L and 5.0 g/L methanol. Alcohols are found to affect the cell membrane by increasing the fluidity of the membrane (Ingram 1976; Kabelitz, Santos, and Heipieper 2003). The intensity of the effect increases with the carbon chain length and the concentration of the aliphatic alcohol (Ingram 1976). The bacteria counteract the increase in fluidity caused by the alcohols by altering the ratio of saturated and unsaturated fatty acid tails in the lipid bilayer of the membrane (Ingram 1976). Methanol, being an aliphatic alcohol of one carbon, is weakly lipophilic and has the least effect on the fluidity of the membrane. The fluidity effects of methanol on bacterial membranes were prominently observed only at

concentrations of 1.0% and higher (Ingram 1976). The effect of methanol at 2.5 g/L (0.25 %) and 5 g/L (0.5 %), which were the concentrations used in the experiments, will have minimal effect on the bacterial growth and metabolism. Hence, there was no inhibition observed in the yield of butanol. Since bacteria counteract the effects of methanol on the fluidity of the membrane by altering the degree of saturation in the fatty acid tails (Ingram 1976) of the lipids, the bacteria undergo certain metabolic changes, which is observed in the form of lowering the yield of 1,3-PDO. In *Clostridia*, it is known that the bacteria alter the ratio of saturated fatty acids to the unsaturated fatty acids in response to the alcohol and solvent stress (Baer *et al.* 1987), but the lipid composition of *C. pasteurianum* and the effects of alcohols and solvents on this strain have not been investigated. Though, the addition of methanol to the media affects the membrane fluidity, its negative effect on product yield is only observed with a slight reduction in the yield of 1,3-PDO. Production of 1,3-PDO is a two-step process from glycerol (Figure 1.1) and as it is the first reaction during glycerol fermentation (Taconi *et al.*) and hence this pathway will be affected due to the presence of impurities and result in a reduction in the amount of 1,3-PDO formed. The fact that the addition of methanol lowers the yield of 1,3-PDO in the reduction pathway, without affecting the yield of the products formed from the oxidation branch of glycerol utilization, might also suggest a presence of an alternate reduction pathway from glycerol that is involved in altering the saturation levels in the fatty acids tail of the lipid membrane. Methanol has been shown to inhibit the production of DHA (docosahexaenoic acid) from crude glycerol using *Schizochytrium limacinum* (Pyle, Garcia, and Wen 2008). However, in the current study

no inhibitory effect on the butanol production and only a small inhibition in 1,3-PDO production were observed.

The addition of salts did not inhibit the growth and yield of butanol. There was a gradual reduction in the yield of 1,3-PDO during the addition of K_2SO_4 and a constant reduction in the 1,3-PDO yield was observed for all concentrations of KCl. Monovalent salts have been described as having a swelling effect on the membrane at high concentrations by weakening the Van der Waals forces between the lipid tails in the membrane (Petrache et al. 2006). This has been observed to affect the energy barrier within the lipid layer leading to alterations in the biochemical processes such as substrate transportation across the membrane. This effect was observed to be higher for KCl as it has a monovalent cation and a monovalent anion, and is reflected in the form of reduction in the yield of 1,3-PDO and less prevalent for K_2SO_4 at lower concentrations.

Table 4.2 Effect of salts on glycerol fermentation in terms of product yield (g/g)

Products / Variants	Acetate (g/g)	1,3-PDO (g/g)	Ethanol (g/g)	Butyrate (g/g)	Butanol (g/g)	Biomass (mg/mL)
Control	0.03 ± 0.007	0.17 ± 0.023	0.02 ± 0.009	0.04 ± 0.019	0.26 ± 0.027	3.07 ± 0.218
2.5% K_2SO_4	0.03 ± 0.009	0.16 ± 0.047	0.03 ± 0.015	0.03 ± 0.022	0.26 ± 0.030	2.94 ± 0.314
5% K_2SO_4	0.03 ± 0.009	0.15 ± 0.025	0.03 ± 0.013	0.02 ± 0.011	0.28 ± 0.011	3.03 ± 0.138
7.5% K_2SO_4	0.02 ± 0.003	0.12 ± 0.032	0.04 ± 0.009	0.02 ± 0.003	0.27 ± 0.001	2.91 ± 0.068
10% K_2SO_4	0.03 ± 0.007	0.12 ± 0.027	0.03 ± 0.011	0.02 ± 0.005	0.27 ± 0.006	3.00 ± 0.036
2.5% KCl	0.01 ± 0.001	0.11 ± 0.004	0.02 ± 0.001	0.03 ± 0.003	0.27 ± 0.003	3.51 ± 0.030
5% KCl	0.02 ± 0.001	0.10 ± 0.004	0.01 ± 0.001	0.02 ± 0.002	0.25 ± 0.013	3.20 ± 0.030
7.5% KCl	0.02 ± 0.002	0.10 ± 0.024	0.01 ± 0.003	0.03 ± 0.010	0.24 ± 0.028	3.21 ± 0.305
10% KCl	0.02 ± 0.002	0.11 ± 0.013	0.02 ± 0.012	0.03 ± 0.003	0.25 ± 0.039	3.354 ± 0.091

4.3.2 The Effect of Fatty acids:

4.3.2.1 NMR Analysis of Crude glycerol:

As shown in Figure 4.2A, the ^{1}H-NMR spectrum of pure glycerol (D_2O, 500 MHz) revealed *H*O-CH_2-CH(O*H*)-CH_2O*H* (s, 4.68 ppm), HO-C*H*$_2$-CH(OH)-C*H*$_2$ OH (m, 3.53 ppm, 3.45 ppm) and HO-CH_2-C*H*(OH)-CH_2OH (m, 3.65 ppm). The analysis of crude glycerol showed all glycerol characteristic peaks as described above and a multitude of low intensity peaks in the chemical shift range between 0.5 ppm and 5.4 ppm (Figure 4.2B). These peaks were assigned to the fatty acid impurities and the spectrum of the partially purified crude glycerol is almost free of these peaks (Figure 4.2C). The presence of fatty acids was further confirmed by the ^{1}H-NMR spectrum taken of the precipitate obtained when the crude glycerol was purified by acid precipitation. The signals of the precipitate coincide with the signal ascribed to the impurities in the crude glycerol (Figure 4.2D). Additionally, a strong signal is observed at 3.23 ppm (C*H*$_3$-OH) and 4.68 ppm (CH_3-O*H*), which are indicative of the protons in methyl and hydroxyl group of methanol, respectively (Figure 4.2B and Figure 4.2C). The acid precipitates from the crude glycerol analyzed by GC/MS revealed the presence of palmitic acid (25 %), stearic acid (16 %), oleic acid (45 %), linoleic acid (12 %) and remaining 2% other fatty acids. The fatty acids in crude glycerol contained a higher percentage (nearly 60 %) of unsaturated fatty acids.

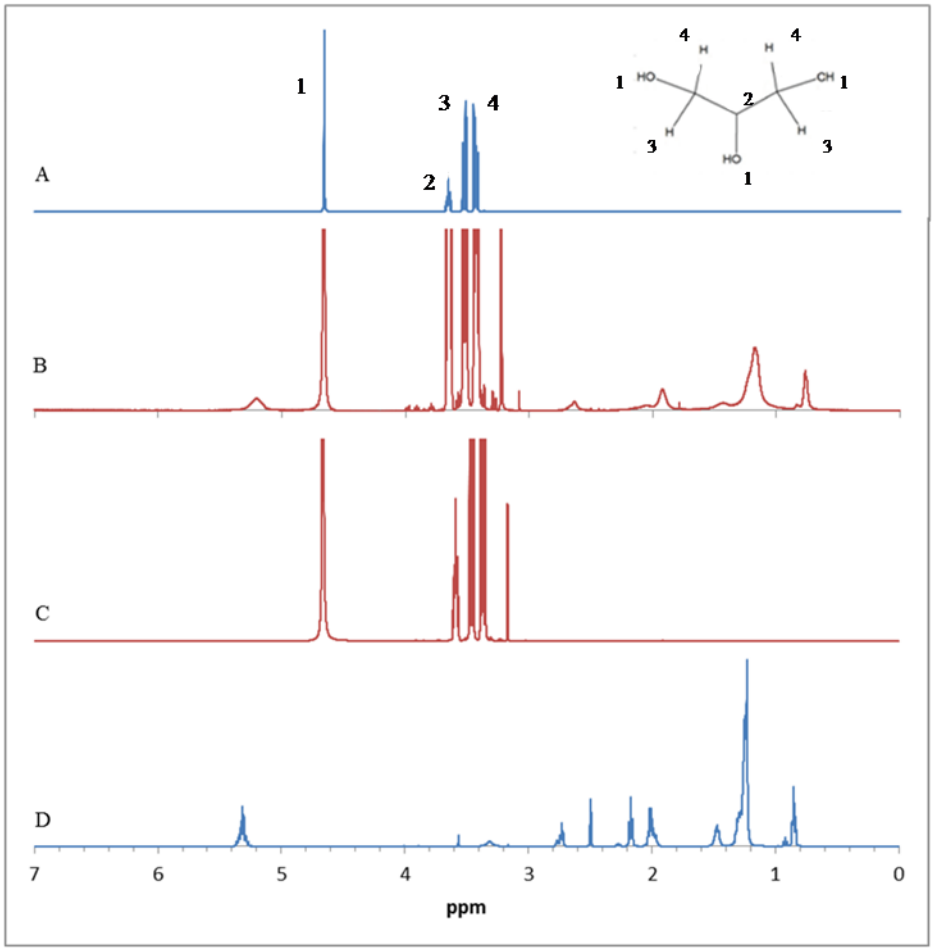


Figure 4.2 ^{1}H-NMR of (A) pure glycerol, (B) crude glycerol, (C) partially purified crude glycerol and (D) fatty acids precipitated from crude glycerol.

Glycerol is characterized by 1(4.68 ppm), 2 (3.65 ppm), 3 (3.51 ppm) and 4 (3.45 ppm). The 1H-NMR of crude glycerol contains an extra peak at 3.23 ppm which corresponds to methanol. Apart from the methanol peak the crude glycerol ^{1}H-NMR spectrum revealed peaks from 0.8 ppm to 5.3 ppm, which is region corresponding to the peaks of hydrogen present on aliphatic hydrocarbons. These peaks disappeared after purification (C). The precipitates were analyzed after they were separated from crude glycerol (D) and correspond to the extra peaks found in crude glycerol.

4.3.3.2 Addition of Fatty acids:

The fatty acids present in the crude glycerol were separated using acid (HCl) precipitation. Fatty acids were removed by centrifugation, weighed and were found to constitute 11.65 ± 1.80 % (n=3; wt of fatty acids/wt of crude glycerol) of crude glycerol. The effect of the presence of fatty acids was studied by using three different fatty acids that had the same carbon chain length, but varied in their number of double bonds. Stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) were added at concentration of 5%, 10% and 15% (wt of fatty acid/wt of initial substrate concentration) of the total glycerol in the media. These concentrations were selected based on the estimation of fatty acids (11.65 ± 1.80%). Two control experiments were performed for the effect of addition of fatty acid, the first control experiment had only 25 g/L of pure glycerol, while the other control experiment was performed with fatty acids as the sole carbon source. The latter control experiments were carried out at a concentration of 1.25 g/L of each of the three fatty acids.

The addition of stearic acid had a negative effect on the yield of 1,3-PDO and butanol. The yield of 1,3-PDO dropped by 29%, 29% and 18% respectively for each of the three concentrations (Table 4.3). Similarly, the yield of butanol showed a steady decrease by 15%, 26% and 30% with a corresponding increase in the concentration of stearic acid added. Despite the slight inhibitory effects observed for product yield, there was no inhibition in growth and metabolism (Figure 4.3). The addition of oleic acid also reduced the product yields of 1,3-PDO and butanol (Table 4.3). The 1,3-PDO concentrations were reduced by 29%, 18% and 24% for 5%, 10% and 15% (wt of fatty

acid/wt of initial substrate concentration) of oleic acid that was added to the media, while the yield of butanol was reduced by 26%, 26% and 30% respectively. The growth and metabolism were also slightly inhibited by the addition of oleic acid (Figure 4.3). The maximum optical density (O.D) value at the end of 72 hours measured at 600 nm decreased gradually from 1.61±0.01 to 0.95±0.18 at 15% (wt of fatty acid/wt of initial substrate concentration) oleic acid addition. The addition of linoleic acid completely inhibited substrate utilization and resulted in the formation of only small amounts of acetate (Table 4.3). The growth was also completely inhibited (Figure 4.3).

The control experiments with fatty acids as the sole carbon source resulted in no growth of the cells. Also, earlier work on the addition of exogenous fatty acids in the growth medium of *Clostridia,* have shown that the fatty acids are incorporated in to lipids of the bacterial membrane (Baumann et al. 1965). It has also been shown that stearic acid, oleic acid and linoleic acid, when added to the growth medium of *C. butyricum*, get incorporated into the acyl chains and alk-1-enyl chains of cellular lipids (Khuller et al. 1975). The addition of stearic acid at 5, 10 and 15% (wt of fatty acids/ wt of glycerol) had a lesser inhibitive effect on the product yield by slightly lowering the yields of 1,3-PDO and butanol alike. This can be explained by the nature of stearic acid as it is a saturated C18 fatty acid and, when added to the media, it will align itself with the fatty acid tails of the membrane (Muranushi et al. 1981). Similarly, the addition of oleic acid reduced the yields of 1,3-PDO and butanol and inhibited the growth at the higher concentrations. Oleic acid differs from stearic acid by containing one double bond. The presence of a double bond in oleic acid introduces a kink in the molecule. The interaction of the bent molecule with the membrane affects its equilibrium by hindering the diffusion

of nutrients and metabolites through the membrane (Furusawa and Koyama 2004; Galbraith and Miller 1973; Thompson, Cockayne, and Spiller 1994). The presence of one double bond in oleic had an inhibitory effect, larger than the effect observed with stearic acid. Linoleic acid has two double bonds, hence, there are two kinks in the molecule. Subsequently there is a more pronounced interaction with the cell membrane, and the diffusion processes are massively hindered (Furusawa and Koyama 2004; Galbraith and Miller 1973; Thompson, Cockayne, and Spiller 1994). Due to the presence of two kinks in the linoleic acid, the effect can also be completely inhibitory to the diffusion as observed here (Desbois and Smith 2010).

Linoleic acid and fatty acids with higher degree of unsaturation are found to be present in most of the triacyl glycerides of plant oils, which are used as the major reactant for the biodiesel production in the from of palm oil, canola oil, corn oil etc. Therefore, fatty acids with two or more double bonds will enter the crude glycerol component, as they fail to undergo transesterification. The ^{1}H-NMR spectrum of crude glycerol revealed additional peaks between 0.8 ppm and 5.3 ppm. A comparison of the ^{1}H-NMR spectrum of the precipitate (Figure 4.2D) obtained from the purification of crude glycerol to the Spectral Database for Organic Compounds (SDBS) confirms that the impurities are indeed fatty acids.

Table 4.3 Effect of fatty acids on glycerol fermentation in terms of product yield (g/g)

Products / Variants	Acetate (g/g)	1,3-PDO (g/g)	Ethanol (g/g)	Butyrate (g/g)	Butanol (g/g)	Biomass (mg/mL)
Control	0.03 ± 0.007	0.17 ± 0.023	0.02 ± 0.009	0.04 ± 0.019	0.26 ± 0.027	4.14 ± 0.040
5% Stearic acid	0.02 ± 0.008	0.12 ± 0.060	0.02 ± 0.009	0.04 ± 0.018	0.23 ± 0.021	3.41 ± 0.021
10% Stearic acid	0.02 ± 0.008	0.12 ± 0.027	0.01 ± 0.010	0.06 ± 0.009	0.20 ± 0.031	3.60 ± 0.024
15% Stearic acid	0.03 ± 0.010	0.14 ± 0.039	0.01 ± 0.022	0.09 ± 0.021	0.19 ± 0.019	3.83 ± 0.165
5% Oleic acid	0.04 ± 0.013	0.13 ± 0.098	0.02 ± 0.003	0.06 ± 0.035	0.20 ± 0.040	3.06 ± 0.279
10% Oleic acid	0.04 ± 0.006	0.22 ± 0.027	0.02 ± 0.001	0.09 ± 0.027	0.19 ± 0.016	3.74 ± 0.083
15% Oleic acid	0.04 ± 0.007	0.12 ± 0.078	0.02 ± 0.001	0.09 ± 0.033	0.19 ± 0.036	2.91 ± 0.556
5% Linoleic acid	0.01 ± 0.00	0	0	0	0	0.51 ± 0.106
10% Linoleic acid	0.01 ± 0.00	0	0	0	0	0.356 ± 0.198
15% Linoleic acid	0.01 ± 0.00	0	0	0	0	0.071 ± 0.830

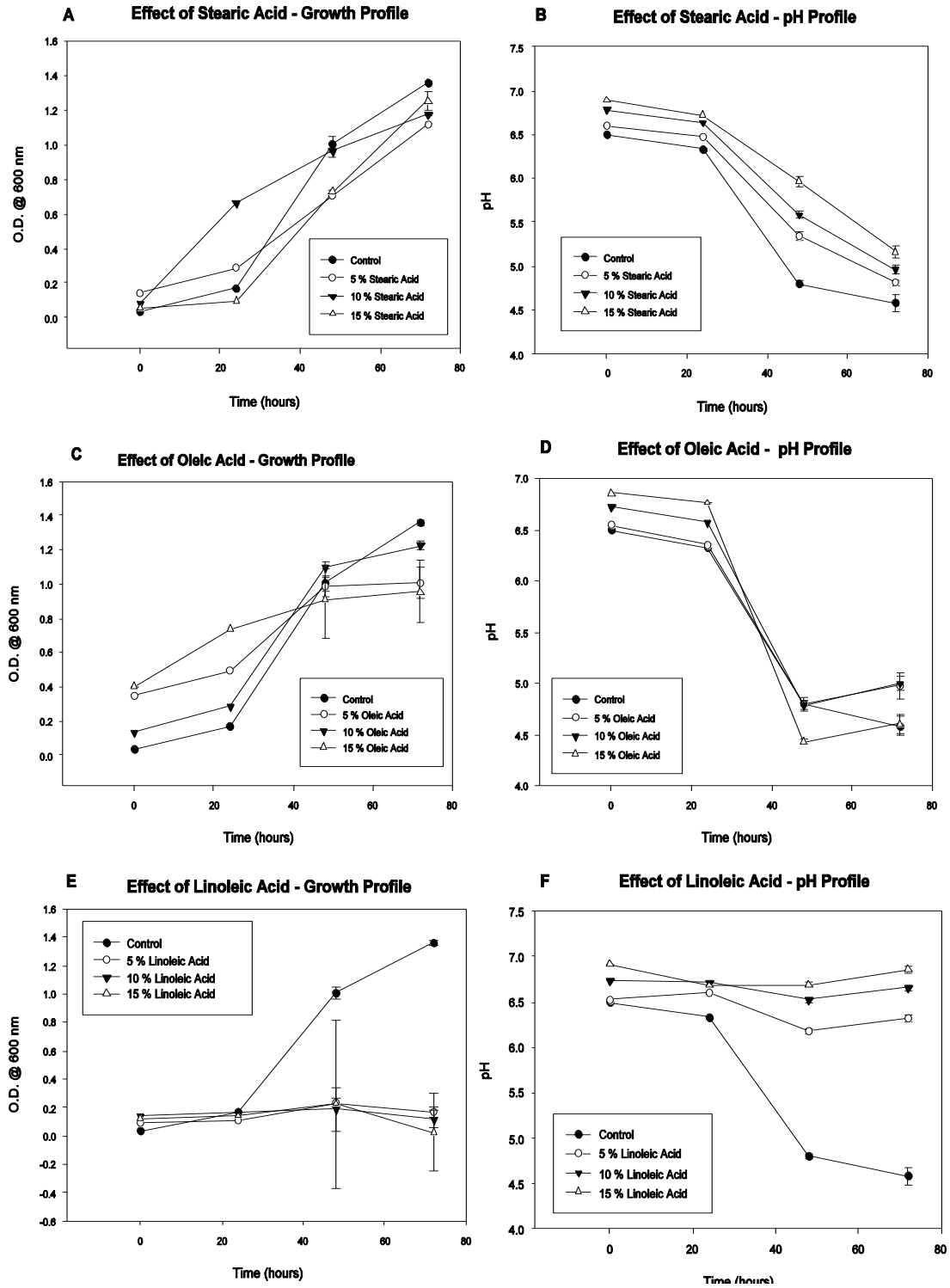


Figure 4.3 Effect of the addition of fatty acids. The growth profile obtained during the addition of stearic, oleic and linoleic acids are shown in (A), (C) and (E), respectively, while the corresponding pH profiles are depicted in (B), (D) and (F), respectively.

4.3.3.3 Partially Purified Crude Glycerol:

The use of acid precipitation to remove the fatty acids from the crude glycerol results in the formation of partially purified crude glycerol, which is devoid of fatty acids (Figure 4.2C). During the purification of crude glycerol, it was estimated that it contained 11.65 ± 1.8 % (weight of fatty acid precipitate/ weight of crude glycerol) and after purification the partially purified crude glycerol was stoichiometrically estimated to contain 6-7 % (weight of NaCl/ weight of purified crude glycerol) NaCl. This partially purified crude glycerol was used in fermentation and compared with pure glycerol. The product yields of partially purified crude glycerol were comparable to those observed for pure glycerol (Table 4.4), with the butanol yield being slightly higher (0.28 g/g) compared to the pure glycerol (0.26 g/g) and crude glycerol (0.21 g/L). The yield of 1,3-PDO was lowered from 0.17 g/g to 0.09 g/g, but higher than 0.03 g/g obtained on crude glycerol. The fermentation of crude glycerol was longer ranging from 14 to 24 days (Figure 4.4 and Figure 2.3) while the fermentation period was just 4-5 days for partially purified crude glycerol (Figure 4.4).

It has been reported that the separation of the fatty acids from crude glycerol makes the partially purified crude glycerol a better substrate (Chi et al. 2007; Liang et al. 2010; Pyle, Garcia, and Wen 2008). The fermentation of the partially purified crude glycerol, which was purified of the fatty acids, was consumed in a manner similar to pure glycerol. Table 4.4 and Figure 4.3 show the impedance of fatty acids in substrate utilization, growth, fermentation time and product yield. Substrate utilization rate and the rate of fermentation are directly related. Also, the purification step removes only the fatty

acids but not the salts and methanol, thereby re-confirming that salts and methanol have a lesser inhibitive role on the yield of butanol, though they show a significant inhibition for 1,3-PDO production. The difference in the pH profiles of the pure glycerol and partially purified crude glycerol suggests that the rate of substrate utilization and acid formation was lower with partially purified glycerol, however, the fermentation periods (complete utilization of glycerol) were similar (Figure 4.4). Also, the removal of fatty acids from crude glycerol results in a faster and a much better utilization of the partially purified crude glycerol (Figure 4.4). These results clearly demonstrate the inhibitive nature of fatty acids on bacterial fermentation of glycerol.

Table 4.4 Product yield (g/g) comparison of pure glycerol, purified crude glycerol and crude glycerol.

Products / Substrate	Acetate (g/g)	1,3-PDO (g/g)	Ethanol (g/g)	Butyrate (g/g)	Butanol (g/g)	Biomass (mg/mL)
Pure Glycerol	**0.03 ± 0.007**	**0.17 ± 0.023**	**0.02 ± 0.009**	**0.04 ± 0.019**	**0.26 ± 0.027**	**4.14 ± 0.040**
Purified crude glycerol	**0.02 ± 0.002**	**0.09 ± 0.008**	**0.03 ± 0.002**	**0.01 ± 0.001**	**0.28 ± 0.022**	**3.825 ± 0.090**
Crude Glycerol[#]	**0.02 ± 0.010**	**0.03 ± 0.009**	**0.04 ± 0.020**	**0.01 ± 0.01**	**0.21 ± 0.04**	**3.45 ± 0.133**

[#]the fermentation period of pure glycerol and partially purified crude glycerol took 4 days while the crude glycerol was utilized in 14-24 days.

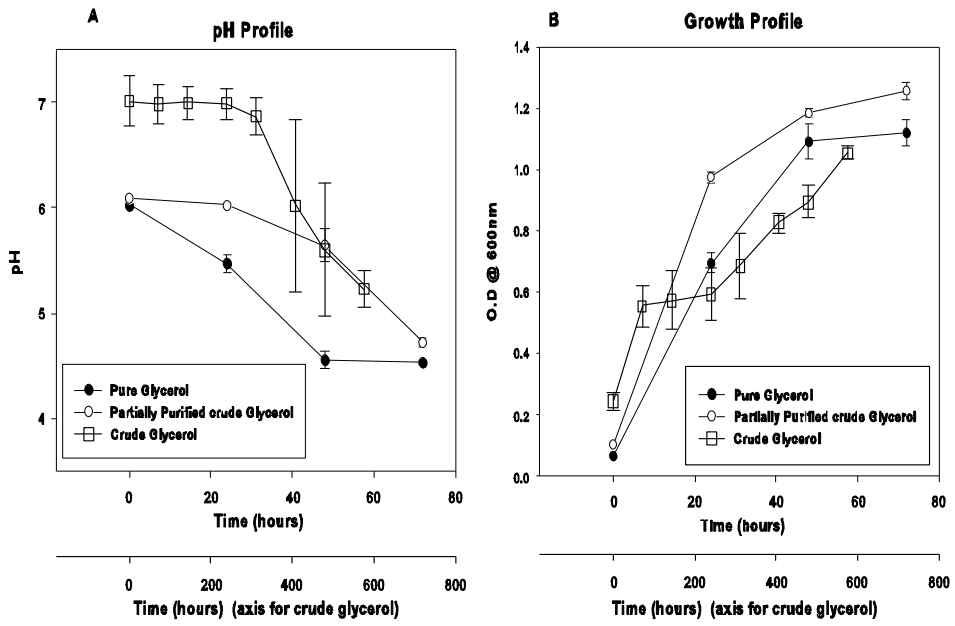


Figure 4.4 Comparison of pure glycerol, partially purified crude glycerol and crude glycerol (A) Growth comparison (B) pH profile. Note: The crude glycerol plots have a secondary x-axis offset to actual x-axis.

4.4 Conclusion

The NMR analysis of crude glycerol, partially purified crude glycerol and the fatty acids precipitated from the crude glycerol clearly show the presence of free fatty acids in crude glycerol and the estimation of the amount of fatty acids, reveal a substantial amount of 11.65 ± 1.8 % (w/w). The longer fermentation period of crude glycerol (14-24 days) can be explained based on the presence of higher percentages of unsaturated fatty acids, leading to a slower metabolism of glycerol. Also, the relatively higher salt concentrations along with presence of methanol and free fatty acids in crude glycerol have lead to a greater reduction in the yield of 1,3-PDO during the metabolism of crude glycerol as the sole carbon source. The removal of the fatty acids from the crude glycerol is an essential step going forward towards glycerol fermentation. The presence of unsaturated fatty acids with two or more double bonds has a greater inhibitory effect in the diffusion of the substrate along the membrane. The fatty acid-free crude glycerol is utilized faster by the bacteria at the same rate of utilization as of the pure glycerol. The results from the current study also show that methanol and salts have a lesser inhibitive role to affect the overall productivity. Hence, for any fermentation process involving crude glycerol, the removal of fatty acids through acid precipitation is the ideal step for improving substrate utilization and product yield.

CHAPTER 5

5. BUTANOL TOXICITY AND TOLERANCE

5.1 Introduction

Butanol is widely considered one of the most promising alternative fuels, at least in the near term. Compared to ethanol, butanol has a higher energy content, better miscibility with petroleum fuels, and lower miscibility with water. Butanol is also a biorefinery platform chemical that can be used for the production of various biobased chemicals.

Species of bacteria, including *Clostridium acetobutylicum* and *Clostridium beijerinckii*, are capable of producing a mixture of acetone, butanol, and ethanol (ABE) via fermentation from a variety of substrates including monosaccharides, oligosaccharides, and polysaccharides that are derived from raw materials such as molasses, whey permeate, and corn. The ratio of acetone:butanol:ethanol in most fermentation mixtures is 3:6:1. *C. acetobutylicum* typically produces 20-25 g/L total solvents, of which 12-13 g/L is butanol. A metabolically engineered strain of *C. acetobutylicum* has been shown to produce up to 17.6 g/L butanol. A mutant strain of *C. beijerinckii* can produce up to 33 g/L total solvents and 19 g/L butanol. Butanol yields are usually 15-20 wt% using sugar substrates (Papoutsakis, 2008). In order for butanol

fermentation to become more economically favorable, it is widely agreed that both the butanol titer must be increased (it has been suggested that increasing concentrations to 19 g/L will decrease separations costs by as much as half (Papoutsakis, 2008)), and the fermentation of lower-cost feedstocks must produce yields comparable to high quality feedstocks such as glucose or molasses.

A third species of clostridia, *Clostridium pasteurianum*, can ferment glycerol as the sole substrate. Glycerol fermentation by *C. pasteurianum* produces a mixture of butanol, ethanol, and 1,3-propanediol (PDO) and does not produce acetone (Dabrock et al., 1992; Biebl, 2001; Taconi et al., 2009). *C. pasteurianum* is of particular interest for butanol production, as it has also been shown to ferment biodiesel-derived crude glycerol (as the sole substrate), producing butanol yields comparable to those when purified glycerol is the substrate (Taconi et al., 2009). Crude glycerol is a promising candidate substrate for butanol fermentation because of the significant amounts that are generated during biodiesel production. For biodiesel plants currently in operation, crude glycerol is generally treated as a waste by-product and presents logistical and financial burdens, as options for glycerol management are limited. Crude glycerol has little commercial value (~$0.05/lb) unless it is refined to a higher purity, and the refining process is costly ($0.15-$0.20/lb) (Taconi and Johnson, 2007). In Europe, rapid and large increases in biodiesel production generated a glycerol glut that ultimately bankrupted some biodiesel producers (Willke and Vorlop, 2004). Crude glycerol is a particularly attractive substrate for solvent production, as there would be little to no cost associated with acquiring or pretreating this feedstock. Additionally, butanol yields from glycerol are higher than those typically

obtained from glucose, starch or lignocellulosic feedstocks (Dabrock et al., 1992; Biebl, 2001; Taconi et al., 2009).

Although crude glycerol shows promise as a low-cost feedstock for butanol production, there is still the issue of low butanol titer. Butanol is toxic to cells, as it accumulates in the cell membrane, affecting both the structural and functional integrity of the cell. The extent of solvent toxicity correlates to the log P value. Solvents with a log P value less than 4 are considered extremely toxic, as much more solvent partitions into the lipid membrane bilayer. Butanol has a log P value of 0.8 and is considered to be one of the most toxic solvents (Sardessai and Bhosle, 2002). When *Clostridia* are exposed to solvents, the solvents exhibit a significant fluidizing effect on the phospholipid bilayer, which causes the organism to alter the lipid composition of the bilayer. To compensate for the fluidizing effects of butanol, *Clostridia* increase the ratio of saturated to unsaturated lipids in the membrane (Vollherbst-Schneck et al., 1984). Butanol also eliminates the ability of the cell to maintain an internal pH and inhibits membrane-bound ATPases and the uptake of glucose (if present), which subsequently inhibits energy generation (Bowles and Ellefson, 1985).

It is widely accepted that most species of *Clostridia* cannot tolerate more than 2 vol % butanol. In an attempt to develop a more butanol tolerant microorganism, efforts have focused on the metabolic engineering of *C. acetobutylicum*. *Escherichia coli* and *Saccharomyces cerevisiae* are also considered good host candidates for metabolic engineering, since the genetic tools for altering these organisms are more advanced than those for *Clostridia*. However, neither *E. coli* nor *S. cerevisiae* has been shown to have a

higher butanol tolerance than *Clostridia*, as the butanol tolerance limit is 2 vol % (Fischer et al., 2008.; Knoshaug and Zhang, 2009).

Research has shown that *Clostridium pasteurianum* may offer several advantages over other organisms that have been considered for solvent production. A wild-type strain of *C. pasteurianum* has been shown to produce as much as 17 g/L butanol from pure glycerol (Biebl, 2001), which suggests that this organism has a natural ability to tolerate higher concentrations of butanol than other species of solventogenic *Clostridia*. Other research efforts have demonstrated that *C. pasteurianum* is capable of producing butanol from crude glycerol, which contains significant amounts of methanol and salts, at yields comparable to those produced from purified glycerol (Taconi et al., 2008). The ability of *C. pasteurianum* to ferment crude glycerol further indicates that this strain may be better able to tolerate solvents and impurities.

The goal of this research was to evaluate the butanol tolerance of *C. pasteurianum* ATCC® 6013™ by subjecting cultures to butanol challenge experiments under increasing concentrations of butanol. Butanol-challenged cells were subsequently evaluated for glycerol metabolism and solvent production to determine if exposure to butanol affects substrate utilization and product yield.

5.2 Materials and Methods

Clostridium pasteurianum ATCC® 6013™ was obtained from the American Type Culture Collection (Manassas, VA). To ensure anaerobic conditions, all cultures were prepared in a vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Freeze dried cultures were reconstituted with 1 mL of media and heat-shocked at 70–

80°C for 10 minutes. The heat-shocked culture was transferred to 75 mL culture vials containing 30–40 mL of nutrient medium containing K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$-$7H_2O$, $CaCl_2$-$2H_2O$, $FeSO_4$-$7H_2O$, $CaCO_3$, yeast extract, and trace element solution, as described previously (Chapter 2) and 8% glucose as the carbon source. All media components were purchased from Fischer Scientific or Sigma-Aldrich. Cultures were allowed to grow to the mid-log phase (O.D. > 0.6 @ 600nm) before being transferred to butanol challenge experiments and to prepare glycerol stock cultures that were stored at -80°C for future use.

Once reaching the mid-log phase, 5 mL of inoculum was transferred into 50 mL of nutrient medium containing 8% glucose, 0.8 mg/L biotin, and variable butanol concentrations ranging from 0, 5, 10, 15, 20, or 25 g/L. Butanol challenge experiments were performed in batch culture using two different approaches. Approach A involved adding cultures to a glucose medium supplemented with specific amounts of butanol ranging from 0-25 g/L. Approach B involved transferring the cultures used in approach A from lower butanol concentration to higher butanol concentration. Cultures initially grown with no butanol were transferred to media containing 5 g/L butanol; cultures grown in 5 g/L butanol were transferred to media containing 10 g/L butanol; and so on until the butanol concentration reached 30 g/L. All experiments were performed in 75 mL culture flasks. Cultures were incubated anaerobically at 35°C and shaken at 140 rpm. Cell growth and fermentation pH were recorded at frequent intervals. The initial pH of the media was approximately 7.0, and the media were not buffered. Cell growth was determined using optical density (OD) at 600 nm. All experiments were performed in triplicate. Once the cells demonstrated growth at a particular concentration of butanol,

they were subsequently subcultured into fresh nutrient media supplemented with the next higher butanol concentration.

Solvent production by strains grown at various butanol concentrations was evaluated by transferring 5 mL of inoculum from the butanol challenge experiments into 50 mL of nutrient media containing 25 g/L purified glycerol as the carbon source. These experiments were also incubated anaerobically at 35°C, shaken at 140 rpm and performed in triplicate. Growth was monitored through optical density measurements and pH was recorded. Liquid samples were analyzed for glycerol, acetate, 1,3-propanediol, ethanol, butyrate and butanol using a high pressure liquid chromatography system equipped with a Bio-Rad Aminex HPX-87H column and a refractive index detector (Varian, Walnut Creek, CA). The mobile phase was 5 mM H_2SO_4, pumped at a flow rate of 0.4 mL/min.

The maximum specific growth rate (hr^{-1}) was calculated via linear regression of a plot of the ln of optical density vs. time in hours. The maximum specific growth rate is the slope of this line. The maximum specific growth rate was determined using data from only the exponential phase of growth and did not include data from the lag or stationary phases. A two-sample t-test using a "pooled estimate" of the sample variance was performed using the average butanol yield data to determine if the difference between the two population means was significant. The t-test was performed at the 95% confidence interval ($\alpha = 0.05$) for ($n_1 + n_2 - 2$) degrees of freedom.

5.3 Results and Discussion

To evaluate the effect of butanol on cell growth, cultures were grown in media containing 8% glucose. Cells were grown in glucose media instead of glycerol media because glucose is a better substrate for cell growth as compared to glycerol (as well as other highly reduced sugars). The results of this research indicate that *C. pasteurianum* does exhibit a higher tolerance to butanol, as growth of cultures was observed in butanol concentrations up to 15 g/L. Figures 5.1 and 5.2, show the optical density of cultures grown in various butanol concentrations up to 25 g/L. Figure 5.1 shows the optical density of cultures grown using Approach A, where cultures were added directly to media containing varying concentrations of butanol. As shown in the figure, a butanol concentration of 10 g/L increased the lag phase from about 5 hours to 20 hours, while the lag phase at higher concentrations of butanol increased to 20-30 hours. Essentially very minimal growth was observed in cultures on 20 g/L and 25 g/L butanol (Figure 5.1).

Figure 5.2 shows the optical density for cultures as they were transferred to incrementally higher butanol concentrations (Approach B). This approach appeared to improve the acclimation process, as subsequent transfers to higher butanol concentrations did not exhibit the long lag phases that were observed in approach A. With the exception of experiments transferred from 10 to 15 g/L butanol, all other experiments exhibited a lag phase of less than 10 hours. Essentially very minimal growth was observed in cultures transferred from 15 g/L to 20 g/L butanol and 20 g/L to 25 g/L (Figure 5.2). Control experiments for butanol evaporation were conducted by adding butanol to Biebl

medium and measuring butanol concentration over time (24 hours and 48 hours). These results are summarized in Figure B.1.

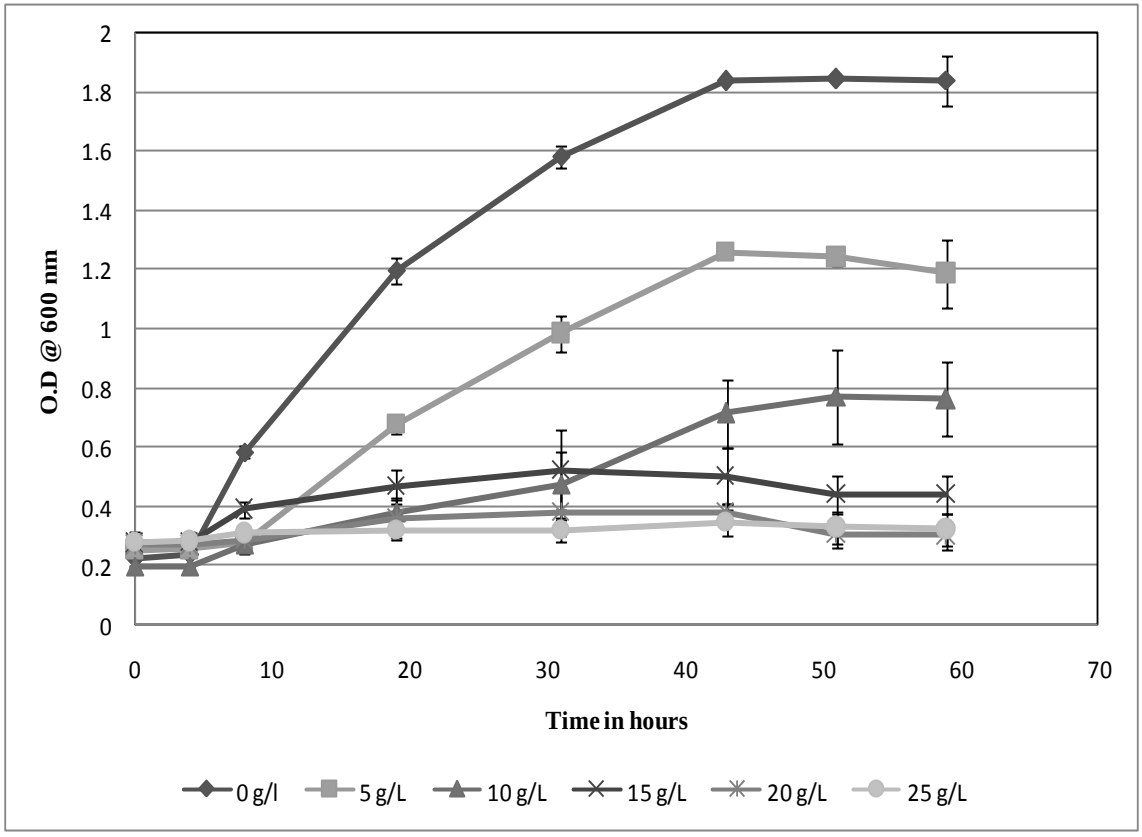


Figure 5.1 Optical densities of cultures grown in various concentrations of butanol using Approach A

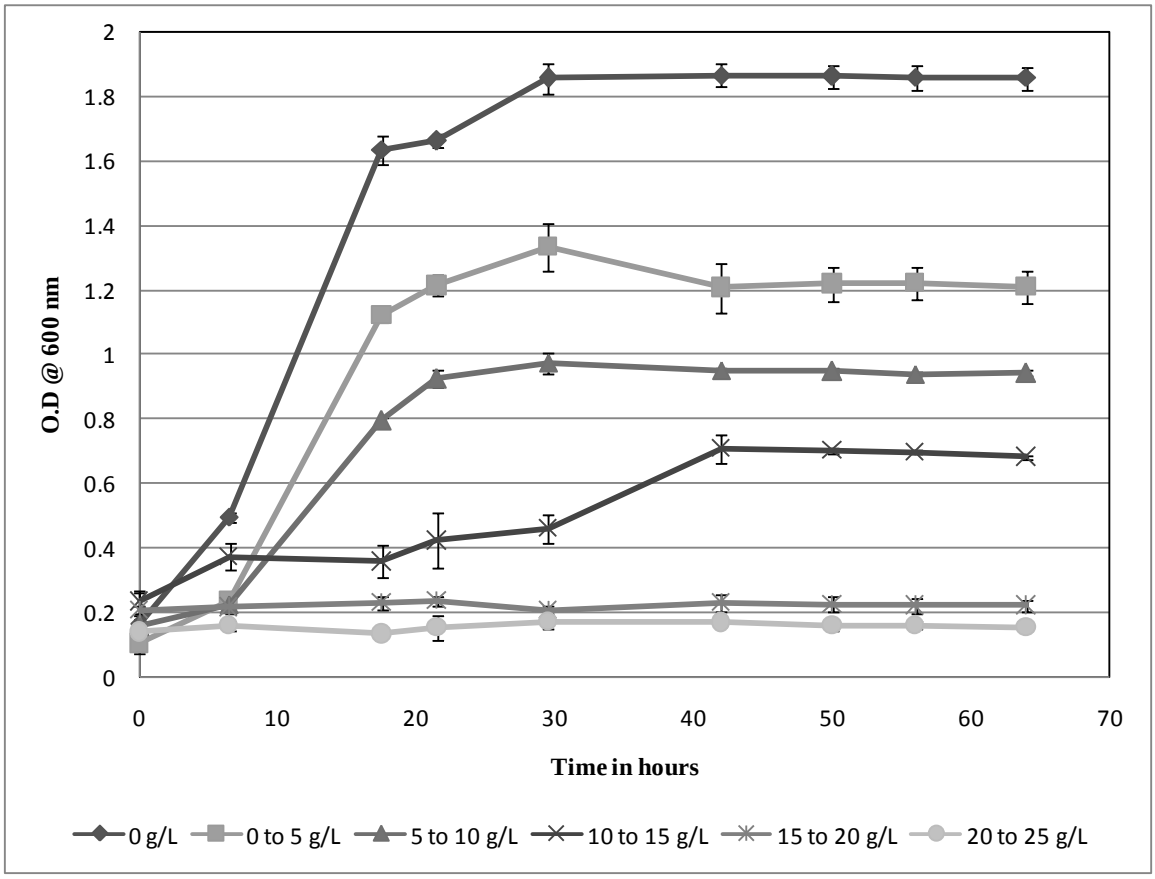


Figure 5.2 Optical densities of cultures grown in various concentrations of butanol using Approach B

Figures 5.3 and 5.4 shows the relative growth of cultures grown in various butanol concentrations. Relative growth was calculated by normalizing the average maximum optical density observed at a specific butanol concentration with respect to the average maximum optical density observed in cultures grown in the absence of butanol. As shown in Figure 5.3, the relative growth of cultures grown in the presence of 5 and 10 g/L butanol was least affected. Increasing the butanol concentration to 15, 20, or 25 g/L decreased the relative growth to about 20%.

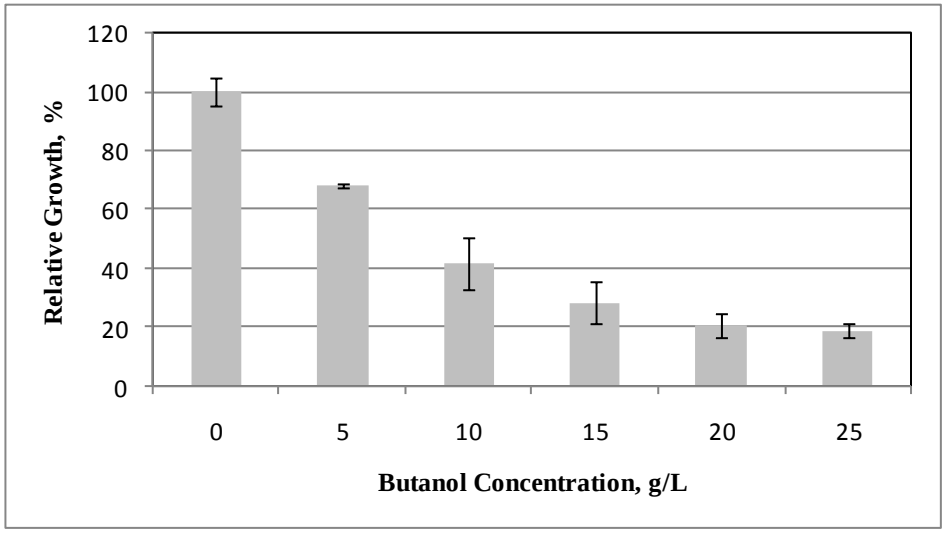


Figure 5.3 Relative growth of cultures grown in various concentrations of butanol using Approach A

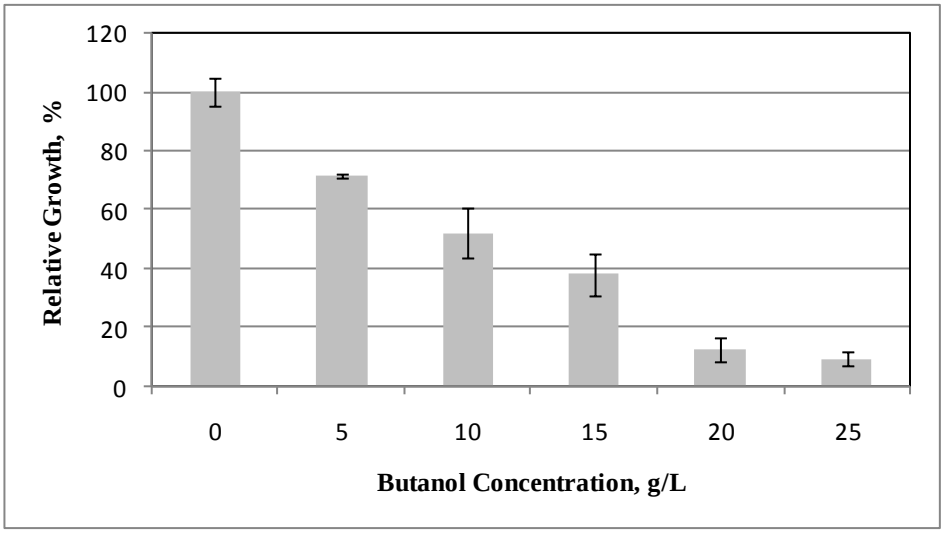


Figure 5.4 Relative growth of cultures grown in various concentrations of butanol using Approach B

Figure 5.4 shows the relative growth for cultures grown using Approach B. With some variability, the relative growth did decrease as cultures were transferred to increasing butanol concentrations. At lower butanol concentrations (5 g/L to 15 g/L), relative growth decreased by about 25%, while at higher concentrations (20 g/L and 25 g/L), relative growth decreased by about 50%. This was likely due to the fact that cultures evaluated using Approach B were exposed to butanol for longer periods of time, acclimatizing to the presence of butanol.

Figure 5.5 shows the normalized maximum specific growth rate for cultures grown in the presence of different butanol concentrations for both Approach A and Approach B. Similar to the relative growth rate, the normalized specific growth rate was determined by comparing the maximum specific growth rate at a specific butanol

concentration to that observed when no butanol was present. As shown in the figure, growing cultures in the presence of 5-10 g/L butanol using Approach A decreased the maximum specific growth rate by only about 10% for an increase in butanol concentration by 5 g/L, while further increases in butanol concentration to 15, 20 and 25 g/L decreased the specific growth rate by 60, 80% and 90%, respectively. Cultures grown using Approach B exhibited a more dramatic decrease in the maximum specific growth rate at higher butanol concentrations. Cultures transferred from 10 to 15 g/L butanol exhibited a decrease in the maximum specific growth rate of almost 60%, while subsequent transfers to 20 and 25 g/L butanol decreased the maximum specific growth rate by 80-90%. The cultures transferred to lower butanol concentrations of 5 g/L and 10 g/L show relatively lower decrease in maximum specific growth rate.

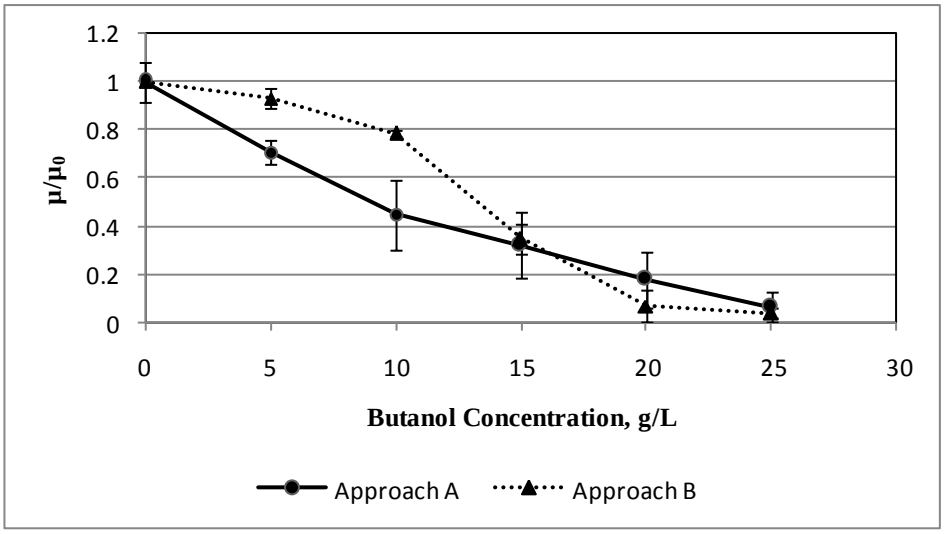


Figure 5.5 Relative specific growth rates of cultures grown in various concentrations of butanol

Although increasing butanol concentration did adversely affect both the relative growth and the maximum specific growth rate of cultures of *C. pasteurianum*, these results are significant in that cultures did exhibit significant growth at butanol concentrations up to 15 g/L. Previous work evaluating various different microorganisms has reported no butanol tolerance above 2% by volume, which is 16.2 g/L butanol ($\rho_{butanol}$ = 0.81 Kg/m^3). Lin and Blaschek (1983) were able to develop a mutant strain of *C. acetobutylicum* ATCC 824 capable of growth in butanol concentrations up to 18.6 g/L butanol. Hermann et al. (1985) observed essentially no growth of both *C. acetobutylicum* wild-type strain 903 and butanol-resistant strain 904 at butanol concentrations of 10 g/L. Liu and Qureshi (2009) reported maximum butanol tolerances of 1.95 and 1.96 vol% (16 g/L) for *C. beijerinckii* strains P260 and BA 101, respectively.

Knoshaug and Zhang (2009) have evaluated the butanol tolerance of a wide variety of organisms including two species of *Candida*, one species of *Pachysolen*, three species of *Lactobacillus*, three species of *Pichia*, two strains of *Zymomonas mobilis*, three strains of *E. coli*, and ten strains of *S. cerevisiae*. Only one species of *Candida* and one strain of *Z. mobilis* exhibited any growth at a butanol concentration of 2 vol%, and the growth rate was less than 20% of cultures grown in the absence of butanol. Three species of *S. cerevisiae* exhibited growth in the presence of 2 vol% butanol, but the growth rate was also decreased by 80% or more. A butanol concentration of 1 vol % (8 g/L) decreased the growth rate of *S. cerevisiae* by 30-40%. No strains of *E. coli* demonstrated growth in the presence of 2 vol% butanol, and 1 vol% decreased the growth rate by as

much as 80%. Two species of *Lactobacillus* were able to grow in 2.5 vol% butanol (20 g/L), and one species grew in 3 vol% butanol (24 g/L). However, since *Lactobacillus* does not have the natural ability to produce solvents, this tolerance could only be exploited if the species were metabolically engineered to produce solvents. Fisher et al. (2008) reported similar results for *S. cerevisiae*, *E. coli*, and *Z. mobilis*, reporting about a 90% reduction in the growth rate in the presence of 1.5 vol% butanol. The results of butanol tolerance for *S. cerevisiae* and *E. coli* are particularly interesting, since these two organisms have been metabolically engineered to produce butanol. The results reported by Knoshaug and Zhang (2009) and Fisher et al. (2008) indicate that these two organisms do not exhibit any better tolerance to butanol than *C. acetobutylicum*. The results from this study indicate that *C. pasteurianum* exhibits similar butanol tolerance as observed in *C. acetobutylicum* and *C. beijerinckii*, and higher butanol tolerance than *E. coli* and *S. cerevisiae*.

In order to further evaluate the effect of butanol on solventogenic cultures of *C. pasteurianum*, the cultures that were grown in glucose media supplemented with butanol were transferred to media containing glycerol as the sole carbon source. The purpose of these experiments was to determine if cultures were still able to produce significant amounts of butanol, ethanol, and PDO even after being exposed to high concentrations of butanol. Solvent production was evaluated for cultures grown in an initial butanol concentration of 15 g/L, as well as for cultures that were grown in 5 g/L butanol and transferred to 10 g/L butanol. Cultures grown in 15 g/L butanol and transferred to 20 g/L butanol, and cultures grown in 20 g/L butanol and transferred to 25 g/L butanol did not grow in glycerol (data not shown), most of the cells died during butanol challenge

experiments. The results were compared to cultures grown in the presence of no butanol. Table 1 shows the product yield results for cultures that were exposed to varying amounts of butanol prior to being transferred to glycerol fermentation experiments.

Table 5.1 Butanol yield from glycerol by cultures previously exposed to various butanol concentrations

Butanol Exposure (g/L)	**Butanol Yield (g/g)**	**Ethanol Yield (g/g)**	**PDO Yield (g/g)**
0	0.34 ± 0.02	0.036 ± 0.003	0.023 ± 0.003
0→5	0.28 ± 0.03	0.029 ± 0.004	0.065 ± 0.01
5→10	0.26 ± 0.002	0.033 ± 0.0006	0.085 ± 0.01
10→15	0.24 ± 0.01	0.037 ± 0.02	0.017 ± 0.01

As shown in the Table 5.1, the butanol yield for cultures that were not exposed to any butanol was 0.345 ± 0.017 g of butanol produced per g of glycerol consumed. Although the butanol yield does decrease for cultures that were exposed to butanol, there is no definitive trend with respect to the concentration of butanol to which cells were exposed. However, the amount of time that cells were exposed to butanol did seem to affect the butanol yield when cultures were transferred to glycerol media. For example, cells that were initially exposed to a butanol concentration of 15 g/L produced statistically the same butanol yield from glycerol as compared to cells that were not

exposed to butanol. Comparatively, cells that were exposed to butanol concentrations that were gradually increased (from 0 to 5, 5 to 10, and 10 to 15 g/L) in subsequent transfers produced a significantly lower butanol yield when transferred to glycerol media.

The butanol yields achieved in this work are comparable to those reported previously for the fermentation of purified and crude glycerol. Ethanol yields are slightly lower than what was reported previously, but still of the same order of magnitude (less than 10 wt%). The PDO yields are notably less in this work than what was observed in previous fermentations (Taconi et al., 2008). However, there is no real trend in PDO yield with respect to the butanol concentration to which cultures were exposed. The decrease in PDO yield is likely attributable to differences in the cultures that were used in this work, rather than any effects due to butanol exposure. Exposure of cells to butanol also did not affect the culture's ability to metabolize butanol, as the average amount of glycerol consumed during fermentation was greater than 90%.

5.4 Conclusions

The results of this work indicate that *C. pasteurianum* exhibits higher butanol tolerance compared to other microorganisms, including other species of *Clostridia* and strains of *E. coli* and *S. cerevisiae*. *C. pasteurianum* was able to grow at butanol concentrations up to 15 g/L (1.875 vol%), which correlates to previous work that reported butanol tolerance only up to 16 g/L (2 vol%). Cultures of *C. pasteurianum* that were exposed to high concentrations of butanol were also able to subsequently produce butanol utilizing glycerol as the sole carbon source at yields comparable to cells that were not

exposed to butanol. These findings are significant, in that the search for a butanol-tolerant microorganism is critical to improving the economic viability of biobutanol production. It is estimated that a butanol concentration of 19 g/L or greater will decrease the cost of separating butanol from the fermentation broth by half. However, few organisms are capable of tolerating or producing this butanol titer.

The fact that *C. pasteurianum* is capable of tolerating up to 15 g/L butanol indicates that this species could be a viable for industrial strain fermentations, following metabolic engineering. Given that this organism already has a high tolerance for butanol, any success via metabolic engineering of *C. pasteurianum* using techniques that have been demonstrated in other species of *Clostridia* could result in an increase in butanol production that substantially improves the economic viability of this fermentation. Even an incremental increase in butanol tolerance and butanol production could break the 19 g/L threshold necessary for reducing separations costs. Utilizing *C. pasteurianum* to produce butanol from crude glycerol that results from biodiesel production presents an opportunity to investigate alternative, low-cost feedstocks for butanol production. Identifying low-cost feedstocks is another critical parameter for improving the economics of butanol fermentation. *C. pasteurianum* is also capable of producing butanol from crude glycerol at yields comparable to purified glycerol, which is another characteristic that makes this species preferable to other species of *Clostridia* for industrial fermentations.

Given the advantages of *C. pasteurianum* with respect to both, butanol tolerance and butanol yield from crude glycerol, this organism should be further investigated from

both applied and fundamental perspectives. Transcriptomic analysis of *C. pasteurianum* may identify genes that impart solvent tolerance and provide insight as to how metabolic engineering may be applied to improve butanol tolerance in this organism as well as other species of *Clostridia*.

CHAPTER 6

6. HOMEOVISCOUS RESPONSE OF *Clostridium pasteurianum* TO BUTANOL

6.1 Introduction

Butanol, as a solvent, is toxic to the cell. The mechanism of butanol toxicity has been discussed earlier in chapters 1 and 5. The toxicity of butanol is tolerated by microorganisms by modifying the composition of the fatty acids in the membrane, thereby altering the fluidity of the membrane and inducing butanol tolerance. The fluidity of lipid membrane is directly proportional to the amount of saturated fatty acids in the tail of the lipid bilayer (Liu and Qureshi 2009). Hence, the bacteria that tolerate more butanol have a much higher ratio of saturated to unsaturated fatty acids in the lipid bilayer (Liu and Qureshi 2009). This has been observed to be an essential biophysical process of tolerating butanol in various butanol producing organisms from the genus *Clostridium* (Lepage et al. 1987; Baer, Bryant, and Blaschek 1989; Vollherbst-Schneck, Sands, and Montenecourt 1984; Baer, Blaschek, and Smith 1987; Isar and Rangaswamy 2012; Baut et al. 1994). Tolerating alcohol toxicity by increasing saturation in the fatty acid tails has also been observed in organisms producing and tolerating other alcohols such as ethanol (Liu and Qureshi 2009; H. J Heipieper, Weber, et al. 1994; Lăzăroaie 2009; Ramos et al. 2002).

Butanol has been shown to affect the membrane by increasing fluidity and hence reducing lipid ordering (Kurniawan et al. 2012; Lepage et al. 1987; Aguilar, Sotomayor, and Lissi 1996; Iiyama et al. 1992). Also, butanol's toxic effects lead to the formation of interdigitated phases and hence phase transition (Cevc and Marsh 1987; Kurniawan et al. 2012). Overall, butanol effects result in compromising the cellular function of the membrane including cell fission, fusion, budding, vesicle formation and cell signaling (Cevc and Marsh 1987; Löbbecke and Cevc 1995). The transport of substrates and products that are transported by either passive or active transport is also affected, along with the structure and function of integral membrane proteins. Membrane bound ATPases are one such examples, which maintain a transmembrane pH for ATP generation. Butanol inhibits the ATPases and reduces the transmembrane pH resulting in lower ATP formation (Papoutsakis 2008).

The objective of this chapter was to study the effect of butanol on the lipid membrane's structure and function using reconstituted membranes from the cells of *C. pasteurianum* grown under different conditions. The effect of butanol on cells during exogenous addition of butanol, endogenous production of butanol and the combinatorial effect of exogenous as well as endogenous butanol was studied using reconstituted membranes. This chapter discusses the studies on the stability and the change in composition of the cell membranes during the presence of butanol in the environment of *C. pasteurianum*.

6.2 Materials and Methods

6.2.1 Effect of Butanol

The effect of butanol on growth of the bacteria and the stability and change in composition of the membrane were studied by adding butanol to the media containing either glucose or glycerol as the sole carbon source. Glucose (50 g/L) in Biebl media (Section 4.2) and RCM (Table 3.1) was used to analyze the effect of exogenous butanol, as *C. pasteurianum* does not produce butanol when growing on glucose. The effect of endogenously produced butanol was studied by varying the glycerol concentration from 10g/L to 50 g/L in Biebl media. The concentration of butanol added to the media varied from 0 to 2% (w/v) (0 g/L to 20 g/L). The cells were allowed to grow in the presence of butanol for 24 hours after which the membrane was extracted. All experiments were conducted with 10% inoculum, pre-grown in RCM.

6.2.2 Extraction of the Cell Membrane

The cell membrane was extracted using the modified protocol of Bligh and Dyer using dichloromethane/methanol mixtures. The cells were harvested (0.5 mL cell suspension) by centrifugation at high speed (13000 rpm) for 15 minutes and the supernatant discarded. The pellets were resuspended in 0.5 mL of sterile 1.0% (w/v) NaCl in a 10 mL glass sample tube with PTFE lined caps. To the resuspended pellets, 2 mL of dichloromethane/methanol mixture (1:2 v/v) was added and shaken vigorously for 15 minutes. It was followed by a 2 hour incubation at RT and later centrifuged at 2500 rpm and the supernatant (S1) was collected in a fresh tube. The pellet was again resuspended in 0.5 mL 1.0% NaCl and 2 mL of a dichloromethane/methanol mixture (2:1

v/v) was added to the resuspended pellet and shaken vigorously for 15 minutes. Following a 2 hour incubation, the samples were centrifuged at 2500 rpm and the supernatant (S2) was collected. Supernatants S1 and S2 were combined and 1 mL of dichloromethane and 1 mL of sterile 1.0% NaCl were added. The top phase (aqueous) was removed and the bottom (organic) phase was retained. The solvent was evaporated under a gentle nitrogen stream. Once a dry film was obtained, headspace was flushed with nitrogen, capped tightly, and stored for further analysis.

6.2.3 NMR Analysis

The dry film of the membrane was dissolved in deuterated chloroform for ^{1}H-NMR analysis. Synthetic lipids such as DPPC (Dipalmitoylphosphotidylcholine) and DOPC (Dioleylphosphotidylcholine) were used as standards for NMR analysis. Various ratios of DPPC and DOPC (1:0, 3:1, 1:1, 1:3 and 0:1) in a total lipid concentration of 10 mM were used for calibration. The synthetic lipids were dried and dissolved in deuterated chloroform ($CDCl_3$). All ^{1}H-NMR spectra were recorded on a Varian Unity Inova 500 (500 MHz) spectrometer equipped with a 5 mm triple resonance inverse detectable probe.

6.2.4 Unilamellar Liposome Preparation and Fluorescence Anisotropy

The (dry film) reconstituted cell membrane and 0.1 mM DPH (1,6-diphenyl 1,3,5-hexatriene) dissolved in chloroform were mixed and co-evaporated under a gentle stream of nitrogen until a dry reconstituted cell membrane/DPH film remained. Vacuum was

used (~ 60 min) to remove residual solvent from the film. The film was then hydrated with distilled water and maintained at 50°C in a water bath for 1 hour before shaking. The reconstituted membrane/DPH film was suspended as multilamellar liposomes by vigorously shaking for approximately 1 hour. The liposomes were sonicated before use for 60 min at 50°C, which has been previously shown to yield unilamellar liposomes. 10 mM of DPPC liposomes generated by the same procedure were used as control.

Fluorescence anisotropy (Perkin Elmer LS 55, Waltham, MA) and melting temperature were measured using the L-format configuration (Figure 6.1) using the hydrophobic bilayer probe diphenylhexatriene (DPH) from 25 to 50°C at a rate of 1°C/min under continuous mixing in Dr. Bothun's laboratory in the University of Rhode Island, Kingston, RI. Steady-state DPH anisotropy within the reconstituted membrane bilayer or DPPC was determined at λ_{ex} = 350 nm and λ_{em}= 452 nm using the expression

$$r = \frac{(I_{VV} - gI_{VH})}{(I_{VV} + 2gI_{VH})} \qquad \textbf{(Eq 6.1)}$$

where *I* represents the fluorescence emission intensity, *V* and *H* represent the vertical and horizontal orientation of the excitation and emission polarizers, and g-factor is dependent on the emission wavelength and it accounts for the sensitivity of the instrument towards vertically and horizontally polarized light.

$$g = \frac{I_{HV}}{I_{HH}} \qquad \textbf{(Eq 6.2)}$$

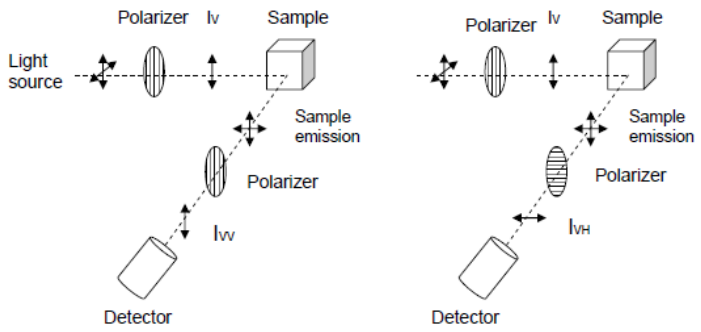


Figure 6.1 Schematic diagram for L-format measurement of fluorescence anisotropy (Dr. Bothun's laboratory, URI)

6.2.5 GC/MS Analysis

The GC/MS analysis was carried out in Dr. Bothun's laboratory at the University of Rhode Island, Kingston, RI. The lipid samples were methylated prior to analysis by GC/MS. The dried lipid samples were first saponified using 1 mL of 3N sodium hydroxide and the samples were heated to 90ºC for an hour and then cooled to RT. The excess sodium hydroxide was neutralized with 1.8 mL of 3.6 N (30 %) hydrochloric acid at 90 ºC for 10 minutes and cooled to RT. The free fatty acids were extracted using 1 mL of hexane and diethyl ether (1:1 v/v). The organic phase was then separated into a clean and dry round bottom flask. The fatty acid hydrolyzates were then dried using a rotoevaporator and stored under Argon in a desiccator.

The dried fatty acid hydrolyzates were then derivatized for GC-MS analysis using 5 mL borontrifluoride-methanol complex at 60 ºC for 5 minutes and then cooled to RT. To the cooled solution, 1 mL water and 1 mL hexane were added and the container shaken multiple times to ensure the transfer of esters into the non-polar solvent. The

upper organic layer (hexane) was removed and transferred into an Erlenmeyer flask that contained 5 g of anhydrous sodium sulfate at the bottom. The flask was covered with parafilm and incubated at RT to dry overnight. The sodium sulfate was filtered and the hexane solution was transferred into a clean round bottom flask and dried in a rotoevaporator. The samples were stored under Argon until the samples were analyzed by GC-MS. The samples were prepared for GC-MS by dissolving them in 250 µL of dichloromethane.

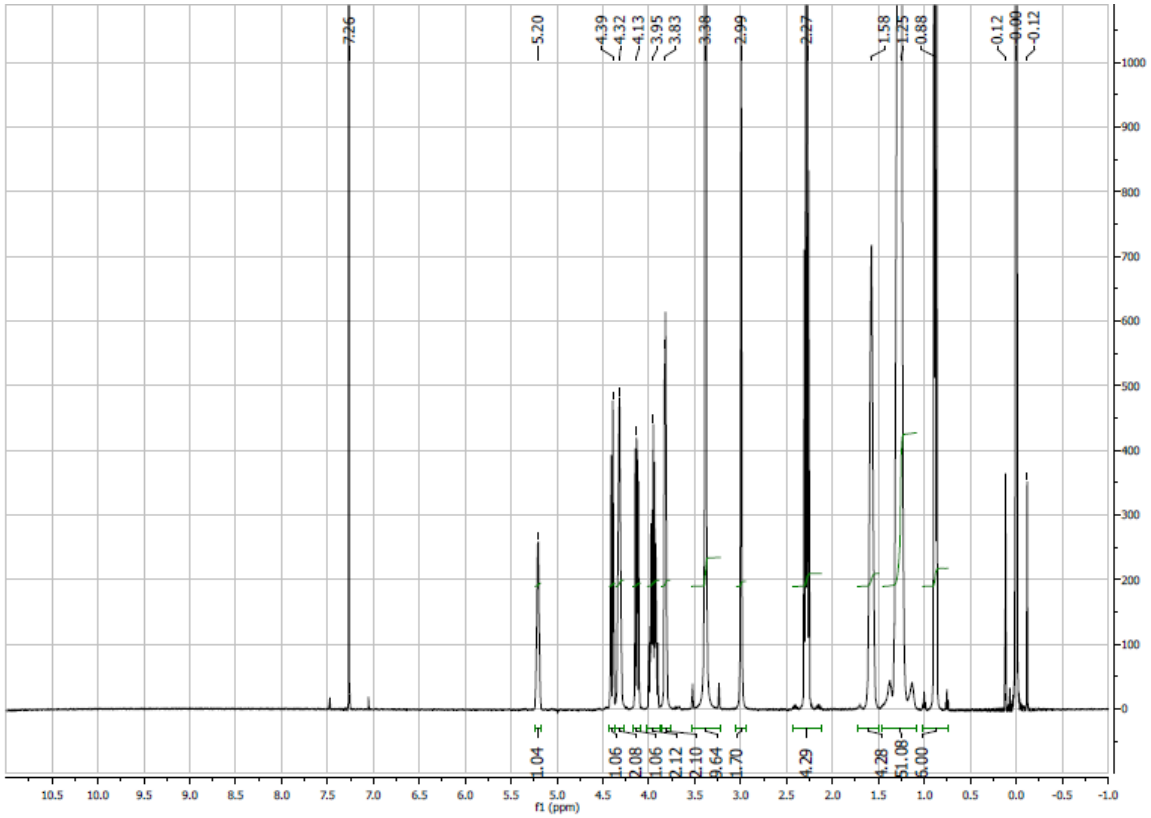
6.3 Results and Discussion

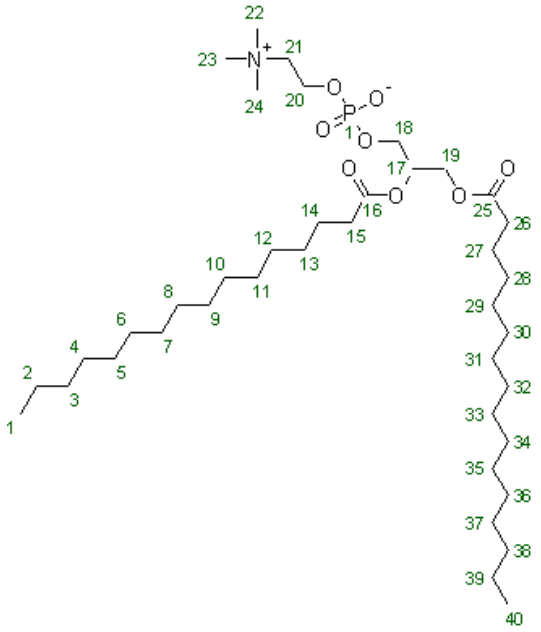
6.3.1 Measurement of Percentage unsaturation using NMR

The unsaturation in the fatty acid tail of the lipids can be analyzed using ^{1}H-NMR. The peaks corresponding to the protons of a double bond were detected at δ=5.35 ppm. A calibration curve for determining the percentage of unsaturation was generated using the integrated ratios of 10 mM of lipids samples varying in the degree of unsaturation. DPPC and DOPC were mixed appropriately to obtain lipid mixtures that will result in the presence of 0, 25, 50, 75 and 100 % of unsaturation. The proton NMR of the samples is shown in Figures 6.2 to 6.6 and the calibration plot for peak area and its corresponding degree of unsaturation is shown in Figure 6.7.

As shown in the Figures (6.2 to 6.6), the peak for the unsaturation (-C*H*=C*H*-) corresponds to the peak observed at 5.35 ppm (Willker and Leibfritz 1998; Sparling et al. 1989). The ^{1}H-NMR of 100 % DPPC, does not contain this peak, as DPPC does not

contain any double bond in its structure (Figure 6.2). The area of the peak progressively increased with an increase in the ratio of DOPC in the samples with respect to DPPC (Figure 6.3 to 6.6). The area of the peak was calculated by normalizing the area of the terminal methyl group (-CH_3) observed at 0.88 ppm to 6 (due to the presence of two fatty acid tails per lipid molecule and hence, two methyl groups with 6 protons). All other peaks between 1.0 ppm and 4.5 ppm correspond to the protons on the backbone carbon (Willker and Leibfritz 1998; Sparling et al. 1989). The peak at 5.20 ppm corresponds to hydrogen on the center carbon of the glycerol molecule and integrates to 1 for the single proton on the carbon (Figure 6.2 to Figure 6.6). A calibration plot (Figure 6.7), based on the area of the proton corresponding to the double bond, resulted in a slope of 24.38 i.e., an integrated peak area of 1 will correspond to 24.38 % of unsaturation in the lipid samples.





Assignment	Shift (ppm)
17	5.20
19	4.39, 4.13
20	4.32
18	3.95
21	3.83
22, 23, 24	3.39
15,26	2.27
14, 27	1.58
2-13,27-39	1.25
1, 40	0.88

Figure 6.2 ^{1}H-NMR of 100 % DPPC (0% unsaturation), DPPC structure and peak assignment.

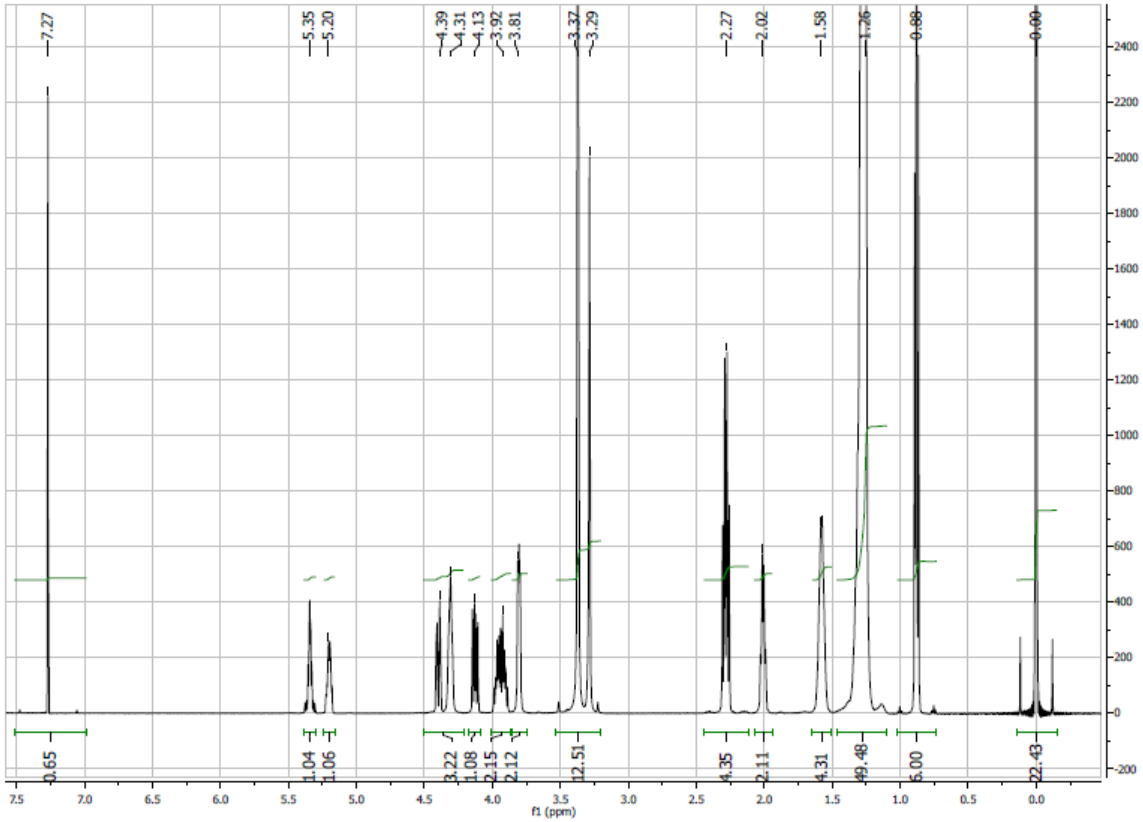


Figure 6.3 ^{1}H-NMR of 3:1 DPPC:DOPC (25% unsaturation).

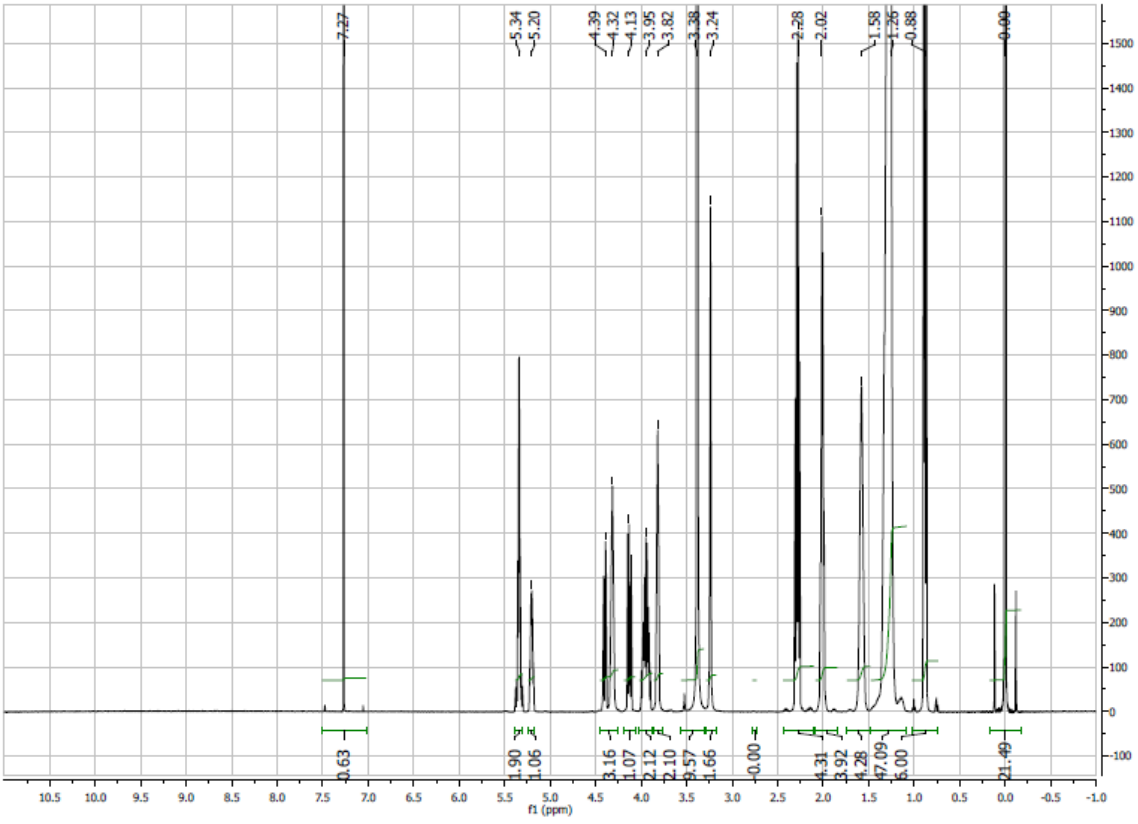


Figure 6.4 ^{1}H-NMR of 1:1 DPPC:DOPC (50% unsaturation).

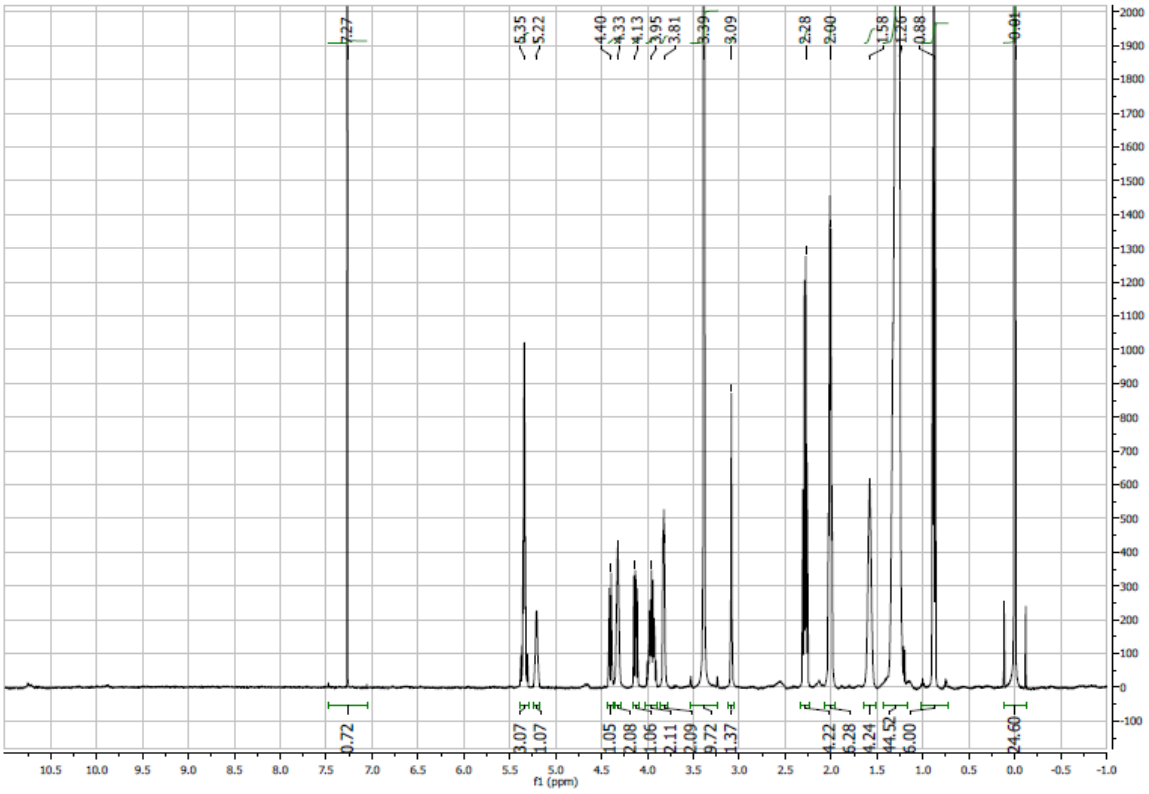
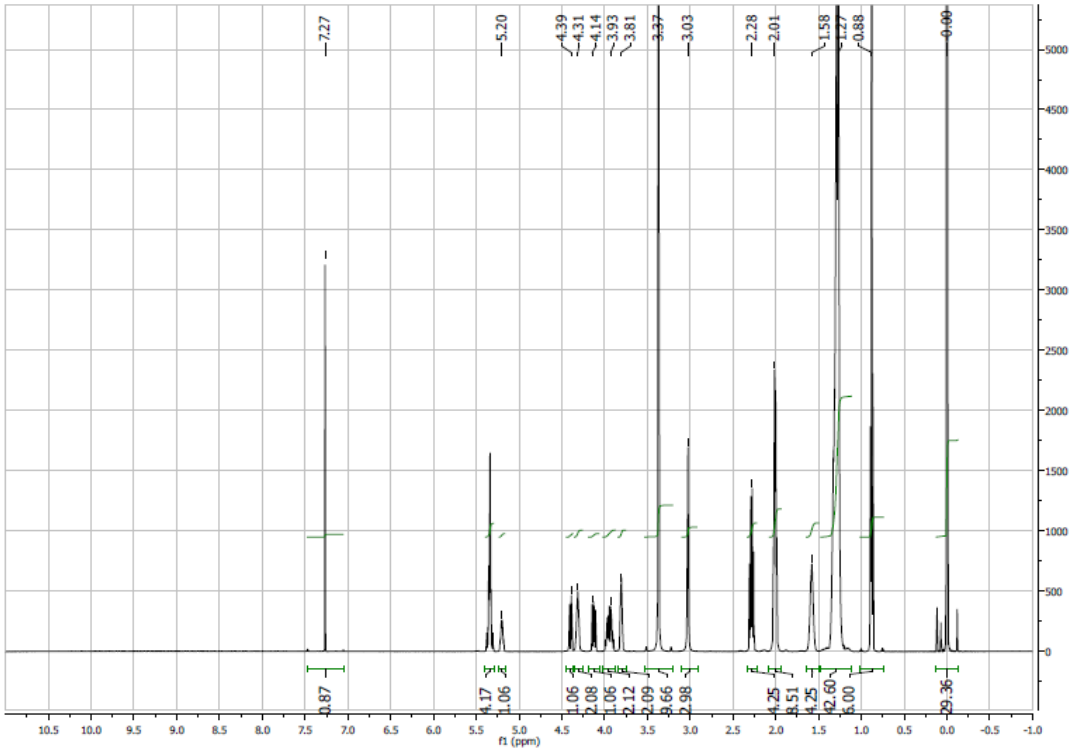
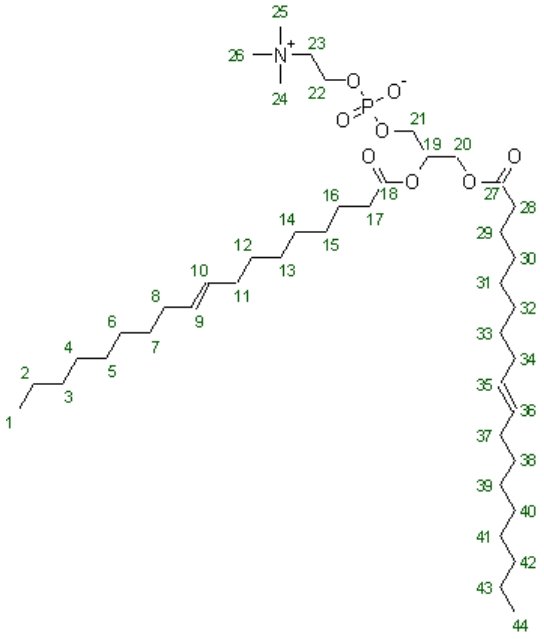


Figure 6.5 ^{1}H-NMR of 1:3 DPPC:DOPC (75% unsaturation).





Assignment	Shift (ppm)
9, 10, 35, 36	5.35
19	5.20
20	4.39, 4.14
22	4.32
21	3.93
23	3.81
24, 25, 26	3.39
17, 28	2.27
16,29	1.58
2-8, 11-15, 37-43, 30-34	1.25
1, 44	0.88

Figure 6.6 ^{1}H-NMR of 100 % DOPC (100% unsaturation), DOPC structure and peak assignment.

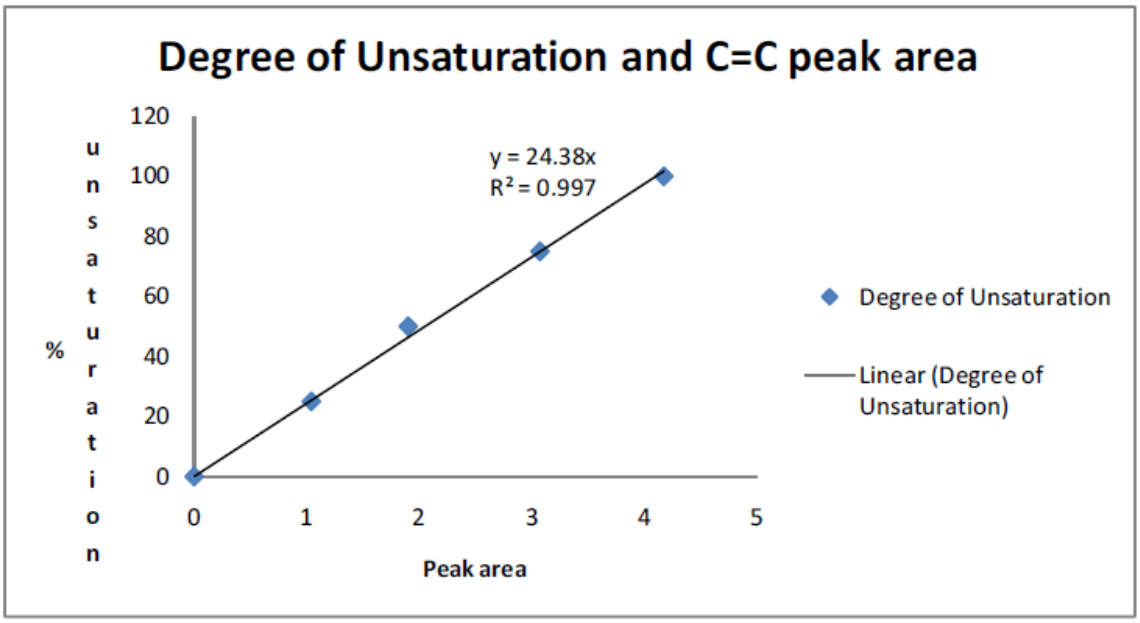


Figure 6.7 Calibration plot for percentage unsaturation and peak area from the proton NMR of DPPC/DOPC standards.

6.3.2 Effect of Endogenous Butanol

The effect of endogenously produced butanol on the bacterial lipid membrane was studied by growing the bacteria in conditions that resulted in the formation of butanol at different concentrations from 0 g/L to 10 g/L (0.134 M). *C. pasteurianum* does not synthesize butanol when grown on glucose as the sole carbon source (Chapter 3). Furthermore, growing in dual carbon source of glycerol and glucose resulted in the formation of higher butanol titers (Chapter 3, Table 3.3). Hence, for the studies on the homeoviscous response during endogenous butanol formation, cells were grown in glucose, glycerol and glycerol-glucose (co-fermentation), resulting in butanol formation between 0 g/L and 10 g/L. The membranes were extracted from the stationary phase cultures and the extracted membranes were analyzed using ^{1}H-NMR and DPH fluorescent anisotropy. The growth curve of the cultures used for studying the homeoviscous response during endogenous butanol formation is shown in Figure 3.1. Figure 6.8 summarizes the results from ^{1}H-NMR and fluorescent anisotropy.

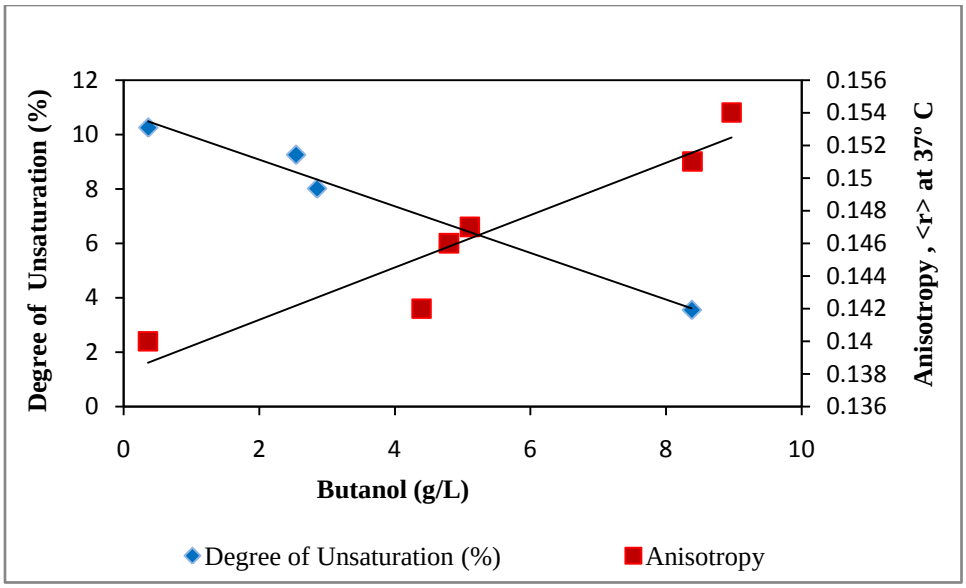


Figure 6.8 Effect of endogenously butanol on the lipid membranes of *C. pasteurianum*

The results in Figure 6.8 show that the lipid membranes of *C. pasteurianum* have a higher anisotropy with higher amounts of butanol being synthesized by the cells. Furthermore, the unsaturation in the fatty acid tails decreases with the corresponding increase in the titers of butanol produced. An increase in the anisotropy indicates a decrease in fluidity and hence, an increase in the rigidity of the membrane. The data on the degree of unsaturation obtained from ^{1}H-NMR, supports the trend observed in the data from the fluorescent anisotropy (Figure 6.8). The ^{1}H-NMR results show a decrease in the degree of unsaturation in the fatty acid tails with an increase in the amount of butanol produced, endogenously. A decrease in unsaturation or an increase in saturation results in lowering the fluidity of the membrane and hence, results in the formation of a more rigid membrane. Altering the membrane composition has been reported to be a universal approach by microorganism towards tolerating the toxic effects of alcohols.

This approach usually involves increasing the ratio of the saturated to unsaturated fatty acids in the lipid membrane, as observed in *C. pasteurianum* during endogenous butanol production.

Homeoviscous response involves membrane alteration that mainly aims at the maintenance of the fluidity through modification of the membrane viscosity (Lepage et al. 1987; Aricha et al. 2004; Baer, Blaschek, and Smith 1987; Vollherbst-Schneck, Sands, and Montenecourt 1984; Dombek and Ingram 1984; Löbbecke and Cevc 1995; Baer, Bryant, and Blaschek 1989; Sinensky 1974; Baut et al. 1994; Lăzăroaie 2009; Hermann J. Heipieper, Weber, et al. 1994; Ramos et al. 2002; Segura et al. 1999). The membrane viscosity can be modified by altering the ratio of saturated to unsaturated fatty acids in the lipid membrane, changes involving cis/trans fatty acid isomerization, changes in the phospholipid head groups and changes in the non-lipid components of the membrane (Baut et al. 1994; Segura et al. 1999; Lăzăroaie 2009; Papoutsakis 2008b; Liu and Qureshi 2009).

Baut *et al.* showed that the homeoviscous response of *C. acetobutylicum* during endogenous butanol production resulted in rigidifying the lipid membrane (Baut et al. 1994). It was also shown that the fluorescence anisotropy of the extracted membrane, measured using DPH, increased in response to the amount of butanol produced by *C. acetobutylicum* (Baut et al. 1994).

The results obtained in this study, during endogenous butanol production by *C. pasteurianum*, are similar to the homeoviscous response reported for *C. acetobutylicum* (Baut et al. 1994). During the production of butanol, the bacteria modify the membrane

to be more rigid and the rigidity in the membrane increases with an increase in the concentration of butanol produced (Figure 6.8). This homeoviscous response is carried out by reducing the percentage of unsaturation in the lipid membrane or in other words, increasing the ratio of saturated to unsaturated fatty acids. Though, the result seem to be incongruous with the fluidizing results reported on the effect of butanol on model membrane (Löbbecke and Cevc 1995; Kurniawan et al. 2012), these results can be explained as the homeoviscous response of *C. pasteurianum* leading to acclimatization to the toxic effects of butanol. The results and their discussion are further summarized in Figure 6.9.

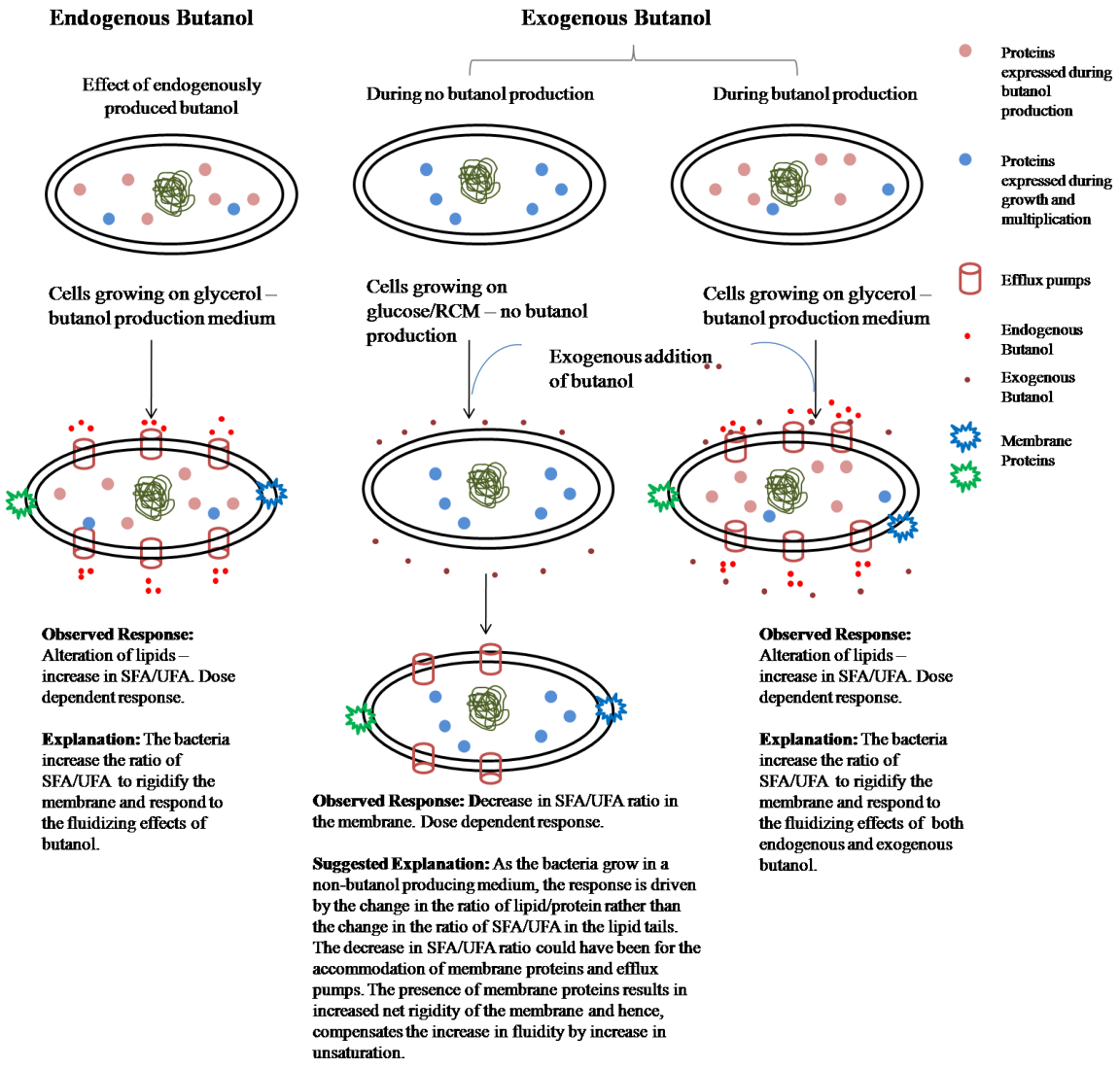


Figure 6.9 Homeoviscous response of *C. pasteurianum* to endogenous, exogenous and endogenous & exogenous butanol.

6.3.3 Effect of Exogenous and Endogenous Butanol

The homeoviscous response of *C. pasteurianum* to the addition of exogenous butanol was studied under two conditions. The first approach involved the addition of external butanol during the production of butanol by *C. pasteurianum* growing on glycerol (exogenous + endogenous). The second approach involved the addition of exogenous butanol during no butanol formation. The second approach was carried out by growing *C. pasteurianum* in RCM or Biebl medium with glucose, as *C. pasteurianum* do not synthesize butanol in these media. This section summarizes the results from the first approach, while the results of the second approach are discussed in the following section.

The effect of exogenous addition of butanol, on the lipid membrane, during endogenous production of butanol was studied by growing the cultures on Biebl medium with 25 g/L of glycerol. Butanol was added at concentrations of 0 g/L, 2.5 g/L (0.0335 M), 5 g/L (0.067 M), 7.5 g/L (0.105 M) and 10 g/L (0.134 M), respectively, once the cells reached mid exponential phase (Figure 6.10). The membranes were extracted after 24 hours of butanol exposure and analyzed using ^{1}H-NMR, fluorescent anisotropy and GC-MS (Figure 6.10). The ^{1}H-NMR and fluorescent anisotropy results are summarized in Figure 6.11, while Table 6.1 summarizes the data from the GC-MS analysis on the fatty acid composition of the membranes.

The homeoviscous response during the presence of exogenous and endogenous butanol on *C. pasteurianum* cultures growing on 25 g/L glycerol resulted in a decrease in the degree of unsaturation in the membrane as determined by ^{1}H-NMR (Figure 6.11). This decrease in unsaturation indicates an increase in the ratio of saturated to unsaturated

fatty acids (SFA/UFA) in the lipid tails. On the other hand, though the unsaturation in the lipids decreased, the anisotropy data show a decrease in anisotropy and hence, an increase in fluidity with increasing concentrations of butanol added. This contrasting data from anisotropy and ^{1}H-NMR, suggests that *C. pasteurianum* is reacting to the fluidizing effect of butanol and altering its membrane composition, but the effect of exogenous butanol is overwhelming, as the fluidity of the membrane is increased with respect to the concentration of exogenous butanol.

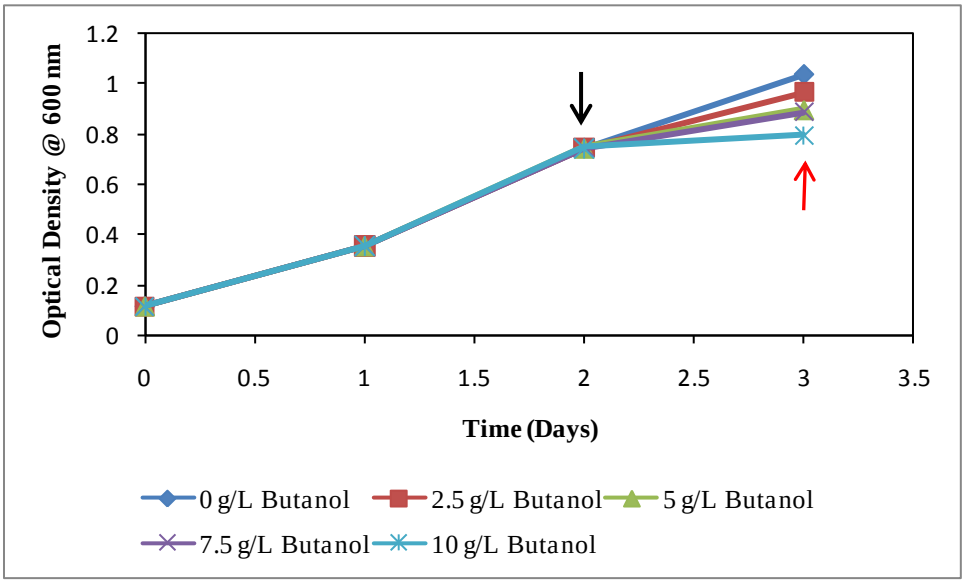


Figure 6.10 Effect of exogenous butanol on the lipid membranes of *C. pasteurianum* endogenously producing butanol on 25 g/L glycerol. The black arrow indicates the addition of butanol and the red arrows indicates the extraction of lipids.

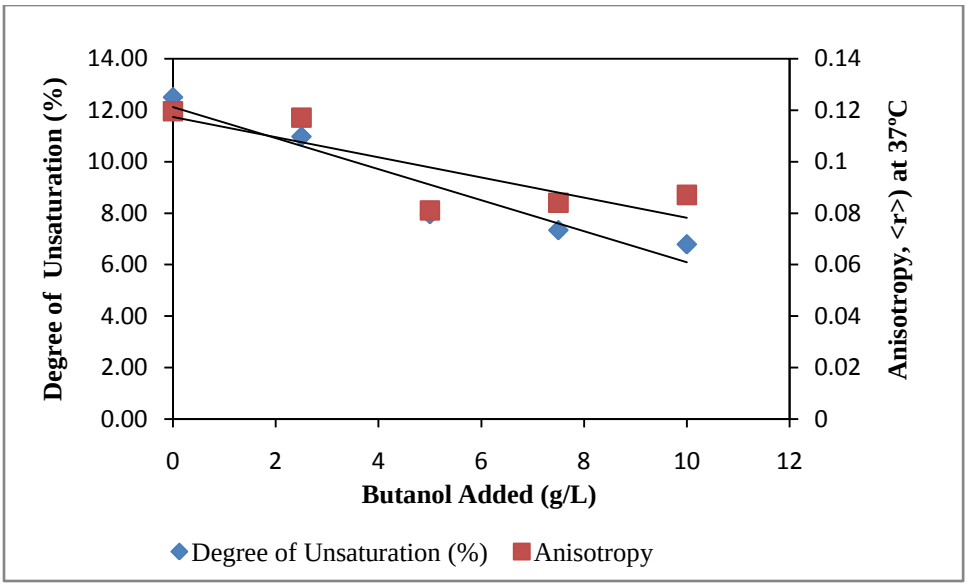


Figure 6.11 Effect of exogenous butanol on the lipid membranes of *C. pasteurianum* endogenously producing butanol on 25 g/L glycerol. The Endogenous butanol concentration, at the time of addition of exogenous butanol was, 1.83 g/L.

Table 6.1 Effect of Exogenous and Endogenous Butanol: Fatty acid composition in %. (Cells grown on glycerol in Biebl medium with additional butanol added)

Fatty Acids	Carbons and Double Bonds	Butanol Concentration added (g/L)				
		0	2.5	5	7.5	10
decanoic	**C10:0**	11.25	12.59	10.78	0.00	0.00
hendecanoic	**C11:0**	9.43	8.61	5.04	2.54	0.39
dodecanoic	**C12:0**	6.88	5.60	3.03	2.78	0.69
tridecanoic	**C13:0**	8.60	7.89	5.88	5.74	1.58
tetradecanoic	**C14:0**	9.00	6.73	1.22	1.59	1.53
pentadecanoic	**C15:0**	14.83	12.81	10.04	9.63	3.83
cis-9-hexadecenoic	**C16:1**	11.86	9.92	4.10	2.00	1.39
hexadecanoic	**C16:0**	27.33	26.61	24.34	19.29	12.03
cis-11-octadecenoic	**C18:1**	0.32	2.04	6.81	7.84	7.59
octadecanoic	**C18:0**	0.49	4.31	14.07	20.69	24.38
nonadecanoic	**C19:0**	0.00	2.88	12.41	16.57	19.58
eicosanoic	**C20:0**	0.00	0.00	2.28	8.92	15.85
heneicosanoic	**C21:0**	0.00	0.00	0.00	2.43	8.66
docosanoic	**C22:0**	0.00	0.00	0.00	0.00	2.50
TOTAL UNSATURATION		**12.18**	**11.96**	**10.91**	**9.84**	**8.98**
TOTAL SATURATION		**87.82**	**88.04**	**89.04**	**90.16**	**91.02**

The data from GC-MS on the fatty acid composition (Table 6.2) of the membranes support the data from ^{1}H-NMR (Figure 6.11). According to the results from GC-MS, not only there is a decrease in the unsaturated fatty acid (C16:1), but a there is also an increase in other higher carbon unsaturated fatty acids (C18:1). Furthermore, there is an increase in the percentage of higher carbon (C19 to C22) saturated fatty acids particularly 18 carbon or higher, which are almost absent in the control samples with no external butanol present. The appearance of longer chain fatty acids in the membrane, with an increase in the concentration of exogenous butanol, signifies a dose dependent response to the presence of exogenous butanol (Table 6.1). A similar trend is also observed in the disappearance or reduction in the shorter chain fatty acids with an increase in the dose of butanol stress (Table 6.1). Moreover, there is also a reduction in the percentage of the lower carbon fatty acids with corresponding increase in exogenous butanol concentrations (Table 6.1). These data clearly suggest that *C. pasteurianum* is modifying its membrane composition (Table 6.1) as determined by ^{1}H-NMR (Figure 6.11), during its homeoviscous response to the exogenous butanol, when the bacteria was grown in a butanol producing media containing glycerol.

The effect of butanol on the lipid composition and the fluidity of the lipid membrane has been reported for *C. acetobutylicum* (Baer, Blaschek, and Smith 1987; Baer, Bryant, and Blaschek 1989; Lepage et al. 1987; Vollherbst-Schneck, Sands, and Montenecourt 1984). *C. acetobutylicum* produces butanol by fermenting glucose and the

effect of butanol challenge was studied during the growth of *C. acetobutylicum* in glucose (butanol producing media) (Baer, Blaschek, and Smith 1987; Baer, Bryant, and Blaschek 1989; Lepage et al. 1987; Vollherbst-Schneck, Sands, and Montenecourt 1984). It has been reported that *C. acetobutylicum* tolerates butanol through a homeoviscous response that predominantly involves the increase in percent saturated fatty acids at the expense of unsaturated fatty acids in the lipid membrane (Baer, Blaschek, and Smith 1987; Baer, Bryant, and Blaschek 1989; Lepage et al. 1987; Vollherbst-Schneck, Sands, and Montenecourt 1984). This increase in the SFA/UFA ratio in the membrane is in response to the fluidizing effects of butanol.

Similar results were also observed in this study, suggesting that *C. pasteurianum* also responds to the presence of exogenous butanol by increasing the SFA/UFA ratio in the membrane. Table 6.1 shows that the percent saturation gradually increases with increase in the exogenous butanol concentration, while the percent unsaturation in lipid membranes decreases. This response of *C. pasteurianum* is not only similar to the homeoviscous response observed during endogenous butanol production, but, also similar to *C. acetobutylicum*'s response to endogenous (Baut et al. 1994) and exogenous butanol (Baer, Blaschek, and Smith 1987; Baer, Bryant, and Blaschek 1989; Lepage et al. 1987; Vollherbst-Schneck, Sands, and Montenecourt 1984).

6.3.4 Effect of Exogenous Butanol

This section summarizes the homeoviscous response of *C. pasteurianum* to exogenous butanol added during non-butanol production. As explained earlier, the effect of externally added butanol on *C. pasteurianum* was studied by growing the bacteria in a

medium that does not support butanol production. The effect of exogenous butanol was studied by adding butanol to the cells during mid exponential phase of the growth curve and the membranes were extracted 24 hours after butanol addition. The concentration of butanol added during this experiment ranged from 5 g/L (0.067 M) to 20 g/L (0.027 M), with an increment of 5 g/L. The membranes were studied using ^{1}H-NMR and fluorescent anisotropy. Figure 6.12 and 6.13 show the growth curve of *C. pasteurianum* during exogenous butanol stress and the results are summarized in Figure 6.14 and 6.15.

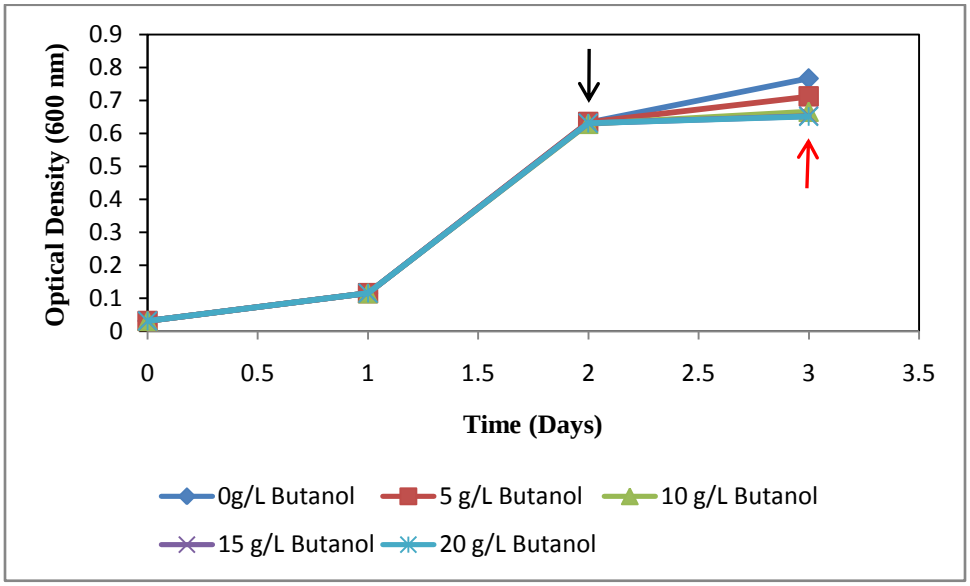


Figure 6.12 Growth curve of *C. pasteurianum* in RCM (exogenous butanol added as indicated by the black arrow, and red arrow indicates the point of membrane extraction)

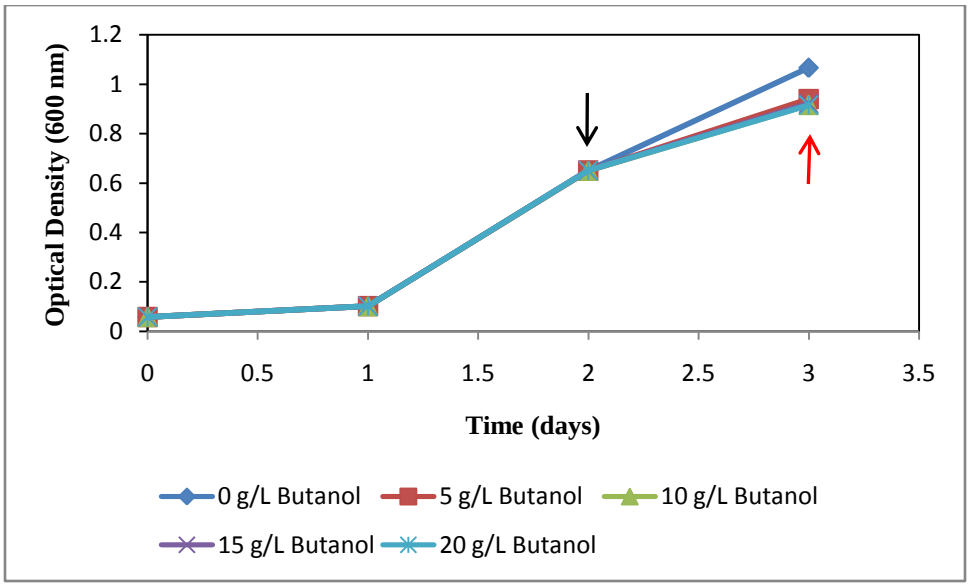


Figure 6.13 Growth curve of *C. pasteurianum* in 50 g/L glucose (exogenous butanol added as indicated by the black arrow, and red arrow indicates the point of membrane extraction)

As shown in the Figure 6.12 and Figure 6.13, the bacteria were allowed to grow until the mid-logarithmic phase of growth and the butanol was added in concentrations from5 g/L to 20 g/L at an optical density between 0.6 and 0.7 at 600 nm and the membranes were extracted 24 hours after the addition of butanol.

Figure 6.14 summarizes the change in the fluidity of the membrane in terms of anisotropy and the degree of unsaturation, as measured using ^{1}H-NMR, in the membranes of *C. pasteurianum* grown on RCM and studied for the effect of exogenous butanol. The results from ^{1}H-NMR reveal an increase in the percentage of unsaturation in the fatty acid tails of the lipid membranes extracted from the cells stressed with exogenous butanol. The unsaturation in the lipid membranes increased proportionately with increase in the concentration of butanol in the media. For an exogenous butanol concentration of 5 g/L,

a small drop in the percentage of unsaturation in the fatty acid tail was observed, in comparison to the control with no external butanol.

The data obtained from fluorescent anisotropy of the reconstituted membrane, using DPH as the probe, supports the data obtained from ^{1}H-NMR (Figure 6.14). The anisotropy, <r>, measured at 37°C decreased gradually with an increase in the concentration of n-butanol in the media. Anisotropy is inversely proportional to the fluidity of the membrane. Hence, a drop in the anisotropy of the membrane with a corresponding increase in the exogenous butanol concentration in the media indicates an increase in fluidity of membranes. Correspondingly, fluidity of the membrane is also directly proportional to the percentage of unsaturation of the lipid membrane's fatty acid tails. Therefore, an increase in unsaturation also indicates an increase in fluidity, indicating that the data from anisotropy and ^{1}H-NMR complement each other. The ^{1}H NMR spectra are shown in the appendix (Figure B.1 to B.5).

The lipid membranes extracted from the cells were also analyzed by GC-MS to determine the constituent fatty acids. Table 6.2 summarizes the results from GC- MS analysis of the cells stressed with exogenous butanol. Thirteen different fatty acids were identified using GC-MS, of which 11 were saturated. The data on the composition of the fatty acids from Table 6.2 show not only an increase in unsaturation in the fatty acids, but also an increase in the percentage of fatty acid length ($\geq$ C16) with an increase in the concentration of exogenous butanol.

Table 6.2 Effect of exogenous butanol during growth on glucose (RCM): Fatty acid composition of membranes (data in %)

Fatty Acids	Carbons and Double Bonds	Exogenous Butanol Concentration				
		0 g/L	5 g/L	10 g/L	15 g/L	20 g/L
Hexanoic	**C6:0**	4.84	4.32	8.18	7.23	2.43
Heptanoic	**C7:0**	8.95	8.05	5.17	6.08	11.18
Decanoic	**C10:0**	11.84	9.50	9.37	11.90	8.36
Dodecanoic	**C12:0**	10.49	10.43	6.45	8.47	2.37
Tridecanoic	**C13:0**	6.56	8.44	6.36	4.85	5.87
Tetradecanoic	**C14:0**	13.61	10.26	5.26	6.35	2.32
Pentadecanoic	**C15:0**	6.28	6.61	12.48	8.38	5.25
7-Hexadecanoic	**C16:1**	4.29	5.13	4.57	7.94	6.78
Hexadecanoic	**C16:0**	7.37	8.82	7.54	8.20	11.01
9-octadecanoic	**C18:1**	4.07	4.15	5.81	4.85	6.78
Octadecenoic	**C18:0**	8.59	8.69	10.93	8.38	10.90
Nonadecanoic	**C19:0**	4.20	4.87	8.37	11.38	14.46
Eicosanoic	**C20:0**	8.91	10.72	9.51	6.00	12.31
TOTAL UNSATURATION		**8.36**	**9.28**	**10.38**	**12.79**	**13.56**
TOTAL SATURATION		**91.64**	**90.72**	**89.62**	**87.21**	**86.44**

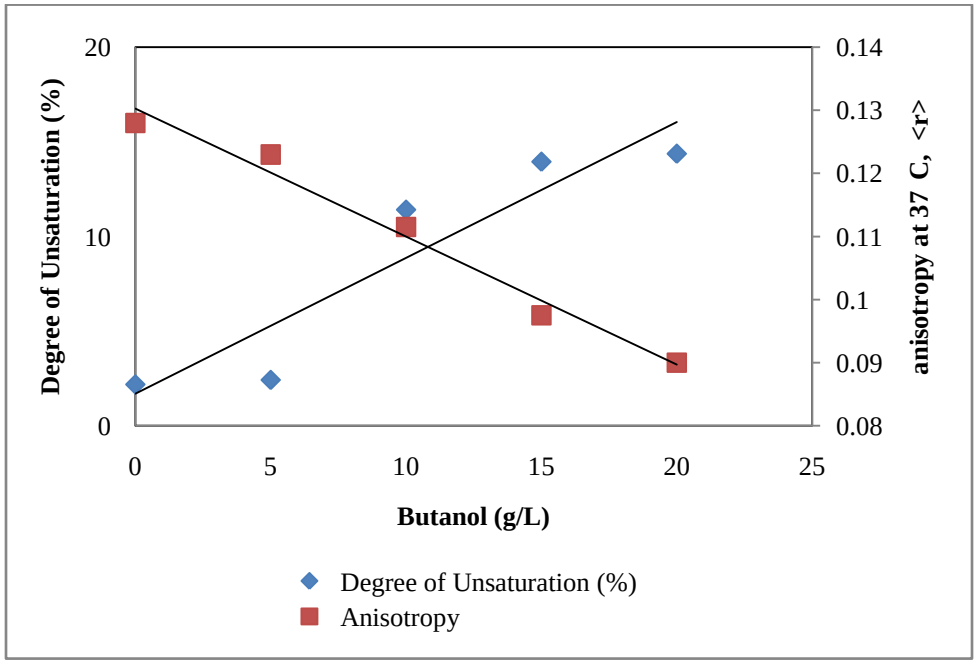


Figure 6.14 Effect of exogenous butanol on the lipid membranes of *C. pasteurianum* grown on RCM

The effect of exogenous butanol was also studied on 50 g/L glucose in Biebl medium to confirm the findings and results obtained from growing the cells on RCM. The ^{1}H NMR spectra of these experiments are shown in the appendix (Figure C.6 to C.11). The growth curve (Figure 6.9) shows that the experiment was carried out in the same manner as in RCM, where the cells were grown until mid-logarithmic phase and butanol was added when the optical density at 600 nm was around 0.6 – 0.7. The membranes were extracted after 24 hours of incubating the cells with butanol. The membranes were analyzed using ^{1}H- NMR to confirm the results obtained from the experiments on RCM on the increase in unsaturation with an increase in exogenous

butanol concentration in a non-butanol producing medium. The ^{1}H-NMR results of the membranes from cells grown on glucose for studying the effect of exogenous butanol is shown in Figure 6.15.

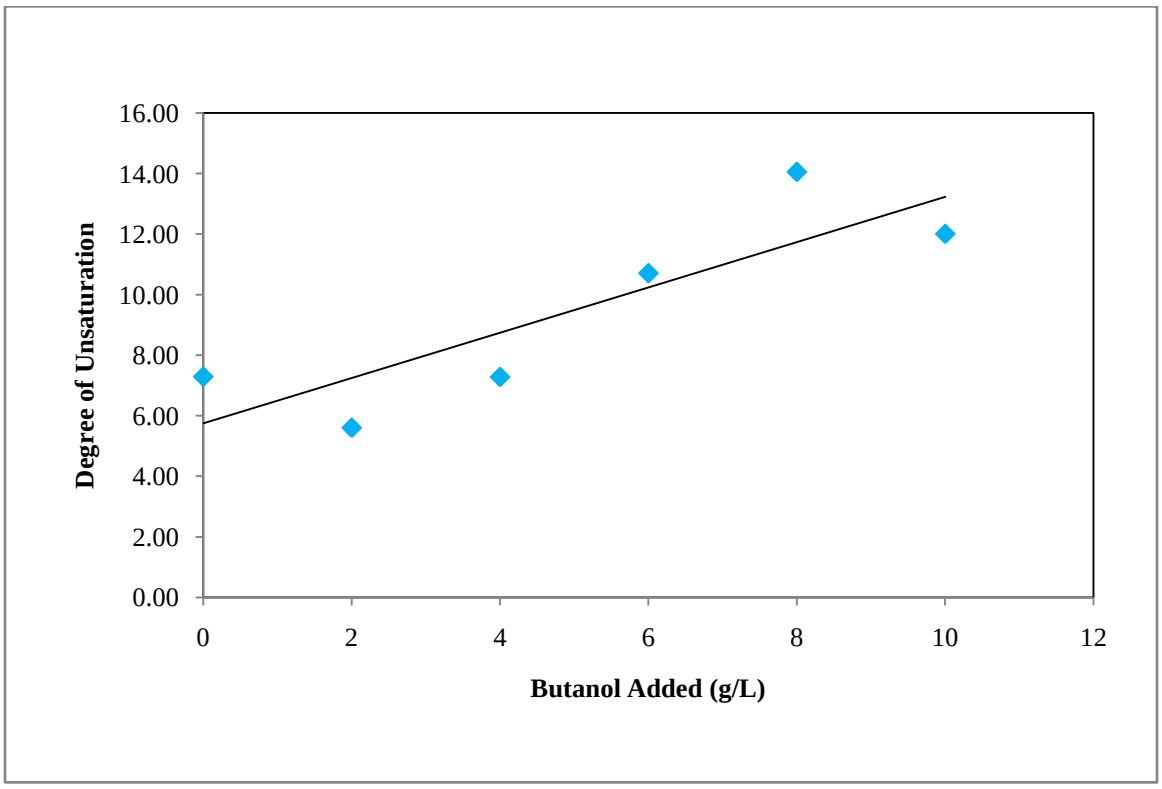


Figure 6.15 Effect of exogenous butanol on the lipid membranes of *C. pasteurianum* grown on 50 g/L glucose.

The ^{1}H-NMR of the membranes extracted from the cells grown in 50 g/L glucose and exogenous butanol, showed a similar pattern (Figure 6.15) as observed from the membranes of the cells grown in RCM (Figure 6.14) with exogenous butanol. An increase in the percentage of unsaturation in the membranes was observed in both the

cases. These results confirm and support the data obtained on RCM, that the unsaturation in the bacterial membrane increases during exogenous butanol addition and hence the fluidity of the membrane increases well. These experiments were conducted to confirm the data obtained in RCM medium and no GC-MS or anisotropy analysis was conducted on these samples.

The results obtained from the effect of butanol on the lipid membrane of *C. pasteurianum* are summarized and compared in Table 6.3. As mentioned earlier and summarized in Table 6.3, during endogenous butanol formation with exogenous butanol present (endogenous + exogenous, cells grown on glycerol), the degree of unsaturation in the membrane decreases with a corresponding increase in C_{BuOH} (butanol concentration). Contrastingly, the degree of unsaturation was found to increase (Figure 6.14, Figure 6.15 and Table 6.2) in the presence of exogenous butanol, but without endogenous butanol production (cells gown on glucose). An increase in unsaturation and the presence of exogenous butanol will result in a decreased anisotropy and an increased fluidity (Table 6.3).

The anisotropy of the liposomes obtained during various concentrations of endogenous butanol production was found to increase and hence, complemented the finding with the decrease in unsaturation (Table 6.3 and Figure 6.8). The decrease in anisotropy during the presence of both exogenous and endogenous butanol (Table 6.3) can be solely explained due to presence of exogenous butanol. Hence, one can conclude that the addition of exogenous butanol results in a fluidized membrane (Table 6.3), but the homeoviscous responses of the bacteria were found to be different and were

dependent on the carbon source in the medium along with an active butanol formation. These results indicate the existence of two different homeoviscous responses which are exerted by the bacteria during solvent production and in the absence of solvent production.

It has been shown that increase in the percentage of saturated lipids in the model liposomes (model membranes from DPPC and DOPC) results in an increase in the anisotropy of the liposomes (Kurniawan *et al.* 2012). Furthermore, the addition of butanol to model membranes comprised of DPPC, DOPC or the mixture of both have shown decrease in anisotropy due to the fluidizing effects of butanol on the lipid bilayer (Löbbecke and Cevc 1995; Kurniawan et al. 2012). Butanol fluidizes the lipid bilayer either by the formation a rippled gel phase at low concentrations or by the formation of interdigitated gel phase at higher concentrations, leading to a fluidized lipid bilayer (Kurniawan *et al.* 2012). The increase in unsaturation in the lipid membrane has been shown to augment the fluidizing effects of butanol (Kurniawan *et al.* 2012). Hence, the results obtained from the addition of exogenous butanol to the cells of *C. pasteurianum* grown on glucose are contradictory and contrasting to results obtained during endogenous butanol production (Section 6.3.2) or the response to exogenous and endogenous butanol (Section 6.3.3). This also leads to question that whether there are other non-lipid entities involved in the homeoviscous adaptation of the membrane.

Table 6.3 Comparison on the effect of butanol on *C. pasteurianum*

Source of Butanol		**Endogenous**	**Endogenous and Exogenous**	**Exogenous**
Carbon Source		Glycerol	Glycerol	Glucose
Degree of Unsaturation		Decreases	Decreases	Increases
Presence of active butanol formation		Yes	Yes	No
Anisotropy		Increases	Decreases	Decreases
Fluidity		Decreases	Increases	Increases
Short Chain Fatty Acids	**<C10**	n.d.	Absent	Present at all butanol concentrations
	C10 to C12	n.d.	Decreases steadily with an increase in exogenous butanol concentration	Decreases, but not steadily with an increase in exogenous butanol concentration
Long Chain Fatty acids (>C19)		n.d.	Newly synthesized only during the addition of exogenous butanol. Percentage increases with an increase in exogenous butanol concentration	Always present. Percentage increases with an increase in exogenous butanol concentration

The fatty acid composition of the cells also varies considerably when the cells are grown on glycerol (Biebl medium) and glucose (RCM), respectively (Table 6.3). The cells grown on glucose have an excess of both shorter (C6 to C10) as well as longer (C19 and greater) fatty acids and were present even during no exogenous butanol (C_{BuOH} = 0g/L). Moreover, the shorter fatty acids did not disappear on addition of butanol (Table 6.3). Unlike the cells grown on glucose, the cells grown on glycerol (Table 6.1 and Table 6.3) were found to have no shorter fatty acids (C6 or C7). The shortest chain length of fatty acids, of cells grown on glycerol, was C10, which eventually disappeared at 10 g/L of exogenous butanol. On glycerol, the longer fatty acids (C19 to C22) were generated in response to the presence of exogenous butanol (Table 6.1).

The reduction in shorter chain fatty acids and an increase in longer chain fatty acids results in increasing the fluidity of the membrane (Baer, Blaschek, and Smith 1987; Lepage et al. 1987). It was also observed in *C. acetobutylicum* that challenging the cells with butanol resulted in the formation of longer chain fatty acids at the expense of shorter chain fatty acids (Baer, Blaschek, and Smith 1987; Lepage et al. 1987).

The increase in unsaturation (exogenous butanol on glucose), observed in all three different analytical methods, does not explain a homeoviscous adaptation, as it leads to an increase in the fluidity of the membrane, complementing the toxic effect of butanol (Figure 6.14, Figure 6.15 and Table 6.2). An increase in unsaturation in the lipid membrane of cells exposed to butanol in RCM can be explained by similar results reported on *E. coli*, during ethanol stress (Hermann J. Heipieper, Weber, et al. 1994; Dombek and Ingram 1984). Dombek and Ingram, also reported that the plasma

membrane in general became more rigid during ethanol challenge experiments, but the extracted lipid membrane exhibited higher fluidity in comparison to the control cells that were unexposed to ethanol. This explains that the rigidity of the membrane is not only dependent on the ratio of saturated to unsaturated fatty acids or the presence of shorter or longer fatty acids, but is also dependent on the lipid to protein ratio in the membrane (Dombek and Ingram 1984). The increase in the proteins and the protein to lipid ratio in the membrane can account for the net increase in fluidity of the membrane and compensates for the loss of fluidity by increase in the unsaturation of the lipid tails (Figure 6.14). An analysis of the fluidity of the whole cells of *C. pasteurianum* exposed to butanol (grown on glucose) should be performed to confirm the finding that *C. pasteurianum*, exhibits a gram-negative like response towards butanol tolerance, when grown on a non-butanol producing media. Hence, we here hypothesize that there might be a homeoviscous response in *C. pasteurianum*, which is driven by the change in lipid:protein ratio rather than SFA:UFA ratio, during the absence of butanol formation.

The general response to exogenous butanol by other butanol producing species of *Clostridia* such as *C. beijerinckii*, *C. acetobutylicum* has been well studied and reported (Lepage et al. 1987; Baer, Blaschek, and Smith 1987; Vollherbst-Schneck, Sands, and Montenecourt 1984; Baer, Bryant, and Blaschek 1989; Isar and Rangaswamy 2012; Baut et al. 1994). These bacteria respond to butanol stress by lowering the percentage of unsaturation in the lipid membrane and hence attempt to neutralize the effects of the butanol. The butanol stress studies in these organisms were carried out by growing the bacteria in a medium containing the carbon source (glucose) that results in butanol formation.

6.4 Conclusion

This study is the first reported study on the analysis of the homeoviscous adaptation to butanol by *C. pasteurianum*. The analysis of the extracted membranes from cells exposed to various concentration of butanol in two different media, butanol producing medium containing glycerol and non-butanol producing medium containing glucose, resulted in two completely different responses with respect to the changes in the composition of the lipids. During butanol production, when stressed with exogenous butanol, *C. pasteurianum* responses by increasing the ratio of the saturated to unsaturated fatty acids in the membrane. This is also the response found in other butanol producing species of C*lostridia*, along with the response exhibited by other microbes towards tolerating toxic organic compounds (Lăzăroaie 2009; Hermann J. Heipieper, Weber, et al. 1994; Ramos et al. 2002; Segura et al. 1999). *C. pasteurianum* exhibits a different response on glucose which can be explained on the hypothesis of altering the ratio of the protein to lipids in the membrane. To confirm this particular hypothesis, further studies need to be conducted to measure the fluidity of the plasma membrane (lipids + protein), as the extracted membrane procedure results in the loss of the protein during the extraction.

During the formation of butanol, the bacteria must be synthesizing butanol efflux pumps in the membrane that serve as butanol transporters to the extracellular environment (Dunlop *et al.* 2011). Dunlop *et al.* have showed that cloning and expressing of efflux pumps from different microorganisms for various solvents resulted in an increase of solvent tolerance. But, over expression of butanol and isobutanol efflux

pumps did not improve butanol tolerance (Dunlop *et al.* 2011). The results from Dunlop *et al.* show that the toleration of butanol is a much more complex phenomenon. There should also be a correlation between the transcriptional regulation of the genes involved in butanol production and the genes responsible for butanol tolerance through homeoviscous adaptation. If there was no correlation, the homeoviscous adaptation of *C. pasteurianum* must be similar during both, endogenous production (from glycerol) and exogenous addition (growth on glucose).

The result from this study substantiates the assumption that a correlation exists between the modes of homeoviscous response, which are in turn, dependent on the activation of butanol production pathway. Furthermore, the existence of two different homeoviscous adaptations to butanol challenge in *C. pasteurianum*, demonstrates the potential of this organism to be studied further in terms of proteomics, functional genomics and metabolic engineering for the development of an industrial strain. The unavailability of the genome sequence and the proteome data must be addressed to explore butanol production and tolerance in *C. pasteurianum*. In the meantime, methods to use genomic data from closely related species can be explored to establish a platform that can be used to perform transcriptional analysis of *C. pasteurianum* during butanol stress.

CHAPTER 7

7. MICROARRAY ANALYSIS OF *Clostridium pasteurianum* – METHOD DEVELOPMENT

7.1 Introduction

The availability of the whole genome sequence of various organisms, eukaryotes and prokaryotes, have resulted in the analysis of whole cell gene expression using high throughput technique such as DNA microarrays (Brown and Botstein 1999) and transcription profiling using next-generation sequencing (Asmann, Wallace, and Thompson 2008). These high throughput techniques offer a measure to analyze the gene expression of the whole cell under various growth conditions. During different growth conditions, the expression of various genes change as expression may be upregulated, down regulated or remain the same.

A gene is expressed in the form of proteins and is guided by the central dogma (DNA to mRNA to proteins). A greater expression of a gene will result in the formation of greater amounts of mRNA (transcripts) during a particular process or growth condition. Thus, the higher the concentration of mRNA, the greater is the expression (translation) of the respective proteins. DNA microarray technology uses the

measurement of the transcripts to analyze the level of expression of various genes. As a high-throughput technique, microarrays are used in profiling the expression of tens of thousands of the genes at the same time, during various processes or growth conditions.

Microarray technology allows for the whole genome expression studies and have been extensively used (Alsaker, Paredes, and Papoutsakis 2005; Alsaker, Spitzer, and Papoutsakis 2004; Tomas et al. 2003; Tomas, Beamish, and Papoutsakis 2004; Borden, Paredes, and Papoutsakis 2005; Paredes et al. 2007; Tummala et al. 2003; Borden and Papoutsakis 2007). The number of genes in bacteria is limited to a few thousands. The use of microarrays to analyze gene expression does not require considerable amount of money and time. The continuous optimization and validation of the microarray technology over the years since its inception in the mid 90's have made microarrays a highly reliable high-throughput technology (Hardiman 2004). Microarrays use the principle of printing cDNA probes on a solid substrate, usually glass, by means of phosphoradite chemistry and using the fluorescent labeled mRNA as the target, which are allowed to hybridize with the probes on the slide (Hardiman 2004). The expression of the mRNA is analyzed using the fluorescence intensity of the hybridized spots (Hardiman 2004). Thus, the expression profiling of mRNA at more than one condition can be analyzed using microarrays. The cDNA probes printed on the microarray are selected on the basis of their specificity to a region of a single gene only (Hardiman 2004). This selection process ensures that a given probe will hybridize only to it respective gene transcript, making this technique highly specific. The hybridized slides are washed to remove any unhybridized RNA and scanned to acquire a TIFF image (8 bit or 16 bit) which is later used for image processing and normalization to get the gene expression

values in terms of the intensity of fluorescence at each spot (Hardiman 2004). Usually the control is labeled with Cy-3 (green) and the sample with Cy-5 (red) (Hardiman 2004). For a particular gene, if the expression is higher in the control, the spot will be green and vice versa. For genes with the same extent of expression under both conditions, the spot will appear as yellow (Hardiman 2004). The steps in the microarray experiments are summarized in Figure 7.1.

The designing of the probes, for the microarray, is dependent on the availability of the genome sequence of the organism under investigation. The lack of genome sequence has resulted in the development of the heterologous microarray hybridization experiments (Buckley 2007; Renn, Aubin-Horth, and Hofmann 2004; Sauer et al. 2004). In heterologous hybridization, the probes from a related species, for instance X, (sequenced) are used to study gene expression for the organism Y (Buckley 2007; Renn, Aubin-Horth, and Hofmann 2004; Sauer et al. 2004). Heterologous hybridization experiments have been conducted not only to analyze gene expression in an organisms with no available sequence information, but also to establish evolutionary relationship among genes in different biochemical pathways (Buckley 2007; Renn, Aubin-Horth, and Hofmann 2004; Sauer et al. 2004). Renn *et al.* studied the gene expression of eight different species of cichlid fish using the microarray probes designed for *Astatotilapia burtoni* and found the consistency in the expression profiles was greater for species that were closely related than the species that was distantly related to *Astatotilapia burtoni* (Renn, Aubin-Horth, and Hofmann 2004). Sauer *et al.* analyzed the gene expression profile of the yeast *Pichia pastoris* using the DNA microarrays designed for *S. cerevisiae* (Sauer et al. 2004). The

heterologous hybridization resulted in 66% positive signals in comparison to the homologous hybridization (Sauer *et al.* 2004).

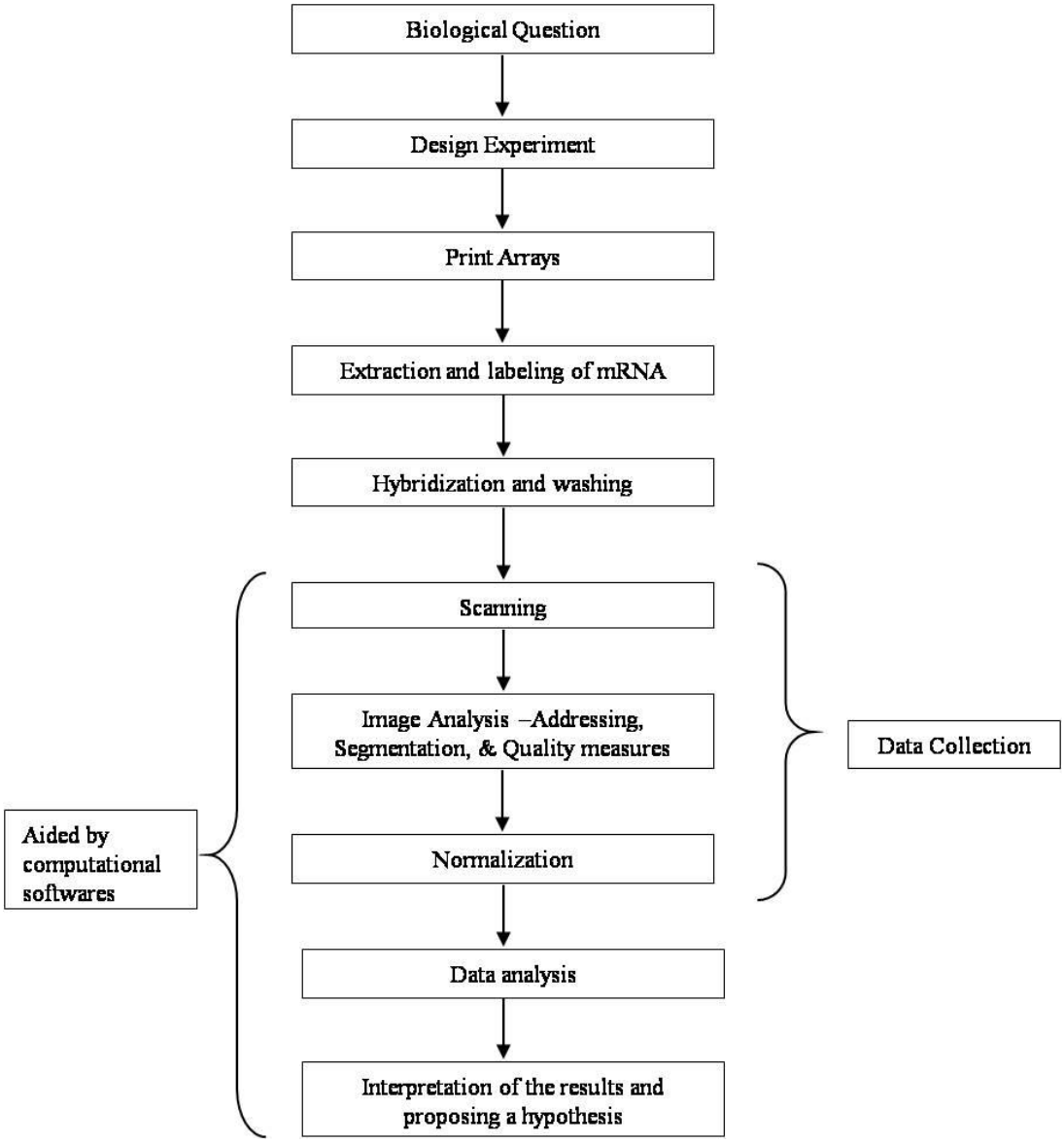


Figure 7.1 Steps involved in a microarray experiment

In this study, we propose to optimize microarray analysis for *C. pasteurianum* using the probes designed for *C. acetobutylicum*. The protocol for conducting microarray experiments in *C. acetobutylicum* has been optimized and well established (Alsaker, Paredes, and Papoutsakis 2005; Alsaker, Spitzer, and Papoutsakis 2004; Tomas et al. 2003; Tomas, Beamish, and Papoutsakis 2004; Borden, Paredes, and Papoutsakis 2005; Paredes et al. 2007; Tummala et al. 2003; Borden and Papoutsakis 2007). *C. pasteurianum* and *C. acetobutylicum* have a GC content of 26 % and 28 %, respectively (Cummins and Johnson 1971). *C. acetobutylicum* has been extensively investigated for solvent production, especially butanol, and this makes the use of *C. acetobutylicum*'s microarray probes suitable to study the gene expression of *C. pasteurianum*.

In order to optimize the conditions for best results, RNA from *C. pasteurianum* grown under three different conditions were used, pure glycerol, partially purified crude glycerol and glucose. Additionally, the differentially gene expression pattern during shift from glucose to pure glycerol and partially purified crude glycerol was also studied at the optimized hybridization conditions.

The stringency of the hybridization of the labeled samples to the cDNA probes is a function of the temperature and salt concentration during post hybridization washing (Korkola et al. 2003). The post hybridization wash is carried out in three steps. The buffers for the three washes for a cDNA microarray usually are 2x saline sodium citrate (SSC) with 1% sodium dodecyl sulfate (SDS), 2x SSC and 0.2x SSC, respectively (Korkola et al. 2003). The first wash is usually carried out at a higher temperature in order to control stringency. Hence, the stringency can be varied by varying the

temperature and the salt concentration. Stringency can be lowered by either lowering the temperature of the first wash or increasing the salt concentration in all the washes or both (Korkola et al. 2003). In heterologous hybridization, lowering the stringency will result in increasing the number of positive signals, although it has the disadvantage of higher non-specific binding. In heterologous hybridization, due to the variation in the sequence of the sample and the probes, probes will be hybridized with samples that do not have 100 % match. These signals will be washed out during high stringent wash, as the melting temperature (Tm) of these signals may be lower than the wash temperature at the given salt concentration (Korkola et al. 2003). Thus, lowering the stringency to increase the number positive signals is an important step in heterologous hybridizations.

7.2 Materials and Methods

7.2.1 *C. acetobutylicum* Microarray

C. acetobutylicum microarrays were purchased from Microarrays Inc. (Huntsville, AL). The arrays were printed on aldehydesilane coated glass slides from Schott, USA. The 5' end of the probes was amino modified and the probes were printed using a robotic arm using amine-aldehyde chemistry. The arrays consisted of 48 blocks, triplicates of 16. Each block contained 19 columns and 16 rows. The arrays contained 4231 probes (60-70 mers) covering 3842 genes and 107 structural RNAs of *C. acetobutylicum*. The information on the sequence and gene list can be accessed at http://www.microarrays.com/docs/CA3001_C_acetobutylcum_genelist.xls. Although, the arrays contained probes for the entire transcriptome of *C. acetobutylicum*, the glycerol utilization genes are absent in *C. acetobutylicum*. To the arrays seven additional probes,

coding for seven genes in *C. pasteurianum* (glycerol utilization), were added and the probe sequence are listed in Table 7.1. These seven additional probes were also amino modified at the 5' end and were obtained from Integrated DNA Technology (Coralville, IA).

Table 7.1 Probe sequence added to the array from *C. pasteurianum*

Gene (accession#)	**Function**	**Sequence (5' to 3')**
dhaB (AF051373)	glycerol dehydratase subunit B	ACCATGCTTACTCAGTGCTTTCACCACATCTATT CCAGTATTCCTCTATTTATCATTTCCTCTGCAGCT
dhaC (AF051373)	glycerol dehydratase subunit C	TCCTGCAATTAACTCTCTCAATATTTTGTCATGAG GTACTCCTACAATGCTCTGATGTTGA
dhaE (AF051373)	glycerol dehydratase subunit E	AGATATTAGTTCAGCAGCTCTTCTAAAATTTCTT GCAATAGCGCATCTACCTGATCCTTCAGCAAC
orfZ (AF051373)	Open reading frame next to *dhaBCE*	CTGTAGCCACTGCATTTCTCGGTCCGTCTAAACCTCT TATATTCCCCTGCCCTGCAACTACACCATATTT
dhaT (AF006034)	PDO dehydrogenase	CTCCAAGGGCTACTGCCTGACGCAGATTGTTAGCT ATTAATTTTATTGCTTGTATAGCTAAGGC
Ferredoxin (AF132673)	Iron-sulfur protein	ATAAATTATTCTTGTACTGGTGCTCCAACTGGACAAA CGTTAGCACAGTTACCACAGTCGATACAAGTAT
ATP (AF283808)	ATP synthase	TCCAAGACCATTTACAACTCTTCCAATCATTTCTTCG CCAACTGGAACTTCAACTACTCTTCCAGTACTC

7.2.2 RNA Sample Preparation

The RNA was isolated from the mid log phase cells (A_{600} = 0.7) of *C. pasteurianum* grown in 25 g/L pure glycerol (Biebl medium), 25 g/L partially purified crude glycerol (Biebl medium) and glucose (RCM). The RNA was also isolated from mid log phase cells of *C. acetobutylicum* grown in 50 g/L glucose (Biebl medium) and this RNA served as a control. The RNA extraction, purification, amplification and labeling was done according to the protocol suggested by the array manufacturer. The total RNA from the above mentioned samples was extracted using Ambion® Ribopure™ Bacteria kit from Life Technologies (Grand Island, NY). The total RNA was tested for its integrity using denatured (formaldehyde) agarose (1%) gel electrophoresis and the concentration was measured using Nanodrop™ spectrophotometer (Thermo Scientific, Wilmington, DE). DNA contamination was removed using the DNase enzyme supplied in the kit. 10 µg of total RNA was subjected to enrichment of the mRNA by removing 16S and 23S rRNA using Ambion® *MICROBExpress*™ Kit from Life Technologies (Grand Island, NY). The integrity and concentration of the purified mRNA was analyzed by gel electrophoresis and Nanodrop™ spectrophotometer, respectively. The purified mRNA was amplified using the Ambion® *MessageAmp*™ II aRNA Amplification kit to generate aminoallyl labeled aRNA, which was labeled with either Cy3 or Cy5. In brief, the protocol involves the generation of a poly-A tail using a poly-A polymerase (PAP). The poly-A tailed mRNA is used to generate a cDNA using Oligo-dT primers and T7 RNA polymerase. The RNA of the mRNA-cDNA duplex was digested with an RNase and the cDNA was purified using the columns provided in the kit. The purified cDNA was used to generate the second strand using random primers. From the second strand,

the aRNA was generated using ArrayScript™ reverse transcriptase. Aminoallyl-UTP:UTP was maintained at 65:35, to incorporate aminoallyl-UTP in the aRNA. The integrity and concentration of the aminoallyl-UTP containing aRNA was analyzed by gel electrophoresis and Nanodrop™ spectrophotometer, respectively. Aliquots (20 µg) of aRNA were prepared and vacuum dried for labeling and storage (-80ºC) purposes. The dried aRNA was labeled with Cy3 and Cy5 and the intensity of the fluorescence and the concentration of labeled aRNA was measured using Nanodrop™ spectrophotometer (Thermo Scientific, Wilmington, DE).

7.2.3 Microarray Hybridization

The slides were pre-warmed to 42ºC using a slide warmer and a cover slip was placed just before loading the samples. The pre-warmed slides were transferred to the bottom half of the Corning™ hybridization chamber. The hybridization chamber was loaded with 10 µL sterile nuclease free water at both ends to maintain humidity. The hybridization samples were prepared by adding 40 pmol of Cy3-labeled samples and 40 pmol of Cy5-labeled samples to a total volume of 50 µL of the hybridization buffer (supplied by Microarray Inc., Huntsville, AL). The samples were pre-warmed to 95ºC to denature the RNA for enhanced hybridization with the probes. The samples were loaded using a micropipette from the end containing the barcode. Once the hybridization sample diffused the entire area of the cover slip, the hybridization chamber was locked using the upper half and the locking supports. The hybridization chamber was incubated in an oven at a temperature of 42ºC for 20-24 hours.

7.2.4 Post hybridization washing

The post hybridization washing was carried out in three steps (Table 7.2). The first step, Wash A, was initially performed at 42ºC and at a salt concentration of 2x SSC and 0.1x SDS, at the highest stringent conditions (Table 7.2). The stringency was later reduced by lowering the temperature to 37ºC at the same salt concentration and later by doubling the salt concentration at 37ºC (Table 7.2). The stringency was further reduced by reducing the temperature to 30 ºC at the doubled salt concentration (4x SSC and 0.2x SDS). The salt concentrations in Wash B and Wash C were maintained proportional to the Wash a (Table 7.2). If the concentration was doubled in Wash A, then the salt concentration was adjusted in Wash B and Wash C, as well. The recommended concentrations for Wash B was 2x SSC, while 0.2x SSC for Wash C. All the washes were carried out in 50 mL Falcon tubes using 50 mL of wash solution per slide. The slides were placed in Boekel Shake 'n' Bake™ hybridization oven for constant mixing. The slides were dried by placing them either on a 50 mL Falcon tube containing Kimwipes™ on the bottom or on a slide wash container with Kimwipes™ on the bottom and centrifuged at 1500 rpm for 3 minutes in an Eppendorf™ 5810 R centrifuge.

7.2.5 Image Scanning and Analysis

The slides were scanned in a GenePix™ 4000 (Molecular Devices, Sunnyvale, CA) scanner and the images were processed using GenePix™ Pro 5.0 (Molecular Devices, Sunnyvale, CA). The data was normalized using ratio of means method for each slide. The data analysis was carried out using Acuity™ 4.0 (Molecular Devices, Sunnyvale, CA).

Table 7.2 Post Hybridization Washing Conditions

Condition	Wash Step	Temperature (ºC)	Salt Concentration	Duration (min)
1	Wash A	42	2x SSC, 0.1x SDS	10 min
	Wash B	RT	2x SSC	10 min
	Wash C	RT	2x SSC	2 min
2	Wash A	37	2x SSC, 0.1x SDS	10 min
	Wash B	RT	2x SSC	10 min
	Wash C	RT	2x SSC	2 min
3	Wash A	37	4x SSC, 0.2x SDS	10 min
	Wash B	RT	4x SSC	10 min
	Wash C	RT	4x SSC	2 min
4	Wash A	30	4x SSC, 0.2x SDS	10 min
	Wash B	RT	4x SSC	10 min
	Wash C	RT	4x SSC	2 min

7.3 Results and Discussion

7.3.1 Optimization of stringency

In order to optimize stringency for the heterologous hybridization of *C. pasteurianum* transcripts to the cDNA microarrays of *C. acetobutylicum*, the wash conditions were changed by lowering stringency to increase the number of positive signals from the samples of *C. pasteurianum* (Table 7.2). To optimize stringency conditions, the experiments were conducted as mentioned in Table 7.3. The names of the samples used for reference are also described in Table 7.3. The use of RNA samples from the cells of *C. pasteurianum* grown in pure glycerol (CPPG) in all the three experiments served to compare the relative percentage of positive signal obtained from the co-hybridization of the samples from *C. acetobutylicum* (homologous hybridization) and *C. pasteurianum* (heterologous hybridization).

The most stringent wash, suggested in the protocol provided by the array manufacturer, was carried out at 42°C and at a salt concentration of 2x SSC and 0.1x SDS. The positive spots that were present in triplicate were only considered to optimize washing stringency. The positive spots were selected based on the criteria that the net intensity of the spot was greater than zero for all three replicates in a slide (Eq 7.1).

$$x_{raw,i} - x_{bg,i} > 1 \qquad \textbf{(Eq 7.1)}$$

where, $x_{raw,i}$ is the raw channel intensity and $x_{bg,i}$ is the background intensity for the corresponding channel. The results for the number of positive spots versus various stringency conditions are summarized in Figure 7.2.

Table 7.3 Samples used for Stringency optimization

S. No.	**Sample 1 (Cy3)**	**Sample 2 (Cy5)**
1.	*C. acetobutylicum* (glucose) CACE-CY3	*C. pasteurianum* (pure glycerol) CPPG-CY5
2	*C. pasteurianum* (partially purified crude glycerol) CPCG-CY3	*C. pasteurianum* (pure glycerol) CPPG-CY5
3.	*C. pasteurianum* (glucose) CPGL-CY3	*C. pasteurianum* (pure glycerol) CPPG-CY5

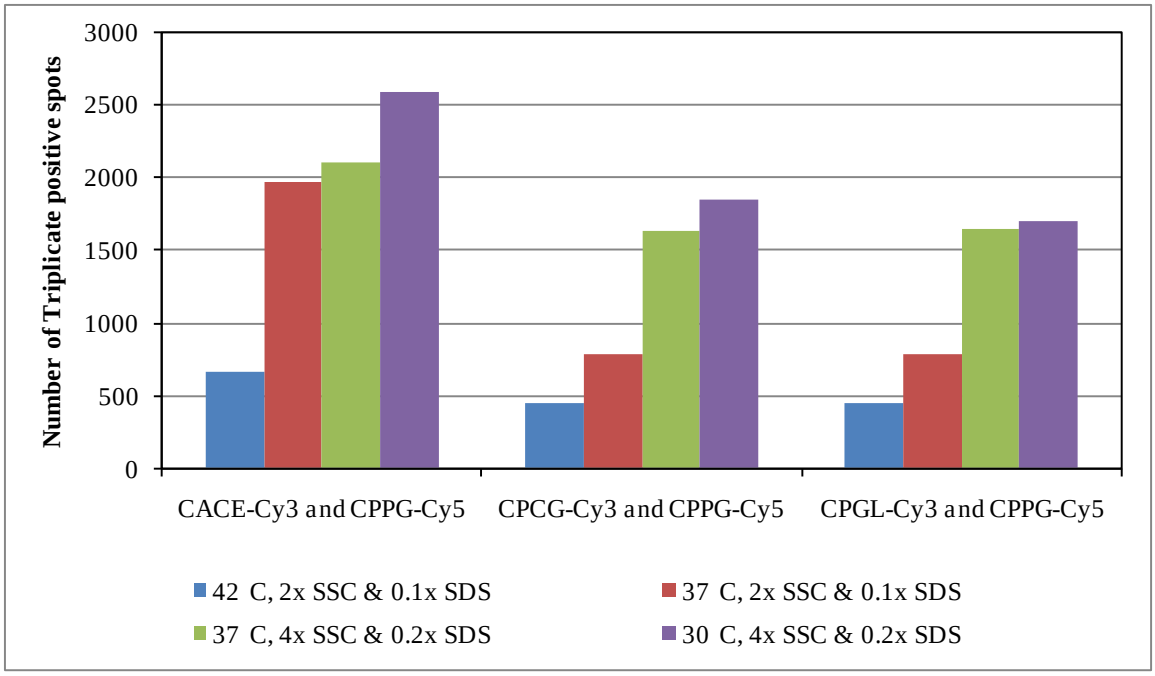


Figure 7.2 Optimization of wash stringency. The number of positive spots (triplicates) increased with a reduction in stringency.

Table 7.4 Optimization of wash stringency. The number of positive spots (triplicates) increased with a reduction in stringency.

Wash Condition	CACE-Cy3 & CPPG-Cy5	CPCG-Cy3 & CPPG-Cy5	CPGL-Cy3 & CPPG-Cy5
42°C, 2x SSC & 0.1x SDS	664	449	450
37°C, 2x SSC & 0.1x SDS	1970	787	778
37°C, 4x SSC & 0.2x SDS	2108	1632	1650
30°C, 4x SSC & 0.2x SDS	2597	1849	1703

At 42ºC and the default concentration of the salts, the hybridization of CACE-Cy3 and CPPG-Cy5 samples resulted in 664 spots out of the total 4231 triplicate spots in the array (Figure 7.2). The number of positive spots for the heterologous hybridization involving CPCG-Cy3 and CPPG-Cy5 was observed to be only 449, while CPGL-Cy3 and CPPG-Cy5 yielded 450 positive spots. The reduction in the positive spots for these two experiments was due to the 100 % heterologous nature of hybridization. In the experiments involving CACE-Cy3 and CPPG-Cy5, the number of positive spots was higher because of the presence of RNA samples from *C. acetobutylicum*, making the experiment partially homologous and hence more positive spots were observed. In spite of the presence of RNA from *C. acetobutylicum*, the percentage of reproducible positive spots was limited to 16 % of the total spots in the array. The percentage of positive spots was much lower (11 %) for the heterologous hybridization involving the RNA samples of *C. pasteurianum*. To increase the number of positive signals, the stringency was lowered by lowering the temperature of the Wash A by 5ºC to 37ºC and the salt concentration was kept constant.

The reduction in the temperature to 37ºC resulted in an increase in the positive signals across all the three experiments. The two experiments with the heterologous hybridizations, CPCG-Cy3 vs CPPG-Cy5 and CPGL-Cy3 vs CPPG-Cy5, had a 75 % and 73 % increase in the overall positive signal, respectively (Figure 7.2 and Table 7.4). The experiment, CACE-Cy3 vs CPPG-Cy5, had a three fold increase in the number of positive spots, covering 46 % (1970/42381) of the total spots in the array (Figure 7.2 and Table 7.4). Though the positive signal improved for the homologous hybridization

experiments, the number positive signals from *C. pasteurianum* samples remained low (18.6 %).

The stringency was further reduced by doubling the salt concentration to 4x SSC and 0.2x SDS and maintaining the temperature at 37ºC. On further reduction of the stringency, the number of positive spots increased for the heterologous hybridization (*C. pasteurianum* RNA samples) to 1632 (CPCG-Cy3 vs CPPG-Cy5) and 1650 (CPGL-Cy3 vs CPPG-Cy5), respectively, increasing the coverage of the spots to 39 %. On the other hand, the positive spots for the homologous sample increased to 2108 (49.5 %).

The stringency was reduced further by lowering the temperature to 30ºC and maintaining the doubled concentration of salts (4x SSC and 0.2x SDS). Under those least stringent conditions, the number of positive signals were increased to 1849 (CPCG-Cy3 vs CPPG-Cy5) and 1703 (CPGL-Cy3 vs CPPG-Cy5), respectively. The positive signals increased to 2597 for the experiment containing the homologous sample (CACE-Cy3 and CPPG-Cy5) and the coverage of the positive spots increased to 61 % of the total spots in the array.

Thus, lowering the stringency during the wash conditions from the highly stringent wash (42ºC, 2x SSC and 0.1x SDS) to the least stringent wash (30ºC, 4x SSC and 0.2x SDS) resulted in an increase in the number of positive signal obtained from the heterologous hybridization of *C. pasteurianum* RNA samples grown in three different carbon source (Figure 7.2 and Table 7.4). Although, reducing the stringency results in an increase in the positive spots, it also results in an increase in non-specific binding and background intensities. The degree of non-specific binding can be measured by

measuring the intensity in various empty or blank spots in the slide. The average of the difference of the channel intensity and the background intensity in the empty spots remained at a value equal or less than zero, for all the slides at all the wash conditions. This indicates that the empty spots had the same amount of non-specific binding as the background glass substrate. This remained zero for all the experiments of varying stringency.

The pair-wise Pearson correlation coefficient (r) was calculated for the three different experiments by comparing the data from the common sample (CPPG-Cy5) between any two of the three experiments. The correlation values are summarized in Table 7.5 and variation of the Pearson correlation among all three slides at each hybridization condition is summarized in Figure 7.3.

Table 7.5 Pairwise Pearson Coefficient for Microarray Optimization

	1 vs 2	**1 vs 3**	**2 vs 3**	**Average**	**Std Dev**
42°C (2x SSC and 0.1x SDS)	0.69	0.76	0.88	0.77	0.10
37°C (2x SSC and 0.1x SDS)	0.86	0.69	0.75	0.76	0.09
37°C (4x SSC and 0.2x SDS)	0.93	0.88	0.90	0.90	0.03
30°C (4x SSC and 0.2x SDS)	0.66	0.58	0.79	0.68	0.11

*1 – CACE-Cy3 vs CPPG-Cy5; 2 – CPCG-Cy3 vs CPPG-Cy5; 3 – CPGL-Cy3 vs CPPG-Cy5

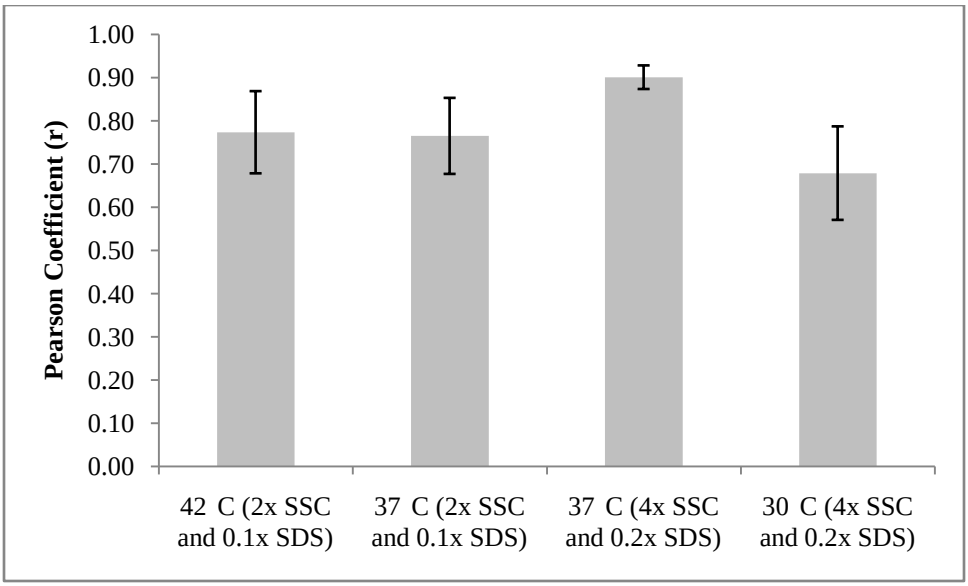


Figure 7.3 Variation of Pearson coefficient (r) over hybridization condition

From Table 7.4 and Figure 7.3, it is evident that Pearson coefficient, based on the intensity of CPPG-Cy5, was very low at the more stringent conditions. Not only was the average value of (r) lower but, it also had a lower reproducibility. At the low stringent conditions of 37ºC and twice the concentration of the salts, the average (r) value was the highest and the variation was also the least. This indicates that although, the least stringent condition at 30ºC and twice the normal concentration of salts resulted in a maximum number of positive spots (Figure 7.2), the (r) value and the reproducibility was however,much higher at 37ºC and twice the concentration of salts (Table 7.4 and Figure 7.3). Based on this result, the analysis of gene expression across the three carbon sources (pure glycerol, crude glycerol and glucose) was carried out at 37ºC and 4x SSC with 0.2x SDS.

7.3.2 Gene Expression Analysis of *C. pasteurianum*

The gene expression analysis of *C. pasteurianum* on three different carbon sources was carried out by hybridizing the Cy3/Cy5 labeled *C. pasteurianum* samples with Cy5/Cy3 labeled *C. acetobutylicum* RNA samples. Thus, the *C. acetobutylicum* RNA sample served as the control for the three sets of experiments. Dye swap experiments were also conducted. The analysis was performed from the average values from both the dye swap experiments.

Each slide was normalized using the ratio of the means method in GenePix Pro software. Following normalization, spots were selected based on the formula by Tomas *et al.* (2005), which was:

$$\frac{x_{raw,i} - x_{bg,i} - \bar{x}_{neg}}{\beta \cdot SD_{bg,i}} > 1 \qquad \text{(Eq 7.2)}$$

where $x_{raw,i}$ is the raw channel intensity, $x_{bg,i}$ is the background for the respective channel, $\bar{x}_{neg}$ is the average non-specific binding, β is a constant and was set for 1.96 for 95 % confidence and 2.81 for 99.5% confidence, $SD_{bg,i}$ is the standard deviation of the background for the respective channel.

Based on this equation each spot was evaluated. The spots that were selected based on 95% confidence level, were scrutinized further for the more stringent 99.5% confidence level. Thus, spots that passed the two selection criteria were only selected. The average non-specific binding was calculated as the average of the difference of

channel intensity and background for the 672 empty spots in every slide. This value was found to be 0.05 ± 0.007 and was hence rounded to zero.

The spots that were selected based on Eq. 7.2, were further filtered based on the reproducibility on all the three spots in each slide and the corresponding three on the dye swap experiments. This filtration further served as an additional quality check for reproducibility upon labeling of either Cy3 or Cy5. Following background subtraction and averaging the net signal intensity between the two dye swap experiments (n = 6), $\log_2$ ratio were calculated and compared among the utilization of three different carbon sources. The $\log_2$ ratios were calculated as the difference of the overall net intensity for *C. pasteurianum* RNA samples and the overall net intensity for the corresponding spot for the *C. acetobutylicum* RNA samples. Thus, a positive $\log_2$ value indicates an over-expression in *C. pasteurianum* while a negative value indicates an over-expression in *C. acetobutylicum*.

The scatter plot for spots that had a positive signal for *C. pasteurianum* samples are shown in Figure 7.4 (Pure glycerol against crude glycerol) and Figure 7.5 (pure glycerol against glucose). Between pure and crude glycerol slides, 295 spots passed all the criteria for selection, while between pure glycerol and glucose there were 361 spots. Among all the three carbon sources, 90 spots were hybridized irrespective of the carbon source. The number of commonly positive spots increased to 94 among the two glycerol carbon sources and the number increased to 113 between glucose and pure glycerol.

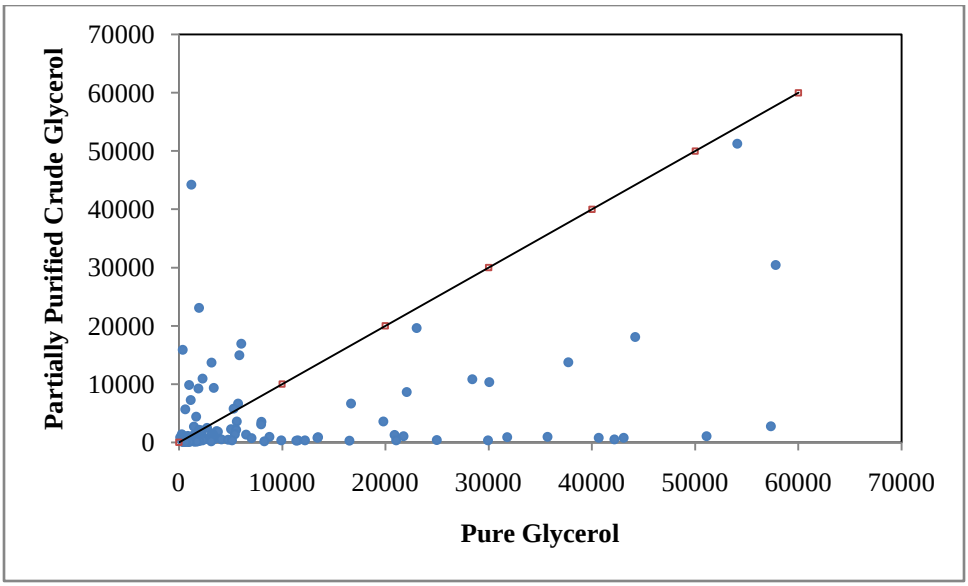


Figure 7.4 Scatter plot of spots between pure and crude glycerol (selected based on the selection criteria by Tomas *et al.* 2005)

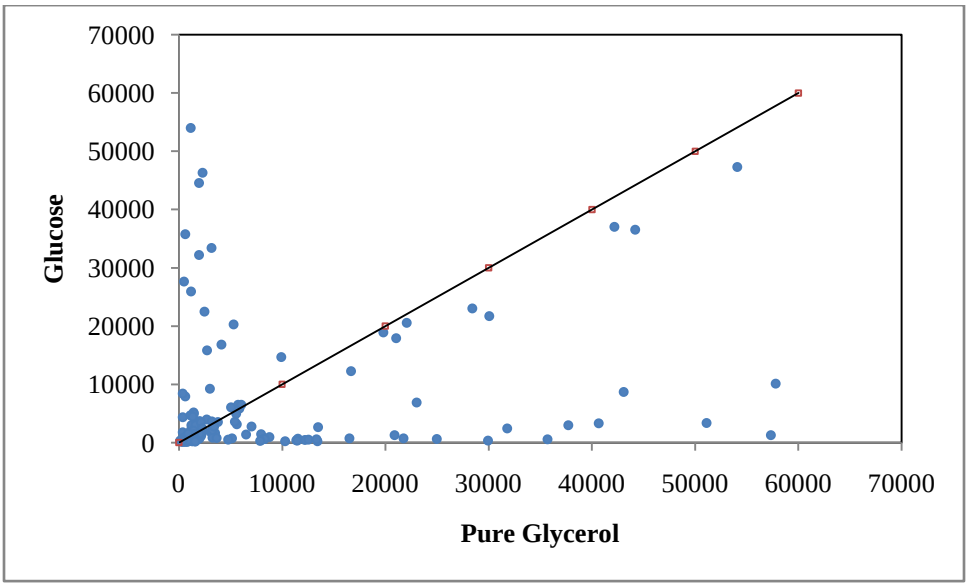


Figure 7.5 Scatter plot of spots between pure glycerol and glucose (selected based on the selection criteria by Tomas *et al.* 2005)

The scatter plot indicates the genes that are expressed differentially expressed (spots far away from the slope of 1) and the genes that are close to the line with a slope of one, have similar expression under both conditions. In the scatter plot of genes between pure and crude glycerol, genes with similar expressions have a higher expression in pure glycerol than crude glycerol. This might be possibly due to the presence of trace amount of impurities (methanol and salts) in the partially purified crude glycerol (Figure 7.4). The genes that were differentially expressed between pure glycerol and partially purified crude glycerol, belonged to categories of genes involved in growth and multiplication of the cells, but had different annotations and possibly multiple copies of genes with similar function. These genes were involved in transcription (transcriptional factors and regulators), translation, tRNAs, membrane transporters and other hypothetical proteins in *C. acetobutylicum*.

The differential expression of genes between glucose and glycerol were also included genes that had similar functions as mentioned above. But, the scatter plot of the genes between glucose and glycerol, indicates that genes with similar expression between the two carbon sources are closer to the line with a slope of 1 (Figure 7.5). This shows that during logarithmic growth phase the genes transcribed in various carbon sources are similar. The differentially expressed genes (along with their annotations) indicate the existence of multiple copies of genes for the same function, which differentially expressed based on the carbon source (glucose or glycerol) or presence of impurities (pure and partially purified crude glycerol).

To further analyze the difference in expression heat maps were drawn for various genes that were commonly expressed among the different carbon sources. Figure 7.6

shows the heat map for genes commonly expressed by *C. pasteurianum* on all the three carbon sources. Figure 7.6 shows the genes that were commonly expressed and Figure 7.7 contains the control spots for sequence comparison and stringency controls.

Only 47 genes out of 3842 genes on the array passed all the criteria for selection and were found to be commonly expressed among cells grown in all three media. Also, investigating the commonly expressed control spots (Figure 7.6), the presence of large number of alien control spots along with stringency control of 50%, indicates a possible difference in the sequence of the two organisms, *C. pasteurianum* and *C. acetobutylicum*. The low number of genes that were detected by the heterologous hybridization of *C. pasteurianum* transcripts to *C. acetobutylicum* arrays can also be a result of the fact that only log phase transcripts were analyzed. As it is know that *C. pasteurianum* solvent production is active only in the stationary phase, analysis of log and stationary phase transcripts should result in a higher number of positive spots.

The noticeable genes expressed in all three conditions are the tRNA transferases for serine, phenyl alanine; heat shock protein – groEL; rubredoxin, cell wall synthesis enzyme – acetyl muramidase; proteins involved in the process of sporulation; transport proteins for nutrients and other transcriptional and translational regulatory protein. The genes having a prefix of CA_P are the genes corresponding to the *pSOL* plasmid of *C. acetobutylicum*. This plasmid contains all the genes (168) in the solvent production pathway. The absence of positive signals from these genes is not surprising as the transcripts are from the log phase of the cells, while most of these genes are active only in the early to late stationary phase. Additionally, the genes added to the microarray from *C. pasteurianum* (Table 7.1), particularly the four *dha* genes involved in glycerol

metabolism were expressed only in the presence of glycerol. The ferredoxin and ATP genes were not detected based on the selection criteria and this indicates that probe designing requires optimization.

Heat map of the genes commonly expressed between pure and partially purified crude glycerol has been summarized in Figure 7.8 and Table 7.7. The list of genes that were exclusively expressed in partially purified crude glycerol and pure glycerol has been summarized in Table 7.8 and Table 7.9 respectively. The genes that were commonly expressed between pure glycerol and partially purified crude glycerol were related to basic cell growth and maintenance. The glycerol utilization genes were found to be expressed only during the growth of *C. pasteurianum* in pure glycerol (Table 7.9). The gene dhaB and orfZ, predominantly involved in the conversion of glycerol into 3-propanaldehyde (Figure 1.1) was found to be expressed in pure glycerol (Table 7.9). Although, the genes that were found to be differently expressed between partially purified crude glycerol and pure glycerol, the differentially expressed genes were responsible for basic cell growth and maintenance and not from the pathways related to glycerol utilization and solvent production.

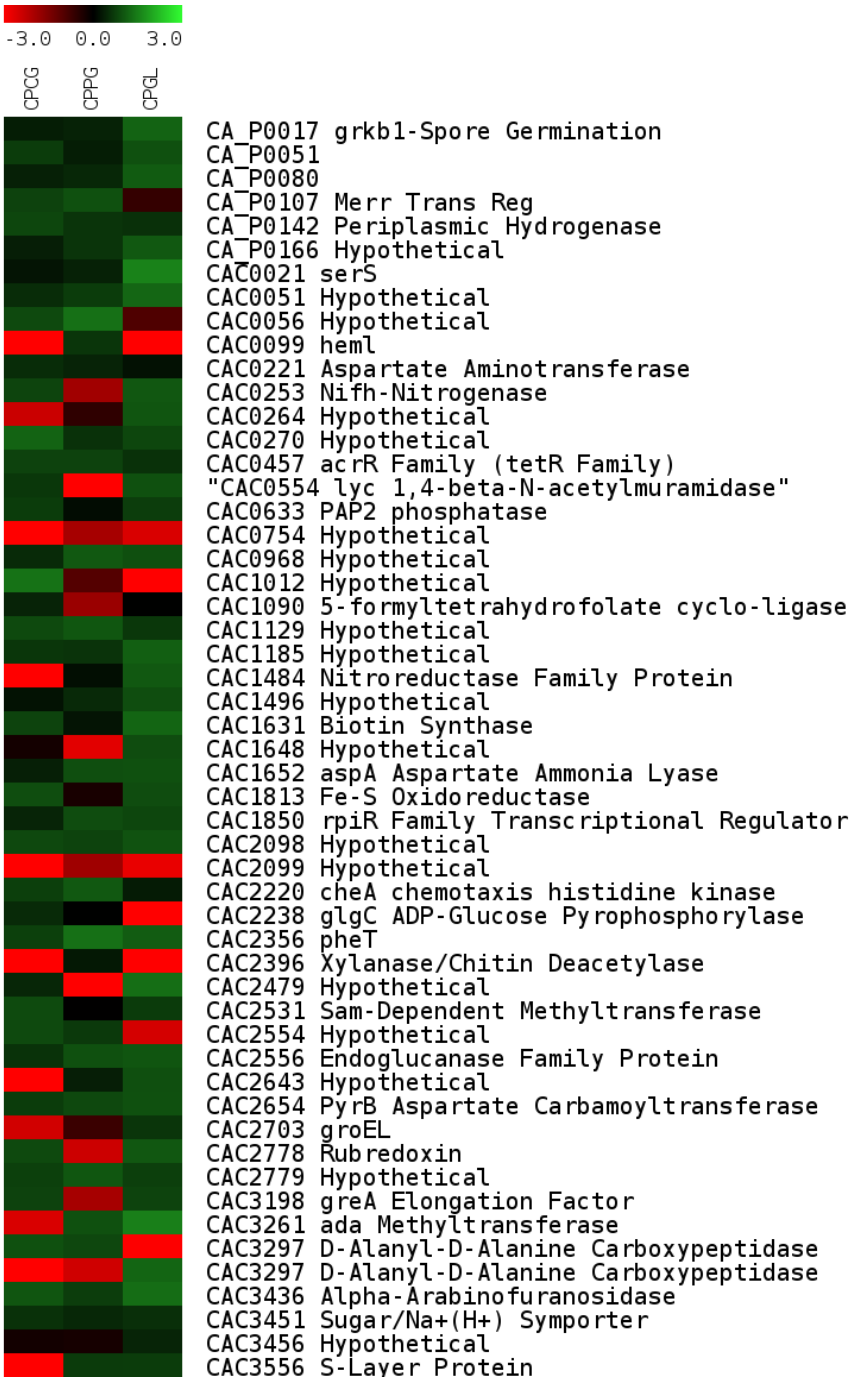


Figure 7.6 Heat map of genes expressed among the cells of *C. pasteurianum* grown on all three carbon sources (CPPG-pure glycerol, CPCG-partially purified crude glycerol and CPGL-glucose).

.

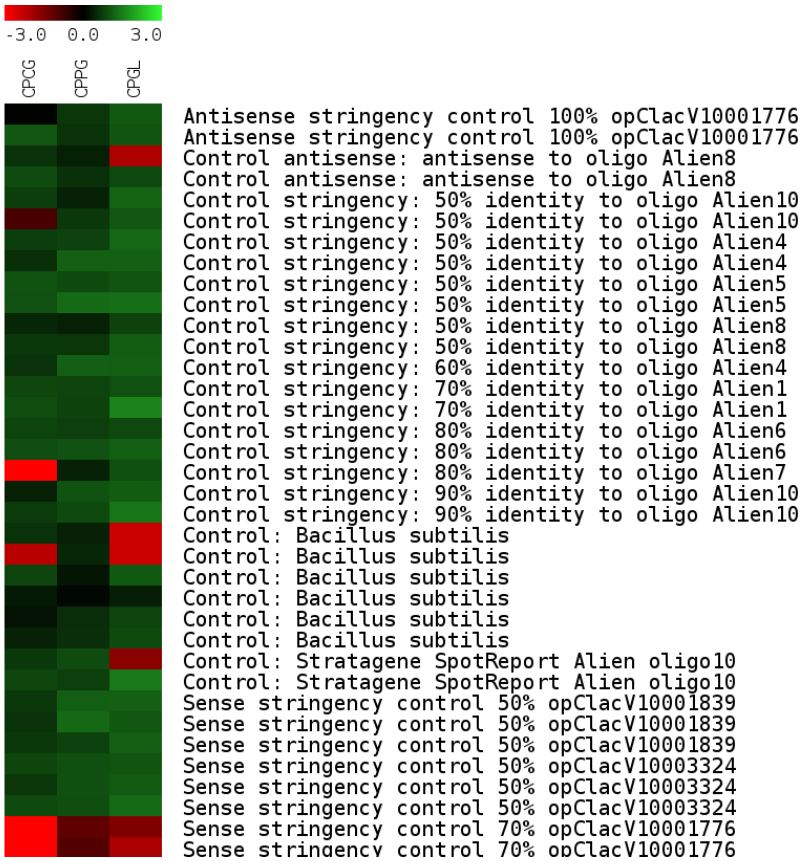


Figure 7.7 Heat map of control spots expressed among the cells of *C. pasteurianum* grown on all three carbon sources (CPPG-pure glycerol, CPCG-partially purified crude glycerol and CPGL-glucose).

Table 7.6 Genes expressed among the cells of *C. pasteurianum* grown on all three carbon sources (CPPG-pure glycerol, CPCG-partially purified crude glycerol and CPGL-glucose).

Gene ID	Expression			Protein ID	Gene	Product
	Crude Glycerol	Glucose	Pure Glycerol			
CA_P0017	786.7	1693.3	1948.0	15004721	grkb1	spore germination protein, GRKB
CA_P0051	365.5	1301.3	1957.7	15004755	-	oxidoreductase
CA_P0080	524.0	1750.5	1429.0	15004784	xerC	site-specific tyrosine recombinase XerC
CA_P0107	1962.5	4691.8	3633.0	15004810	-	MerR family transcriptional regulator
CA_P0142	23092.2	44527.3	51100.0	15004845	mbhl	periplasmic hydrogenase large subunit, dehydrogenase
CA_P0166	2039.2	6530.0	5861.8	15004869	-	hypothetical protein
CAC0021	734.2	2180.2	1613.0	15893319	serS	seryl-tRNA synthetase
CAC0051	2694.7	5206.2	5592.7	15893349	-	hypothetical protein
CAC0056	806.5	3299.3	3350.0	15893353	-	hypothetical protein
CAC0099	9230.7	20273.8	24991.0	15893395	hemL	glutamate-1-semialdehyde aminotransferase
CAC0221	907.8	2241.0	2189.8	15893513	-	aspartate aminotransferase
CAC0253	701.0	2284.7	5421.7	15893545	nifH	nitrogenase iron protein (nitrogenase component II) gene nifH
CAC0264	162.5	532.7	1517.8	15893556	-	hypothetical protein
CAC0270	953.5	2679.2	3252.2	15893562	-	hypothetical protein
CAC0457	3107.8	6875.5	7971.2	15893748	-	AcrR family transcriptional regulator
CAC0554	656.5	1466.7	2120.2	15893844	lyc	1,4-beta-N-acetylmuramidase
CAC0633	276.7	755.2	1137.2	15893921	-	phosphatase
CAC0754	614.8	1147.2	2137.7	15894041	-	hypothetical protein
CAC0968	7258.2	14696.3	23012.0	15894255	-	hypothetical protein
CAC1012	180.8	558.2	879.7	15894299	-	hypothetical protein
CAC1090	592.7	847.3	1869.0	15894375	-	5-formyltetrahydrofolate cyclo-ligase
CAC1129	3613.3	8728.0	8761.3	15894413	-	hypothetical protein
CAC1185	13736.0	32169.5	37734.7	15894468	-	hypothetical protein

CAC1484	584.5	2468.7	2459.3	15894763	-	nitroreductase
CAC1496	367.2	1443.7	1094.3	15894775	-	hypothetical protein
CAC1631	718.3	2121.7	2704.2	15894909	-	biotin synthase
CAC1648	342.2	645.2	1316.5	15894925	-	hypothetical protein
CAC1652	5696.0	10113.8	13461.5	15894929	aspA	aspartate ammonia-lyase
CAC1813	530.0	789.0	1963.0	15895089	-	Fe-S oxidoreductase
CAC1850	1375.7	3973.0	3136.3	15895125	-	RpiR family transcriptional regulator
CAC2098	1489.0	4972.2	4120.5	15895368	-	hypothetical protein
CAC2099	260.8	709.8	1963.2	15895369	-	hypothetical protein
CAC2220	5785.2	15817.5	21766.5	15895488	cheA	chemotaxis histidine kinase CheA
CAC2238	345.7	1308.2	968.0	15895506	glgC	ADP-glucose pyrophosphorylase
CAC2356	10866.8	23004.0	28414.3	15895623	pheT	phenylalanyl-tRNA synthetase subunit beta
CAC2396	3607.3	3373.8	5123.0	15895662	-	xylanase/chitin deacetylase
CAC2479	422.7	940.7	1161.7	15895744	-	hypothetical protein
CAC2531	347.8	749.7	610.3	15895794	-	S-adenosylmethionine-dependent methyltransferase
CAC2554	970.3	2765.7	3237.7	15895816	-	hypothetical protein
CAC2556	6677.7	16814.2	20898.5	15895818	-	endoglucanase
CAC2643	14930.3	33428.0	43074.3	15895901	-	hypothetical protein
CAC2654	1055.0	2999.2	3382.5	15895912	pyrB	aspartate carbamoyltransferase
CAC2703	349.3	1623.3	2273.5	15895960	groEL	molecular chaperone GroEL
CAC2778	453.7	757.5	1443.7	15896033	-	rubredoxin
CAC2779	682.5	1889.3	1997.7	15896034	-	hypothetical protein
CAC3198	815.2	1764.0	5292.0	15896445	greA	transcription elongation factor GreA
CAC3261	1351.5	3507.8	3123.2	15896506	ada	methylated-DNA--protein-cysteine methyltransferase
CAC3297	2765.0	5827.5	6504.3	15896541	-	D-alanyl-D-alanine carboxypeptidase
CAC3299	552.2	1388.0	3002.5	15896543	bdhA	NADH-dependent butanol dehydrogenase
CAC3436	16942.2	35759.8	40653.8	15896677	-	alpha-arabinofuranosidase
CAC3451	969.0	1987.8	2324.5	15896692	-	sugar/Na+(H+) simporter
CAC3456	437.0	1479.3	1132.7	15896696	-	hypothetical protein
CAC3556	1262.8	3617.2	3499.5	15896792	-	S-layer protein

Figure 7.8 Genes expressed by *C. pasteurianum* on growth in both pure glycerol and partially purified crude glycerol

Table 7.7 Genes expressed by *C. pasteurianum* on growth in both pure glycerol and partially purified crude glycerol

Gene ID	Expression in Crude Glycerol	Expression in Pure Glycerol	Protein ID	Gene	Product
CA_P0017	786.7	1948.0	15004721	grkb1	spore germination protein, GRKB
CA_P0051	365.5	9914.0	15004755	-	oxidoreductase
CA_P0080	524.0	4120.5	15004784	xerC	site-specific tyrosine recombinase XerC
CA_P0107	1962.5	3633.0	15004810	-	MerR family transcriptional regulator
CA_P0142	23092.2	1957.7	15004845	mbhl	periplasmic hydrogenase large subunit, dehydrogenase
CA_P0166	2039.2	2189.8	15004869	-	hypothetical protein
CAC0021	734.2	2120.2	15893319	serS	seryl-tRNA synthetase
CAC0051	2694.7	1443.7	15893349	-	hypothetical protein
CAC0056	806.5	3350.0	15893353	-	hypothetical protein
CAC0099	9230.7	1869.0	15893395	hemL	glutamate-1-semialdehyde aminotransferase
CAC0221	907.8	13461.5	15893513	-	aspartate aminotransferase
CAC0253	701.0	2459.3	15893545	nifH	nitrogenase iron protein (nitrogenase component II) gene nifH
CAC0264	162.5	1517.8	15893556	-	hypothetical protein
CAC0270	953.5	8761.3	15893562	-	hypothetical protein
CAC0371	80.7	634.5	15893662	-	response regulator
CAC0457	3107.8	7971.2	15893748	-	AcrR family transcriptional regulator
CAC0554	656.5	3499.5	15893844	lyc	1,4-beta-N-acetylmuramidase
CAC0599	3528.2	7980.0	15893888	-	GntR family transcriptional regulator
CAC0633	276.7	1316.5	15893921	-	phosphatase
CAC0754	614.8	2137.7	15894041	-	hypothetical protein
CAC0968	7258.2	1137.2	15894255	-	hypothetical protein
CAC1012	180.8	3123.2	15894299	-	hypothetical protein
CAC1090	592.7	3230.2	15894375	-	5-formyltetrahydrofolate cyclo-ligase
CAC1129	3613.3	19808.3	15894413	-	hypothetical protein
CAC1185	13736.0	37734.7	15894468	-	hypothetical protein
CAC1484	584.5	2693.8	15894763	-	nitroreductase

CAC1496	367.2	1094.3	15894775	-	hypothetical protein
CAC1554	434.8	651.7	15894832	-	heavy metal-binding domain-containing protein
CAC1631	718.3	1429.0	15894909	-	biotin synthase
CAC1648	342.2	5123.0	15894925	-	hypothetical protein
CAC1652	5696.0	610.3	15894929	aspA	aspartate ammonia-lyase
CAC1813	530.0	42192.2	15895089	-	Fe-S oxidoreductase
CAC1850	1375.7	5421.7	15895125	-	RpiR family transcriptional regulator
CAC2098	1489.0	3237.7	15895368	-	hypothetical protein
CAC2099	260.8	1963.2	15895369	-	hypothetical protein
CAC2220	5785.2	5292.0	15895488	cheA	chemotaxis histidine kinase CheA
CAC2238	345.7	1161.7	15895506	glgC	ADP-glucose pyrophosphorylase
CAC2356	10866.8	28414.3	15895623	pheT	phenylalanyl-tRNA synthetase subunit beta
CAC2396	3607.3	5592.7	15895662	-	xylanase/chitin deacetylase
CAC2479	422.7	24991.0	15895744	-	hypothetical protein
CAC2531	347.8	21028.0	15895794	-	S-adenosylmethionine-dependent methyltransferase
CAC2554	970.3	35706.2	15895816	-	hypothetical protein
CAC2556	6677.7	5721.3	15895818	-	endoglucanase
CAC2643	14930.3	5861.8	15895901	-	hypothetical protein
CAC2654	1055.0	51100.0	15895912	pyrB	aspartate carbamoyltransferase
CAC2703	349.3	2273.5	15895960	groEL	molecular chaperone GroEL
CAC2778	453.7	1250.7	15896033	-	rubredoxin
CAC2779	682.5	1997.7	15896034	-	hypothetical protein
CAC3198	815.2	43074.3	15896445	greA	transcription elongation factor GreA
CAC3261	1351.5	6504.3	15896506	ada	methylated-DNA--protein-cysteine methyltransferase
CAC3297	2765.0	57358.5	15896541	-	D-alanyl-D-alanine carboxypeptidase
CAC3299	552.2	3002.5	15896543	bdhA	NADH-dependent butanol dehydrogenase
CAC3436	16942.2	6022.3	15896677	-	alpha-arabinofuranosidase
CAC3451	969.0	2324.5	15896692	-	sugar/Na+(H+) simporter
CAC3456	437.0	1132.7	15896696	-	hypothetical protein
CAC3556	1262.8	20898.5	15896792	-	S-layer protein

Table 7.8 Genes expressed by *C. pasteurianum* on growth in partially purified crude glycerol

Gene ID	Expression Value	Protein ID	Gene	Product
CA_P0084	172.5	15004788	-	hypothetical protein
CA_P0111	415.0	15004814	-	NH2-acetyltransferase
CAC0035	352.3	15893333	-	Serine/threonine phosphatase (inactivated protein)
CAC0165	568.0	15893459	-	ABC transporter permease
CAC0349	234.5	15893640	-	hypothetical protein
CAC0354	997.5	15893645	-	hypothetical protein
CAC0515	100.2	15893806	-	hypothetical protein
CAC0527	124.7	15893817	-	permease
CAC0572	197.0	15893862	-	hypothetical protein
CAC0587	281.7	15893876	-	flavodoxin
CAC0687	565.8	15893975	cysE	Serine acetyltransferase
CAC0727	1110.8	15894014	yifK	amino acid permease
CAC0848	276.3	15894135	-	hypothetical protein
CAC0937	199.8	15894224	hisD	histidinol dehydrogenase
CAC0973	134.5	15894260	argG	argininosuccinate synthase
CAC0981	389.3	15894268	pflA	pyruvate-formate-lyase-activating enzyme
CAC1039	456.0	15894326	-	membrane protein, TerC-like protein
CAC1058	208.5	15894345	-	hypothetical protein
CAC1188	139.7	15894471	-	hypothetical protein
CAC1288	282.0	15894570	rpsU	30S ribosomal protein S21
CAC1422	233.2	15894701	-	hypothetical protein
CAC1632	133.0	15894910	-	endonuclease IV
CAC1802	716.2	15895078	infB	translation initiation factor IF-2
CAC2011	157.8	15895281	fabH	3-oxoacyl-ACP synthase
CAC2032	320.8	15895302	-	hypothetical protein
CAC2228	709.2	15895496	-	hypothetical protein
CAC2303	501.3	15895570	-	stage V sporulation AE, SpoVAE (fragment)
CAC2341	331.3	15895608	-	collagenase protease
CAC2694	197.7	15895952	-	hypothetical protein
CAC2849	467.7	15896103	-	proline/glycine betaine ABC-type transport system, permease component fused to periplasmic component
CAC3123	208.2	15896373	rplN	50S ribosomal protein L14
CAC3163	282.5	15896411	parB	anthranilate synthase component I
CAC3191	393.3	15896439	-	hypothetical protein
CAC3415	410.7	15896656	-	ABC-type multidrug/protein/lipid transport system, ATPase component
CAC3441	83.7	15896682	-	DNA/RNA helicase, SNF2
CAC3486	180.3	15896723	-	multimeric flavodoxin WrbA family protein
CAC3538	797.0	15896774	-	metallo-beta-lactamase superfamily hydrolase
CAC3671	670.5	15896903	amyD	sugar ABC transporter permease
CA_P0084	172.5	15004788	-	hypothetical protein
CA_P0111	415.0	15004814	-	NH2-acetyltransferase
CAC0035	352.3	15893333	-	Serine/threonine phosphatase
CAC0165	568.0	15893459	-	ABC transporter permease
CAC0349	234.5	15893640	-	hypothetical protein

Table 7.9 Genes expressed by *C. pasteurianum* on growth in pure glycerol

S.No.	Gene ID	Expression Value	Protein ID	Gene	Product
1	CA_P0038	928.0	15004742	-	hypothetical protein
2	CA_P0173	773.3	15004876	-	Fe-S oxidoreductase
3	CAC0134	947.2	15893429	-	hypothetical protein
4	CAC0488	289.2	15893779	-	hypothetical protein
5	CAC0505	457.8	15893796	-	cell division membrane protein
6	CAC0567	1449.0	15893857	-	methyltransferase
7	CAC0577	1172.8	15893866	-	endo-arabinase-like protein
8	CAC0595	536.2	15893884	-	glutamine amidotransferase subunit PdxT
9	CAC0648	530.2	15893936	-	molecular chaperone
10	CAC0654	371.8	15893942	-	sensory transduction histidine kinase
11	CAC0741	1302.7	15894028	-	methyl-accepting chemotaxis protein
12	CAC0940	693.8	15894227	hisA	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase
13	CAC1024	361.2	15894311	nadB	L-aspartate oxidase
14	CAC1190	605.8	15894473	-	Fe-S-cluster redox protein
15	CAC1198	268.0	15894481	recJ	single-stranded-DNA-specific exonuclease (recJ)
16	CAC1469	843.7	15894748	-	iron-dependent transcription repressor
17	CAC1647	1184.7	15894924	-	hypothetical protein
18	CAC1671	497.3	15894948	-	recombination factor protein RarA
19	CAC1715	889.8	15894992	-	PLP-dependent aminotransferase
20	CAC1775	113.0	15895051	-	hypothetical protein
21	CAC1994	654.7	15895264	moaB	molybdopterin biosynthesis protein MoaB
22	CAC2042	595.5	15895312	-	ABC transporter permease
23	CAC2165	554.3	15895434	flgB	flagellar basal-body rod protein FlgB
24	CAC2237	1222.8	15895505	glgC	glucose-1-phosphate adenylyltransferase
25	CAC2352	405.3	15895619	-	hypothetical protein
26	CAC2410	574.0	15895676	recX	recombination regulator RecX
27	CAC2436	472.7	15895701	-	hypothetical protein
28	CAC2439	618.2	15895704	-	hypothetical protein
29	CAC2561	1558.7	15895823	-	acetyltransferase
30	CAC2621	934.0	15895879	-	cell wall hydrolase
31	CAC2685	880.8	15895943	-	maltose phosphorylase
32	CAC2739	376.2	15895996	-	acetyltransferase
33	CAC3035	814.3	15896286	-	HAD family phosphatase
34	CAC3054	1314.2	15896305	-	phosphoheptose isomerase
35	CAC3147	8254.8	15896395	rplA	50S ribosomal protein L1
36	CAC3293	1017.8	15896538	-	hypothetical protein
37	CAC3552	1739.7	15896788	-	lactate dehydrogenase
38	CAC3676	808.0	15896908	-	hypothetical protein
39	dhaB	1570.3	3360389	dhaB	glycerol dehydratase subunit B
40	orfZ	376.5	3360392	orfZ	similar to ORFZ of *Citrobacter freundii*

7.4 Conclusion:

The heterologous hybridization of *C. pasteurianum* transcripts (log phase) form three different carbons sources was analyzed on a *C. acetobutylicum* array. Stringency was optimized to maximize positive spots by reducing the temperature and increasing the salt concentration during post hybridization washing. Though, maximum positive spots were observed at 30°C and twice the normal salt concentration, the reproducibility was found to be higher for the experiments conducted at 37°C and twice the normal salt concentration. The transcripts (from the log phase) of *C. pasteurianum* grown on three different carbon sources were analyzed at 37°C and twice the salt concentration.

Using the selection criteria of Tomas et al. only 10 % of the entire genome had positive signals from the transcripts of *C. pasteurianum*. This low number of positive signal after filtration using the equation from Tomas *et al.* can account for a variety of reasons. Analysis of only log phase transcripts, without using transcripts from other phases of growth and product formation can be one of the reasons. Also, there could be a possible difference in the sequence information of *C. acetobutylicum* and *C. pasteurianum.* Apart from these two reasons, the success of a microarray gene expression experiment is dependent on every single step from the printing of the arrays, sample preparation and labeling, hybridization, washing, and image and data analysis. All these steps have their own individual share in introducing variations (Hardiman 2004). The reduction of the variations or reproducibility of the experiments has been optimized over the years for commercially available platforms such as Agilent, Affymetrix, Nimblegen etc., (Hardiman 2004). Most of these commercial platforms contain more than one probe per gene as a quality measure (Hardiman 2004). Use of

these commercial platforms to analyze the gene expression of *C. pasteurianum* on a *C. acetobutylicum* slide may result in a large number of positive spots due to the presence of more than one probe per gene. The presence of more than one probe per gene can be used to confirm the possible existence of sequence dissimilarity.

Moreover, the results of microarray experiments are dependent on the hybridization efficiency between the probes in the slide and the labeled samples. The hybridization depends on probe design, printing of the probes, sample preparation and labeling and the hybridization conditions. Microarray technique is although inexpensive and has the availability of a established informatics, it has the disadvantages of higher background resulting in lower sensitivity(Asmann, Wallace, and Thompson 2008). These disadvantages can be overcome by using Next Generation Sequencing technique such as RNAseq, which are highly sensitive, but are expensive and require a huge informatics infrastructure (Asmann, Wallace, and Thompson 2008).

CHAPTER 8

8. CONCLUSION

The major objective of this study was to develop a process that will result in the conversion of the biodiesel-derived crude glycerol waste stream into a value added stream. The inherent ability of *C. pasteurianum* to grow on glycerol as a carbon source was extended to the use of crude glycerol as the sole carbon, successfully. The use of crude glycerol as a sole carbon, without purification however, resulted in process inhibition. This led to question the effect of impurities in crude glycerol on glycerol fermentation by *C. pasteurianum*.

A detailed analysis of the effect of each impurity in the crude glycerol resulted in the finding that the fatty acids in the crude glycerol, which were not transesterified, had an inhibitory effect. The inhibitory effect of the fatty acids was found to affect glycerol (substrate) transportation through the plasma membrane and hence, affects the overall process by slowing down the utilization of glycerol by the bacteria. The unsaturated fatty acids, particularly linoleic acid, which had a higher degree of unsaturation, had the most detrimental effect by completely inhibiting substrate utilization.

Effects of methanol and salts, the other impurities in crude glycerol, on the fermentation process were found to be negligible. The removal of the fatty acids, without the removal of methanol and salts, by acid precipitation resulted in the formation of

partially purified crude glycerol. The fermentation of the partially purified crude glycerol was both effective and efficient as the fermentation of pure glycerol. This further confirmed the harmful effects of fatty acids in fermentation of crude glycerol by *C. pasteurianum*.

Following the utilization of partially purified crude glycerol in a manner as efficient as pure glycerol, methods to increase butanol yield in glycerol fermentation were also successfully investigated. Use of a secondary carbon source for co-fermentation with glycerol, addition of by-products during glycerol-to-butanol fermentation and addition of vitamins such as biotin were the various methods that were employed, in an effort to increase butanol yields. These experiments led to the findings that glucose when added as a supplementary carbon source for co-metabolism with glycerol, at a ratio of 1:2 (glucose:glycerol), resulting in the doubling of butanol titers and hence, butanol yield. This is a significant discovery in terms of process improvement. This result will open gateways for future research involving the use of glucose from the hydrolysis of lignocellulosic materials, another promising renewable source of raw material.

Butanol at higher concentrations is found to be toxic to the cells and hence, the other objective of this research was to analyze and investigate the homeoviscous response of *C. pasteurianum* towards the harmful toxic effects of butanol on the cell's lipid membranes. Studies of the homeoviscous response of *C. pasteurianum* to exogenous butanol lead to the discovery of the presence of two differential homeoviscous responses in *C. pasteurianum*. This was an astounding discovery, as this is the first report of the presence of more than one homeoviscous response in bacteria towards a toxic organic

compound. In this study, proof of concept for using the microarrays designed for *C. acetobutylicum* to analyze the gene expression of *C. pasteurianum* was established.

Overall, this study resulted in a detailed evaluation of crude glycerol as a carbon source for producing butanol through anaerobic fermentation using cultures of *C. pasteurianum*. Process improvements in the fermentation of crude glycerol were achieved by a comprehensive investigation of the effect of the impurities in the crude glycerol, on crude glycerol fermentation. This led to the establishment that unsaturated fatty acids, with higher degrees of unsaturation, need to be removed from crude glycerol for an effective and efficient fermentation. The effects of the toxic product, butanol, and the tolerance response by the *C. pasteurianum*, were studied and reported for the first time. To further understand the complex link between the changes in various metabolic responses and pathways, and the changes in gene expression, microarray analysis was optimized.

CHAPTER 9

9. FUTURE WORK

The advancements of the high-throughput technology in functional genomics has led to the analysis and understanding of the complex correlations between changes in metabolic activities of cells, under various stress conditions, and changes in gene expression. The presence of two homeoviscous responses in *C. pasteurianum*, introduces new question regarding the genes, proteins and metabolic pathways that are differentially activated and expressed. These questions can be addressed and the complex correlation network can be comprehended by developing a genome-scale model to link the changes in genotype and phenotype. This can be achieved by the use of microarray gene expression analysis and other high throughput techniques such as Next Generation Sequencing, whole cell protein analysis, metabolic flux analysis and metabolic pathway construction.

The use of both experimental biology and functional genomics to analyze and link the changes between genotype and phenotype, can lead to a comprehensive understanding of various genes involved in butanol tolerance, enhanced butanol production and also engineering new industrial strains for sustainable production of butanol. Although, it is feasible to use the sequence information from *C. acetobutylicum* to conduct these studies, the availability of the sequence information of *C. pasteurianum* will result in a much more comprehensive investigation and development of a genome-scale model.

The genome scale model can be further used to develop an industrial strain of *C. pasteurianum*, which will not only be capable of producing butanol at higher concentrations, but will also tolerate butanol at higher concentrations. The techniques for genetically engineering *Clostridia* are well established. Based on the results of the gene expression studies, *C. pasteurianum* can be genetically engineered by extending these established methods of molecular biology. Genetic engineering can result in the development of new strains that will be able to utilize the substrate efficiently, synthesize high titers of butanol and tolerate butanol efficiently by modifying the components of cellular membrane and over expressing proteins that augment butanol tolerance. In short, the development of a genome-scale model will result in a correlating genotype to phenotype during butanol production and butanol stress tolerance. Furthermore, it can also be extended to other microbial fermentation processes involving the synthesis of toxic organic compounds. Thus, the use of *C. pasteurianum* to produce butanol from the fermentation of the biodiesel-derived crude glycerol will lead to the development of an integrated biorefinery.

Appendix A

EFFECT OF KCl

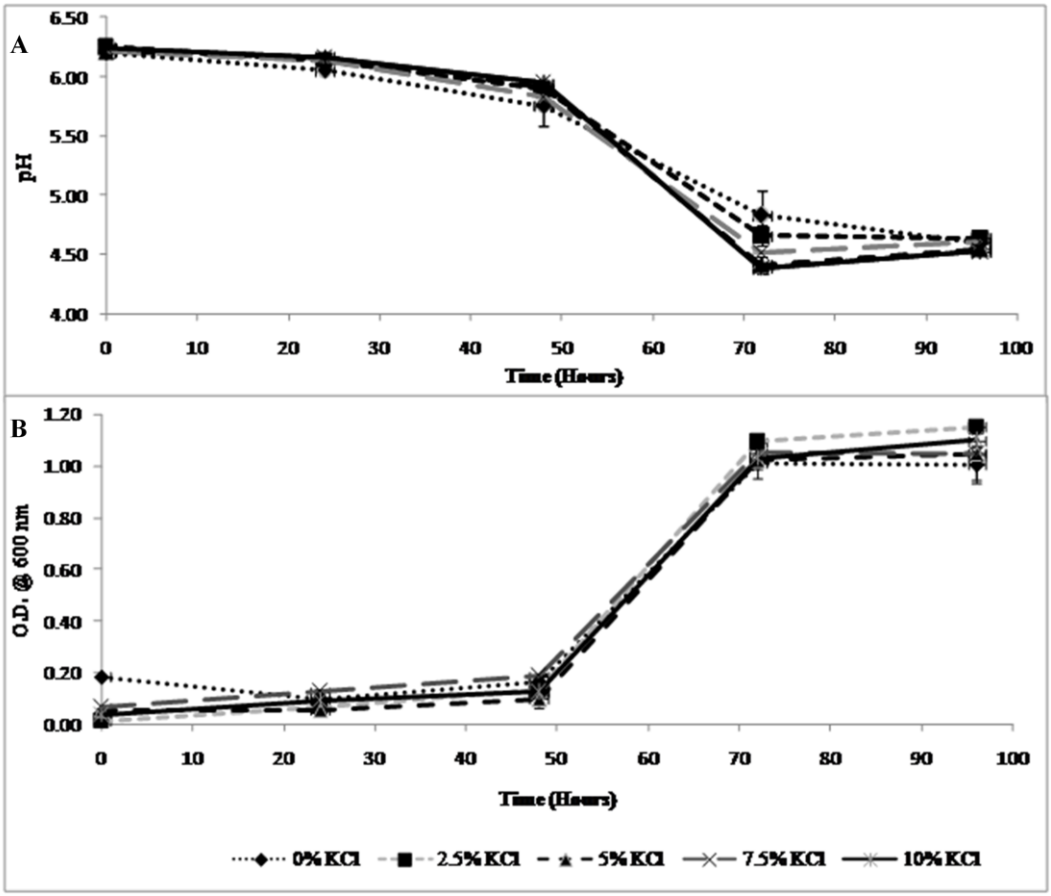


Figure A.1 Effect of potassium chloride. The effect of KCl was studied from 2.5% to 10%. (A) pH profile. (B) Growth.

Appendix B

BUTANOL EVAPORATION DATA

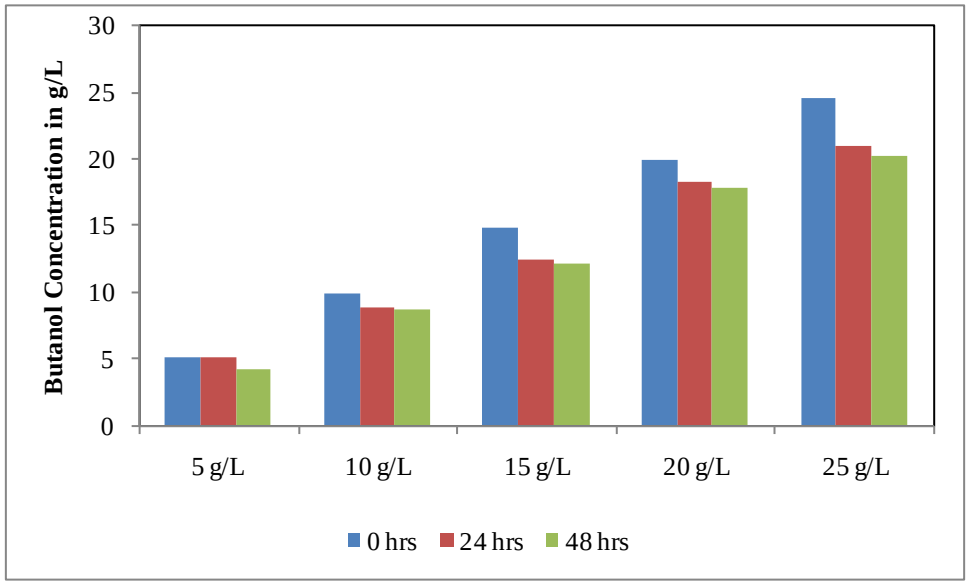


Figure B.1 Butanol evaporation data in Biebl medium from 5 g/L to 25 g/L

Appendix C

NMR SPECTRA

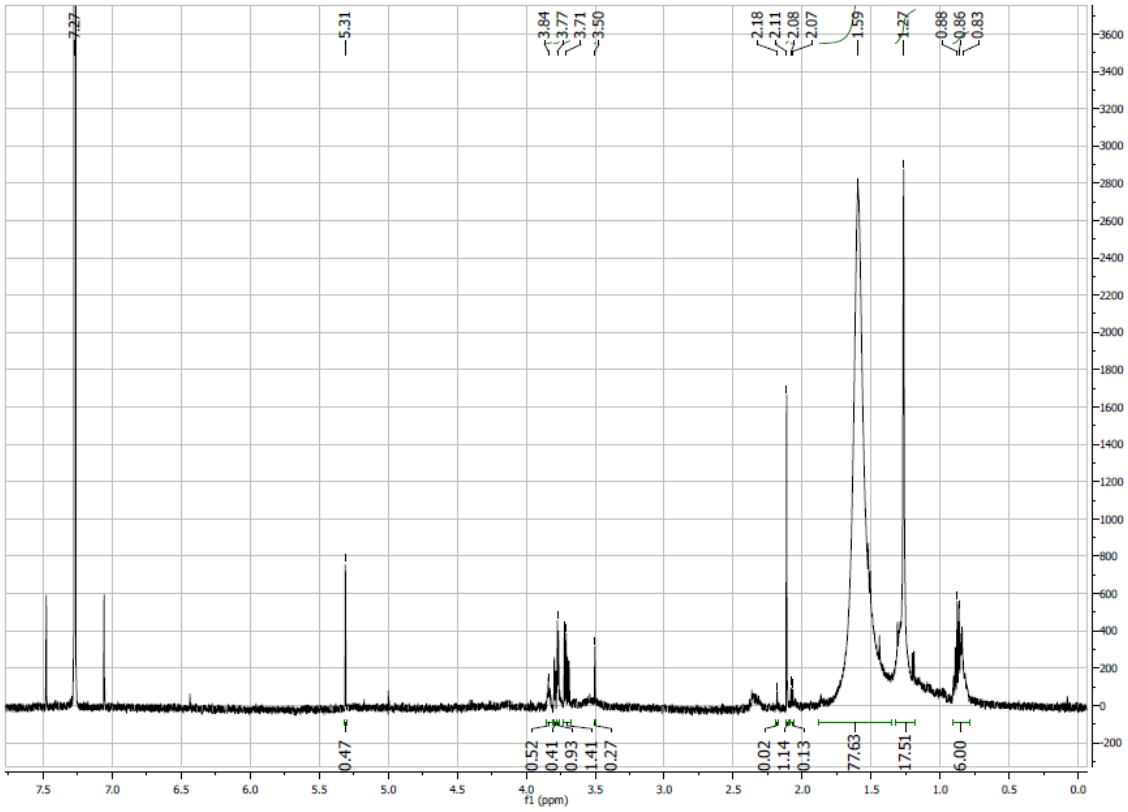


Figure C.1 ^{1}H spectrum of exogenous butanol (0 g/L) on *C. pasteurianum* grown in RCM

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

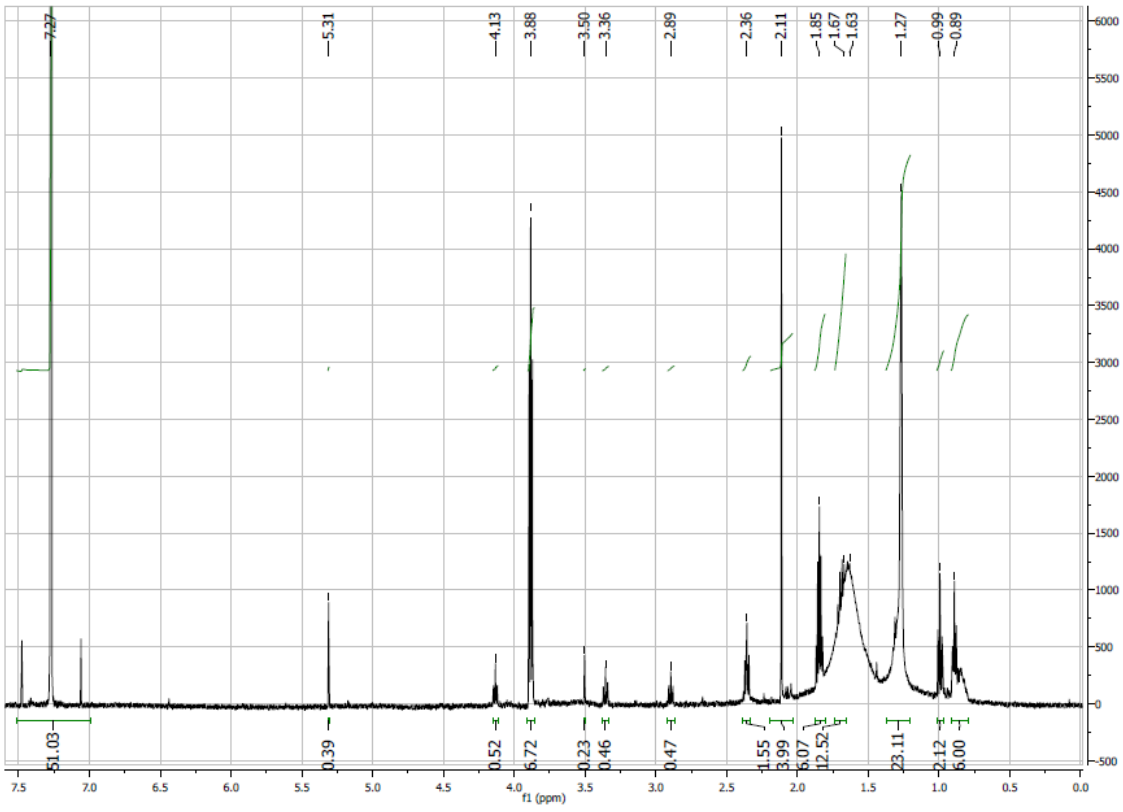


Figure C.2 ^{1}H spectrum of exogenous butanol (5 g/L) on *C. pasteurianum* grown on glucose (RCM)

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

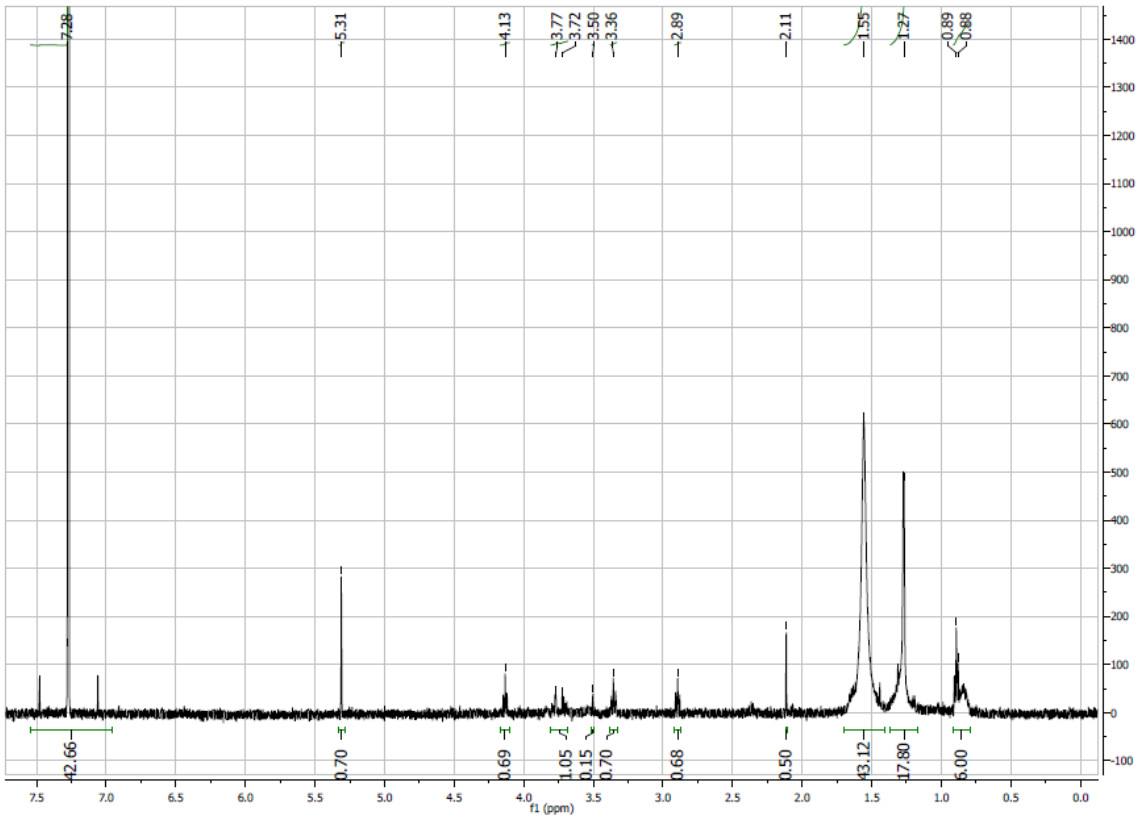


Figure C.3 ^{1}H spectrum of exogenous butanol (10 g/L) on *C. pasteurianum* grown on glucose (RCM)

Parameters

Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

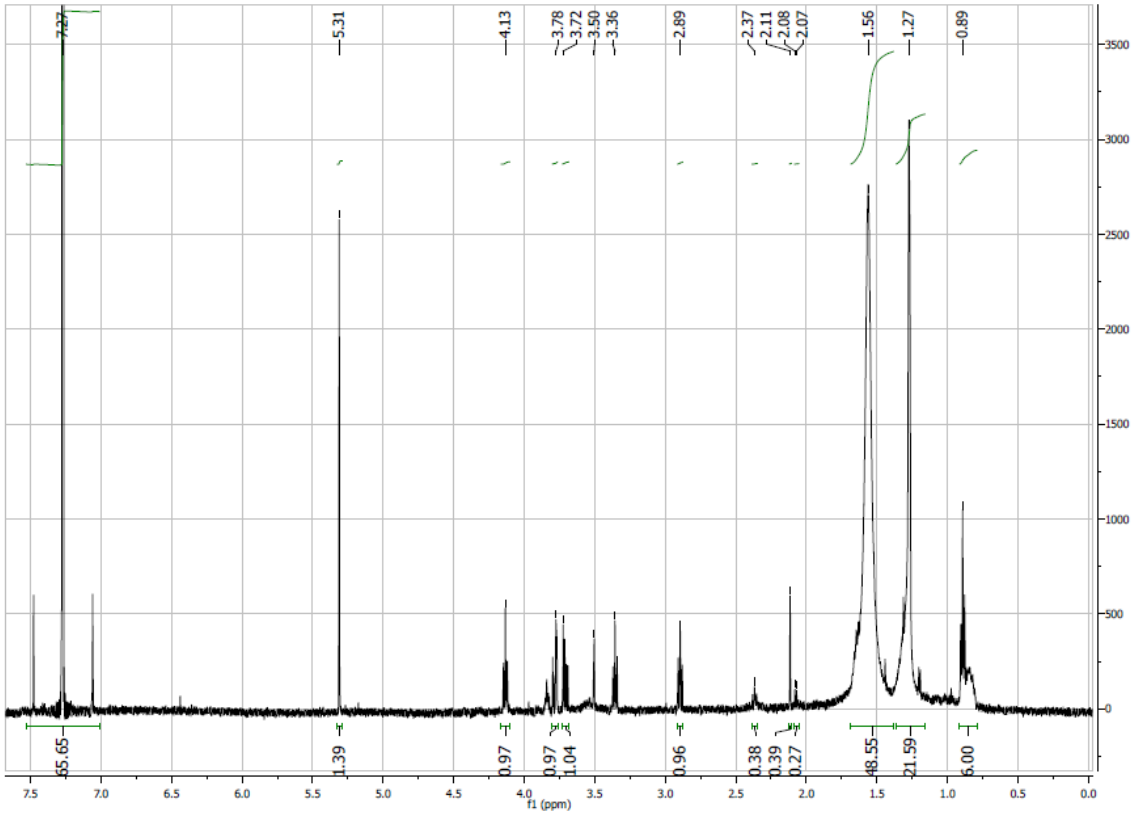


Figure C.4 ^{1}H spectrum of exogenous butanol (15 g/L) on *C. pasteurianum* grown on glucose (RCM)

Parameters

Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

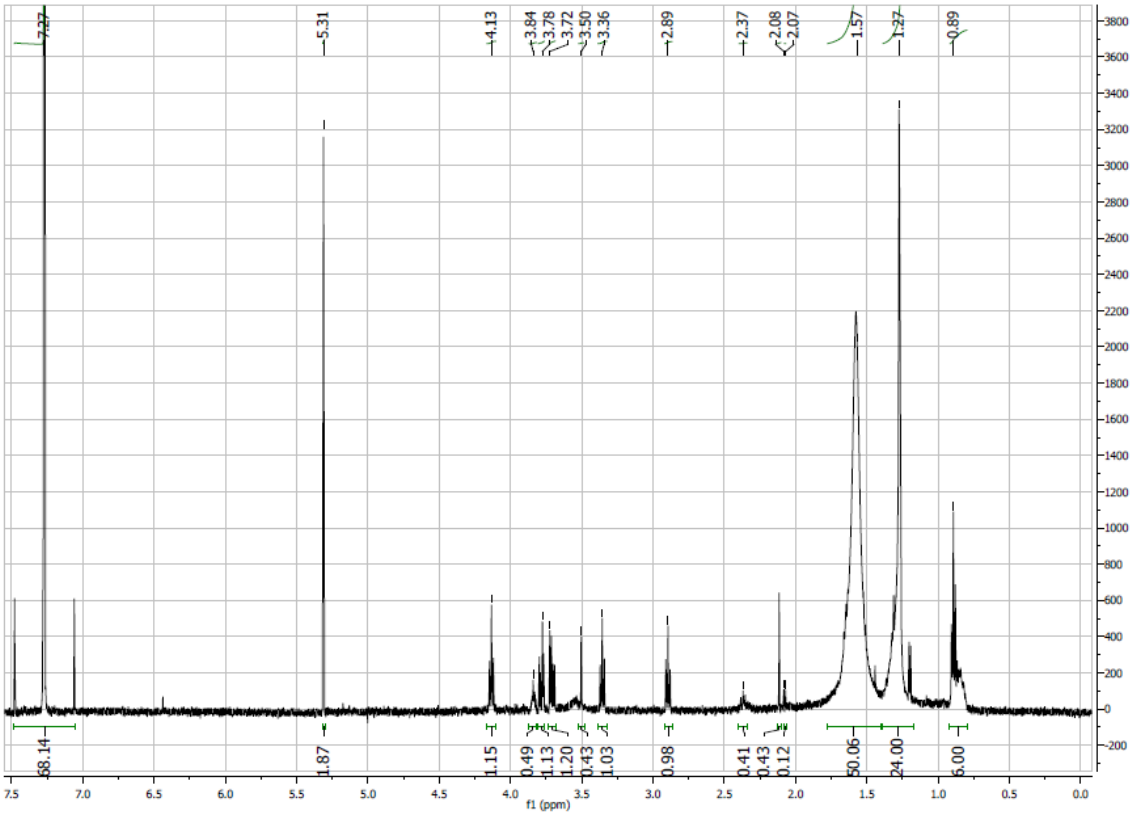


Figure C.5 ^{1}H spectrum of exogenous butanol (20 g/L) on *C. pasteurianum* grown on glucose (RCM)

Parameters

Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

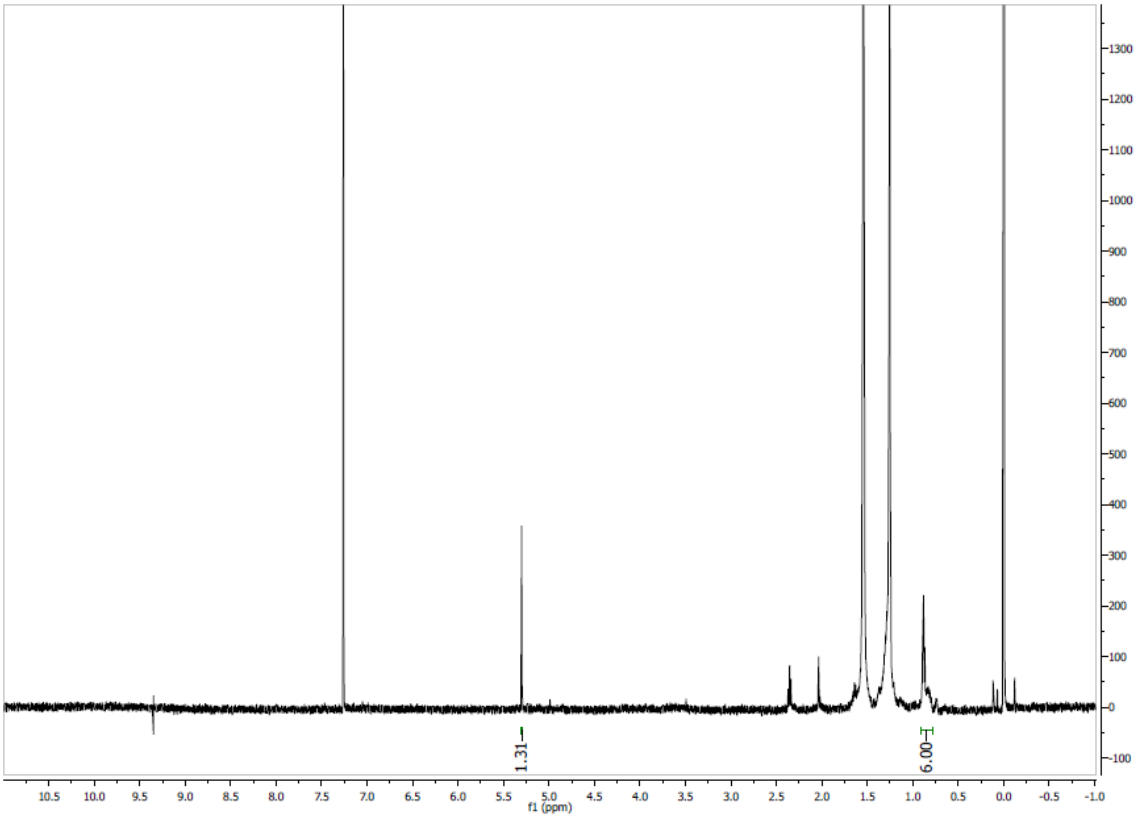


Figure C.6 ^{1}H spectrum of exogenous butanol (0 g/L) on *C. pasteurianum* grown in 50 g/L glucose

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

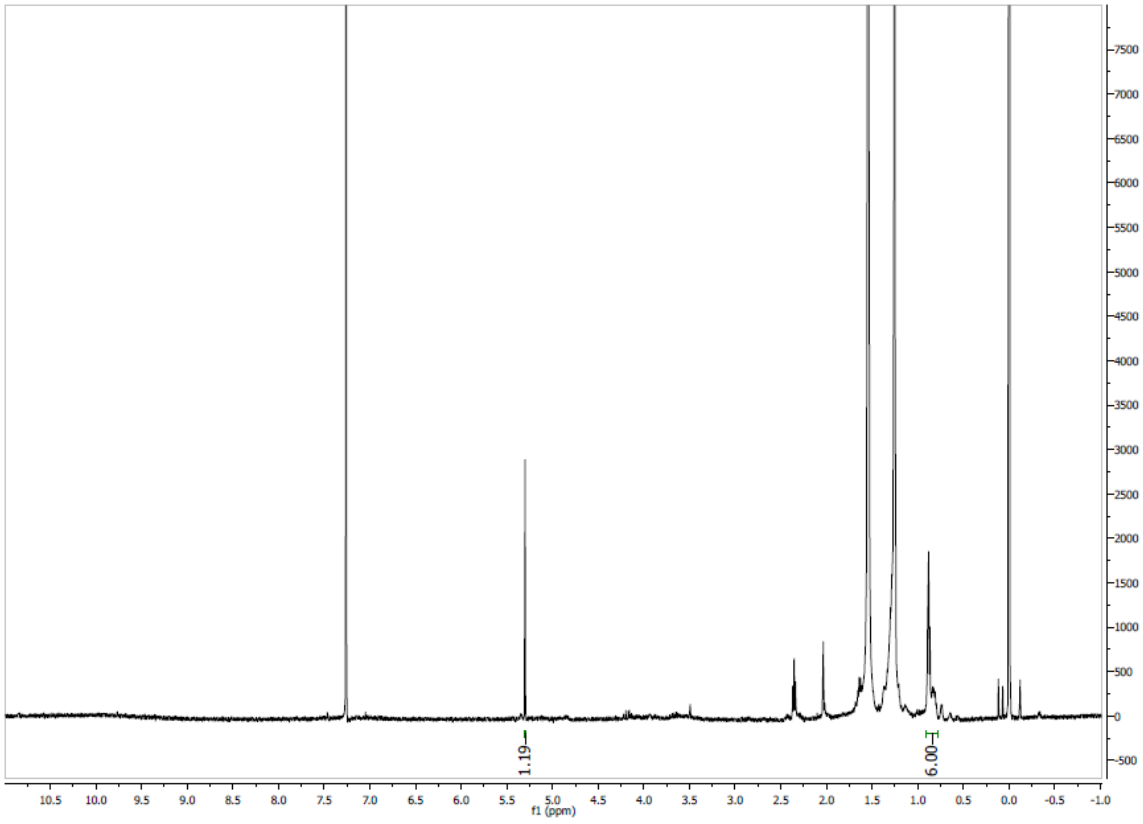


Figure C.7 ^{1}H spectrum of exogenous butanol (2 g/L) on *C. pasteurianum* grown in 50 g/L glucose

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

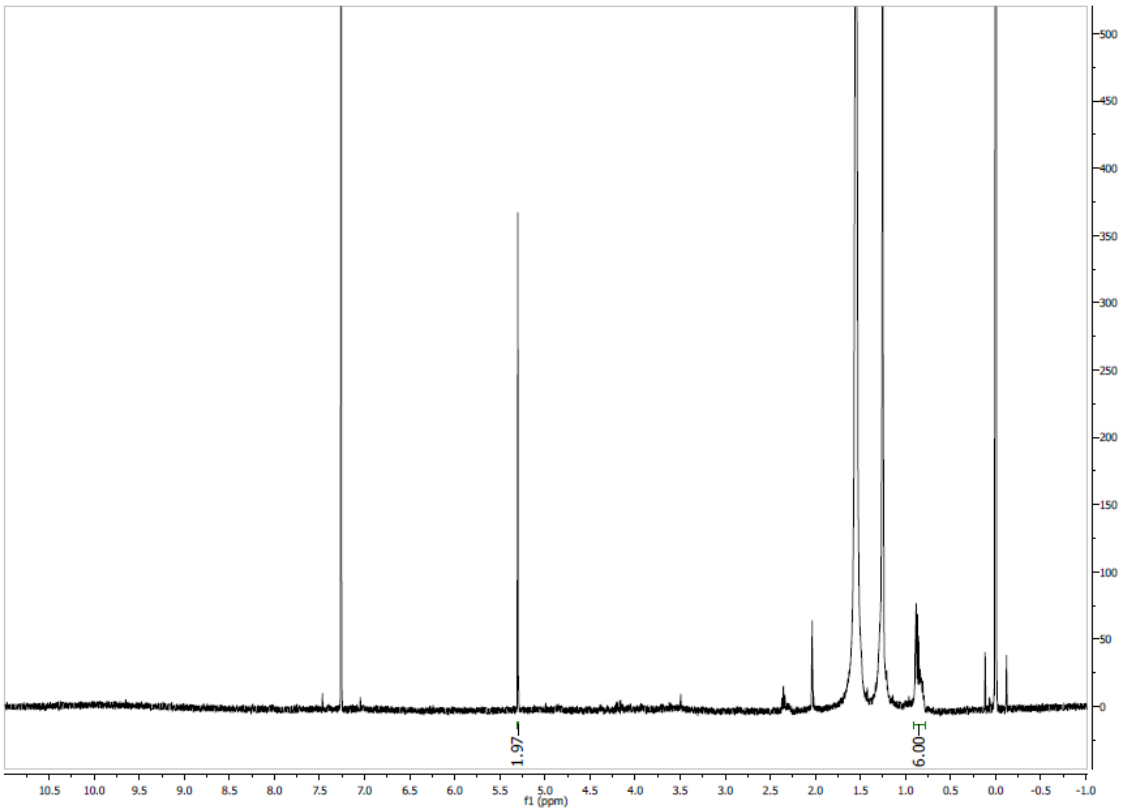


Figure C.8 ^{1}H spectrum of exogenous butanol (4 g/L) on *C. pasteurianum* grown in 50 g/L glucose

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

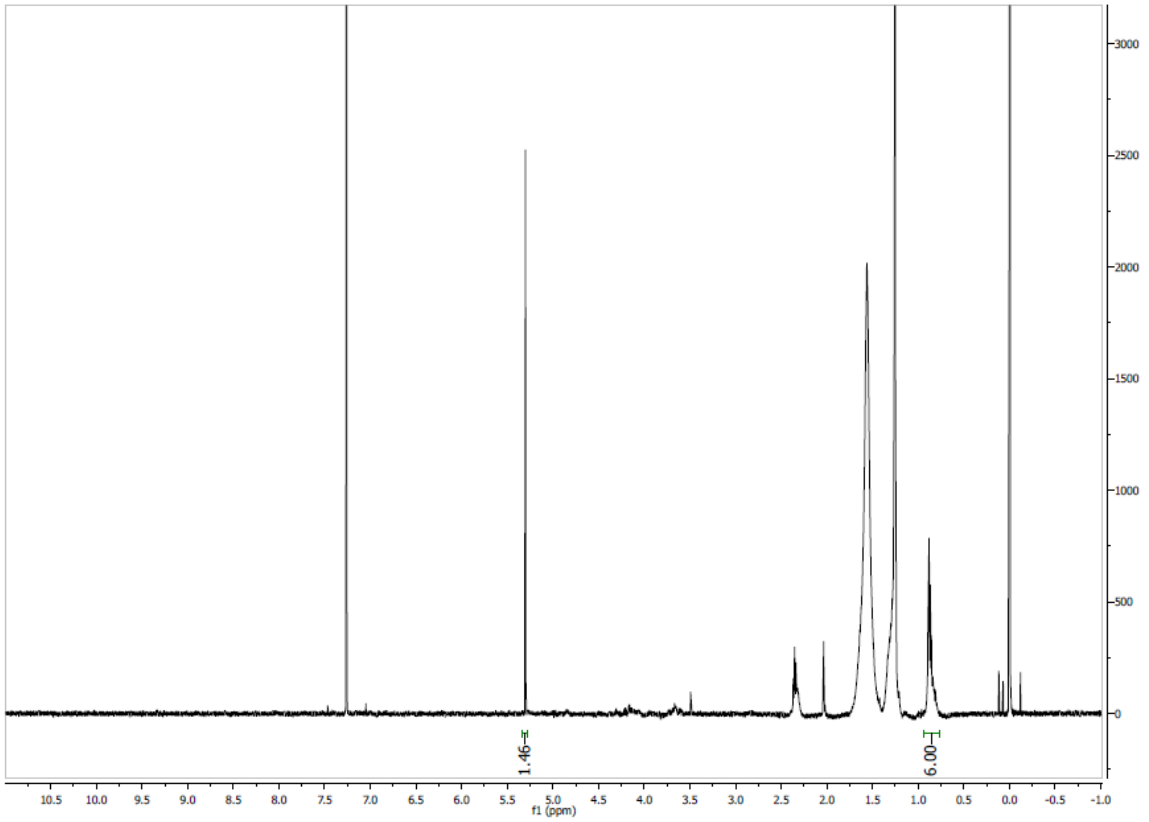


Figure C.9 ^{1}H spectrum of exogenous butanol (6 g/L) on *C. pasteurianum* grown in 50 g/L glucose

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

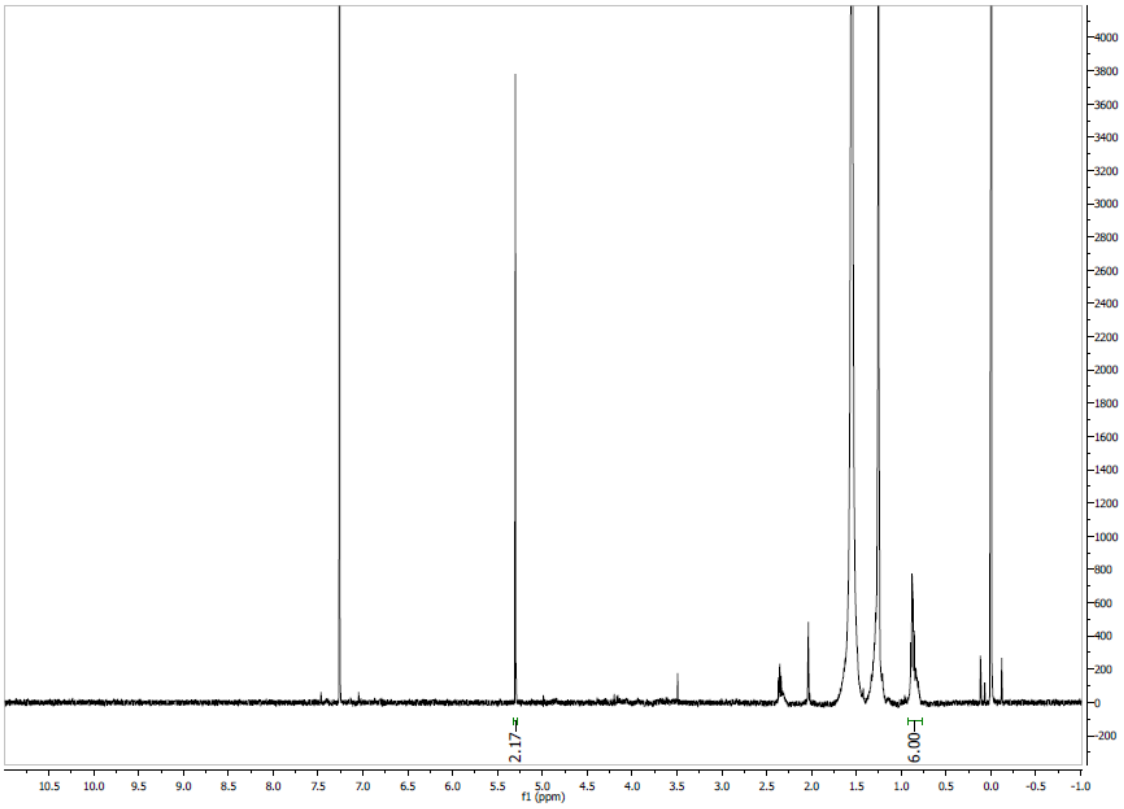


Figure C.10 ^{1}H spectrum of exogenous butanol (8 g/L) on *C. pasteurianum* grown in 50 g/L glucose

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

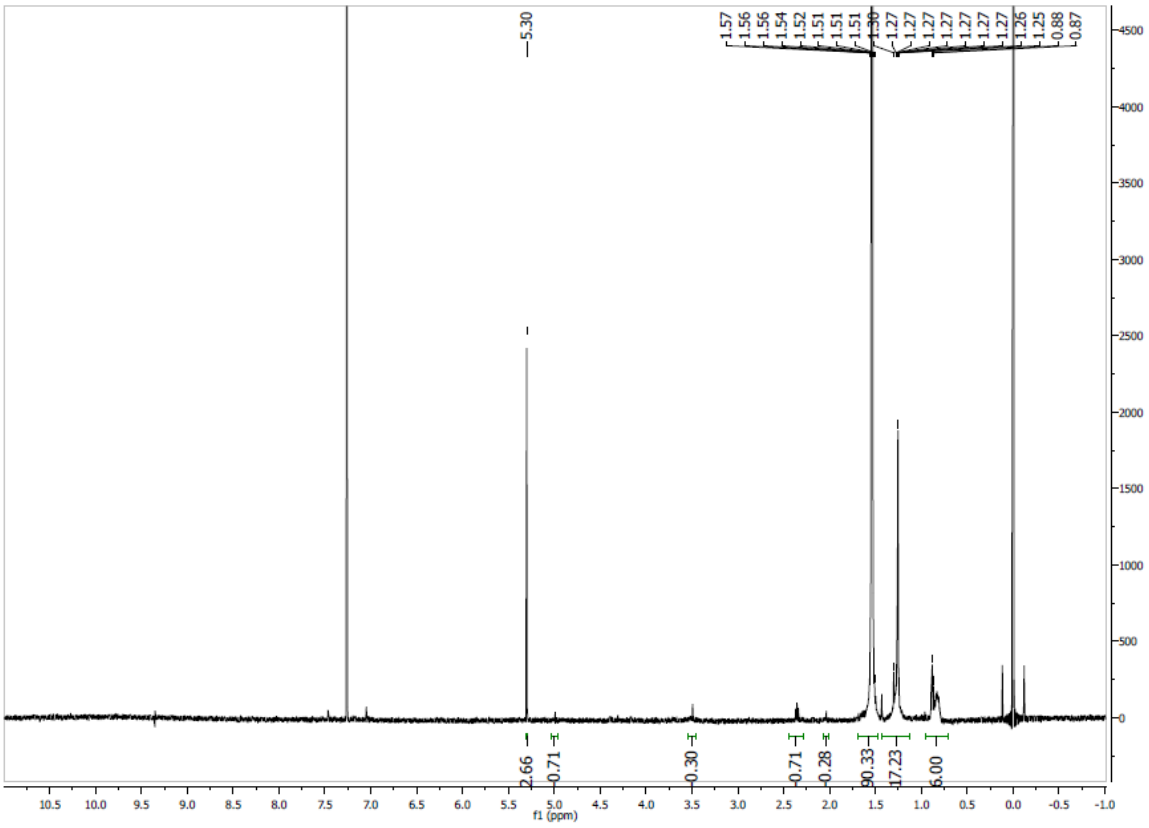


Figure C.11 ^{1}H spectrum of exogenous butanol (10 g/L) on *C. pasteurianum* grown in 50 g/L glucose

Parameters
Frequency: 500 MHz
Experiment: ^{1}H
Temperature: 25ºC
Solvent: $CDCl_3$
Scans: 32

REFERENCES

Abbad-Andaloussi, S., Amine, J., Gerard, P., and Petitdemange, H. 1998. "Effect of Glucose on Glycerol Metabolism by *Clostridium butyricum* DSM 5431." *Journal of Applied Microbiology* 84 (4): 515–522.

Aguilar, L. F., Sotomayor, C. P., and Lissi, E. A. 1996. "Main Phase Transition Depression by Incorporation of Alkanols in DPPC Vesicles in the Gel State: Influence of the Solute Topology." *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 108 (2-3): 287–293.

Alsaker, K. V., Paredes, C. J., and Papoutsakis, E. T. 2005. "Design, Optimization and Validation of Genomic DNA Microarrays for Examining the *Clostridium acetobutylicum* Transcriptome." *Biotechnology and Bioprocess Engineering* 10 (5): 432–443.

Alsaker, K. V., Spitzer, T. R., and Papoutsakis, E. T. 2004. "Transcriptional Analysis of spo0A Overexpression in *Clostridium acetobutylicum* and Its Effect on the Cell's Response to Butanol Stress." *Journal of Bacteriology* 186 (7): 1959.

Annous, B. A., and Blaschek, H.P. 1991. "Isolation and Characterization of *Clostridium acetobutylicum* Mutants with Enhanced Amylolytic Activity." *Applied and Environmental Microbiology* 57 (9): 2544.

Aricha, B., Fishov, I., Cohen, Z., Sikron, N., Pesakhov, S., Khozin-Goldberg, I., Dagan, R., and Porat, N. 2004. "Differences in Membrane Fluidity and Fatty Acid Composition Between Phenotypic Variants of *Streptococcus pneumoniae*." *Journal of Bacteriology* 186 (14): 4638–4644.

Asmann, Y. W., Wallace, M. B., and Thompson, E. A. 2008. "Transcriptome Profiling Using Next-generation Sequencing." *Gastroenterology* 135 (5): 1466–1468.

Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., Chou, K. J.Y., Hanai, T., and Liao. J. C. 2008. "Metabolic Engineering of *Escherichia coli* for 1-butanol Production." *Metabolic Engineering* 10 (6): 305–311.

Baer, S. H., Blaschek, H. P., and Smith. T. L.1987. "Effect of Butanol Challenge and Temperature on Lipid Composition and Membrane Fluidity of Butanol-tolerant *Clostridium acetobutylicum*." *Applied and Environmental Microbiology* 53 (12): 2854.

Baer, S. H., Bryant, D. L., and Blaschek, H. P. 1989. “Electron Spin Resonance Analysis of the Effect of Butanol on the Membrane Fluidity of Intact Cells of *Clostridium acetobutylicum*.” *Applied and Environmental Microbiology* 55 (10): 2729.

Baut, F., Fick, M., Viriot, M. L., Andre, J. C., and Engasser, J. M. 1994. “Investigation of Acetone-butanol-ethanol Fermentation by Fluorescence.” *Applied Microbiology and Biotechnology* 41 (5): 551–555.

Biebl, H. 2001. “Fermentation of Glycerol by *Clostridium pasteurianum*—batch and Continuous Culture Studies.” *Journal of Industrial Microbiology and Biotechnology* 27 (1): 18–26.

Borden, J. R., and Papoutsakis, E. T. 2007. “Dynamics of Genomic-library Enrichment and Identification of Solvent Tolerance Genes for *Clostridium acetobutylicum*.” *Applied and Environmental Microbiology* 73 (9): 3061.

Borden, J. R., Paredes, C. J., and Papoutsakis, E. T. 2005. “Diffusion, Mixing, and Associated Dye Effects in DNA-microarray Hybridizations.” *Biophysical Journal* 89 (5): 3277–3284.

Bowles, L. K., and Ellefson, W. L. 1985. “Effects of Butanol on *Clostridium acetobutylicum*.” *Applied and Environmental Microbiology* 50 (5): 1165.

Brown, P. O., and Botstein, D. 1999. “Exploring the New World of the Genome with DNA Microarrays.” *Nature Genetics* 21 (1 Suppl): 33–37.

Buckley, B. A. 2007. “Comparative Environmental Genomics in Non-model Species: Using Heterologous Hybridization to DNA-based Microarrays.” *Journal of Experimental Biology* 210 (9): 1602–1606.

Cevc, G., and Marsh, D. 1987. *Phospholipid Bilayers: Physical Principles and Models*. Vol. 5. Wiley.

Chen, C. K, and Blaschek, H. P. 1999. “Acetate Enhances Solvent Production and Prevents Degeneration in *Clostridium beijerinckii* BA101.” *Applied Microbiology and Biotechnology* 52 (2): 170–173.

Chi, Z., Pyle, D., Wen, Z., Frear, C., and Chen, S. 2007. “A Laboratory Study of Producing Docosahexaenoic Acid from Biodiesel-waste Glycerol by Microalgal Fermentation.” *Process Biochemistry* 42 (11) (November): 1537–1545. doi:doi: 10.1016/j.procbio.2007.08.008.

Colin, T., Bories, A., Lavigne, C., and Moulin, G. 2001. “Effects of Acetate and Butyrate During Glycerol Fermentation by *Clostridium butyricum*.” *Current Microbiology* 43 (4): 238–243.

Cummins, C. S., and Johnson, J. L. 1971. “Taxonomy of the Clostridia: Wall Composition and DNA Homologies in *Clostridium butyricum* and Other Butyric Acid-producing Clostridia.” *Journal of General Microbiology* 67 (1): 33.

Dabrock, B., Bahl, H., and G. Gottschalk. 1992. “Parameters Affecting Solvent Production by *Clostridium pasteurianum*.” *Applied and Environmental Microbiology* 58 (4): 1233.

Desbois, A. P., and V. J Smith. 2010. “Antibacterial Free Fatty Acids: Activities, Mechanisms of Action and Biotechnological Potential.” *Applied Microbiology and Biotechnology* 85 (6): 1629–1642.

Dharmadi, Y., Murarka, A., and Gonzalez, R. 2006. “Anaerobic Fermentation of Glycerol by Escherichia Coli: a New Platform for Metabolic Engineering.” *Biotechnology and Bioengineering* 94 (5): 821–829.

Dombek, K. M., and Ingram, L. O. 1984. “Effects of Ethanol on the Escherichia Coli Plasma Membrane.” *Journal of Bacteriology* 157 (1): 233–239.

Dunlop, M. J., Dossani, Z.Y., Szmidt, H.L., Chu, H.C., Lee,T.S., Keasling, J.D., Hadi, M.Z., and Mukhopadhyay, A. 2011. “Engineering Microbial Biofuel Tolerance and Export Using Efflux Pumps.” *Mol Syst Biol* 7 (May 10). doi:10.1038/msb.2011.21. http://dx.doi.org/10.1038/msb.2011.21.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. 2004. “Butanol Fermentation Research: Upstream and Downstream Manipulations.” *The Chemical Record* 4 (5): 305–314.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. 2007. “Bioproduction of Butanol from Biomass: From Genes to Bioreactors.” *Current Opinion in Biotechnology* 18 (3): 220–227.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. 2004. “Acetone Butanol Ethanol (ABE) Production from Concentrated Substrate: Reduction in Substrate Inhibition by Fed-batch Technique and Product Inhibition by Gas Stripping.” *Applied Microbiology and Biotechnology* 63 (6): 653–658.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. 2007. “Butanol Production from Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation.” *Biotechnology and Bioengineering* 97 (6): 1460–1469.

Fischer, C. R., Klein-Marcuschamer, D., and Stephanopoulos, G. 2008. “Selection and Optimization of Microbial Hosts for Biofuels Production.” *Metabolic Engineering* 10 (6): 295–304.

Furusawa, H., and Koyama, N. 2004. “Effect of Fatty Acids on the Membrane Potential of an Alkaliphilic Bacillus.” *Current Microbiology* 48 (3): 196–198.

Galbraith, H., and Miller, T. B.. 1973. “Effect of Long Chain Fatty Acids on Bacterial Respiration and Amino Acid Uptake.” *Journal of Applied Microbiology* 36 (4): 659–675.

Gonzalez-Pajuelo, M., Andrade, J. C., and Vasconcelos, I. 2004. “Production of 1, 3-propanediol by *Clostridium butyricum* VPI 3266 Using a Synthetic Medium and Raw Glycerol.” *Journal of Industrial Microbiology and Biotechnology* 31 (9): 442–446.

Hardiman, G. 2004. “Microarray Platforms-comparisons and Contrasts.” *Pgs* 5 (5): 487–502.

Heipieper, H. J., Weber, F. J., Sikkema, J., Keweloh, H., and de Bont, J. A. M. 1994. “Mechanisms of Resistance of Whole Cells to Toxic Organic Solvents.” *Trends in Biotechnology* 12 (10): 409–415.

Homann, T., Tag, C., Biebl, H., Deckwer, W. D., and Schink, B. 1990. “Fermentation of Glycerol to 1, 3-propanediol by *Klebsiella* and *Citrobacter* Strains.” *Applied Microbiology and Biotechnology* 33 (2): 121–126.

Iiyama, S., Toko, K., Murata, T., Ichinose, H., Suezaki, Y., Kamaya, H., Ueda, I., and Yamafuji, K. 1992. “Cutoff Effect of N-alkanols in an Excitable Model Membrane Composed of Dioleyl Phosphate.” *Biophysical Chemistry* 45 (2) (December): 91–100. doi:10.1016/0301-4622(92)87001-Y.

Ingram, L. O. 1976. “Adaptation of Membrane Lipids to Alcohols.” *Journal of Bacteriology* 125 (2): 670.

Isar, J., and Rangaswamy, V. 2012. “Improved N-butanol Production by Solvent Tolerant *Clostridium beijerinckii*.” *Biomass and Bioenergy* 37 (0) (February): 9–15. doi:10.1016/j.biombioe.2011.12.046.

Jin, P., Lu, S., Huang, H. Luo, F., and Li, S. 2011. “Enhanced Reducing Equivalent Generation for 1, 3-Propanediol Production Through Cofermentation of Glycerol and Xylose by *Klebsiella pneumoniae*.” *Applied Biochemistry and Biotechnology*: 1–11.

Johnson, D. T., and Taconi, K. A. 2007. “The Glycerin Glut: Options for the Value Added Conversion of Crude Glycerol Resulting from Biodiesel Production.” *Environmental Progress* 26 (4): 338–348.

Jones, D. T., and Woods, D. R. 1986. “Acetone-butanol Fermentation Revisited.” *Microbiology and Molecular Biology Reviews* 50 (4): 484.

Kabelitz, N., Santos, P. M., and Heipieper, H. J. 2003. “Effect of Aliphatic Alcohols on Growth and Degree of Saturation of Membrane Lipids in *Acinetobacter calcoaceticus*.” *FEMS Microbiology Letters* 220 (2): 223–227.

Knoshaug, E. P, and Zhang, M. 2009. “Butanol Tolerance in a Selection of Microorganisms.” *Applied Biochemistry and Biotechnology* 153 (1): 13–20.

Korkola, J. E., Estep, A. L. H., Pejavar, S., De Vries, S., Jensen, R., and Waldman, F. M. 2003. “Optimizing Stringency for Expression Microarrays.” *Biotechniques* 35 (4): 828–839.

Kurniawan, Y., Bothun, G. D., Venkataramanan, K.P., and C. Scholz. 2012. “n-Butanol Partitioning and Phase Behavior in DPPC/DOPC Membranes.” *Journal of Physical Chemistry B*.

Lăzăroaie, M. M. 2009. “Mechanisms Involved In Organic Solvent Resistance in Gram-Negative Bacteria.” *World Academy of Science, Engineering and Technology*.

Lee, J. Y., Yang, K. S., Jang, S. A., Sung, B. H., and Kim, S. C. “Engineering Butanol Tolerance in *Escherichia coli* with Artificial Transcription Factor Libraries.” *Biotechnology and Bioengineering*.

Lepage, C., Fayolle, F., Hermann, M., and Vandecasteele, J. P. 1987. “Changes in Membrane Lipid Composition of *Clostridium acetobutylicum* During Acetone-butanol Fermentation: Effects of Solvents, Growth Temperature and pH.” *Journal of General Microbiology* 133 (1): 103.

Liang, Y., Sarkany,N., Cui, Y., and Blackburn, J.W. 2010. “Batch Stage Study of Lipid Production from Crude Glycerol Derived from Yellow Grease or Animal Fats Through Microalgal Fermentation.” *Bioresource Technology* 101 (17) (September): 6745–6750. doi:doi: 10.1016/j.biortech.2010.03.087.

Liu, S., and Qureshi, N. 2009. “How Microbes Tolerate Ethanol and Butanol.” *New Biotechnology* 26 (3-4): 117–121.

Liyanage, H., Young, M., and Kashket, E. R. 2000. “Butanol Tolerance of *Clostridium beijerinckii* NCIMB 8052 Associated with Down-regulation of gldA by Antisense RNA.” *Journal of Molecular Microbiology and Biotechnology* 2 (1): 87–93.

Löbbecke, L., and Cevc, G. 1995. “Effects of Short-chain Alcohols on the Phase Behavior and Interdigitation of Phosphatidylcholine Bilayer Membranes.” *Biochimica Et Biophysica Acta (BBA)-Biomembranes* 1237 (1): 59–69.

Luers, F., Seyfried, M., Daniel, R., and Gottschalk, G. 1997. “Glycerol Conversion to 1, 3 Propanediol by *Clostridium pasteurianum*: Cloning and Expression of the Gene Encoding 1, 3 Propanediol Dehydrogenase.” *FEMS Microbiology Letters* 154 (2): 337–345.

Macis, L., Daniel, R., and Gottschalk, G. 1998. “Properties and Sequence of the Coenzyme B12 Dependent Glycerol Dehydratase of *Clostridium pasteurianum*.” *FEMS Microbiology Letters* 164 (1): 21–28.

Malaoui, H., and Marczak, R. 2001. “Influence of Glucose on Glycerol Metabolism by Wild-type and Mutant Strains of *Clostridium butyricum* E5 Grown in Chemostat Culture.” *Applied Microbiology and Biotechnology* 55 (2): 226–233.

Mitchell, W. J., Roohi, M. S., Mosely, M. J., and Booth, I. R. 1987. "Regulation of Carbohydrate Utilization in *Clostridium pasteurianum*." *Microbiology* 133 (1): 31.

Monot, F., Engasser, J. M., and Petitdemange, H. 1984. "Influence of pH and Undissociated Butyric Acid on the Production of Acetone and Butanol in Batch Cultures of *Clostridium acetobutylicum*." *Applied Microbiology and Biotechnology* 19 (6): 422–426.

Muranushi, N., Takagi, N., Muranishi, S., and Sezaki, H. 1981. "Effect of Fatty Acids and Monoglycerides on Permeability of Lipid Bilayer." *Chemistry and Physics of Lipids* 28 (3): 269–279.

Narberhaus, F., and Bahl, H. 1992. "Cloning, Sequencing, and Molecular Analysis of the groESL Operon of *Clostridium acetobutylicum*." *Journal of Bacteriology* 174 (10): 3282.

Pagliaro, M., Ciriminna, R., Kimura, H., Rossi, M., and Della Pina, C. 2007. "From Glycerol to Value Added Products." *Angewandte Chemie International Edition* 46 (24): 4434–4440.

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., and Marc, I. 2002. "*Yarrowia lipolytica* as a Potential Producer of Citric Acid from Raw Glycerol." *Journal of Applied Microbiology* 92 (4): 737–744.

Papanikolaou, S., Ruiz-Sanchez, P., Pariset, B., Blanchard, F., and Fick, M. 2000. "High Production of 1, 3-propanediol from Industrial Glycerol by a Newly Isolated *Clostridium butyricum* Strain." *Journal of Biotechnology* 77 (2-3): 191–208.

Papoutsakis, E. T. 2008. "Engineering Solventogenic Clostridia." *Current Opinion in Biotechnology* 19 (5): 420–429.

Paredes, C. J, Senger, R. S., Spath, I. S., Borden, J. R., Sillers, R., and Papoutsakis, E. T. 2007. "A General Framework for Designing and Validating Oligomer-based DNA Microarrays and Its Application to *Clostridium acetobutylicum*." *Applied and Environmental Microbiology* 73 (14): 4631.

Petitdemange, E., Dürr, C., Andaloussi, S. A., and Raval, G. 1995. "Fermentation of Raw Glycerol to 1, 3-propanediol by New Strains of *Clostridium butyricum*." *Journal of Industrial Microbiology and Biotechnology* 15 (6): 498–502.

Petrache, H. I., Tristram-Nagle, S., Harries, D., Ku erka, N., Nagle, J. F., and Parsegian, V. A. 2006. "Swelling of Phospholipids by Monovalent Salt." *Journal of Lipid Research* 47 (2): 302.

Pyle, D. J., Garcia, R. A., and Wen, Z. 2008. "Producing Docosahexaenoic Acid (DHA)-rich Algae from Biodiesel-derived Crude Glycerol: Effects of Impurities on DHA Production and Algal Biomass Composition." *Journal of Agricultural and Food Chemistry* 56 (11): 3933–3939.

Qureshi, N., and Blaschek, H. P. 2001. “ABE Production from Corn: a Recent Economic Evaluation.” *Journal of Industrial Microbiology and Biotechnology* 27 (5): 292–297.

Ramos, J.L., Duque, E., Gallegos, M., Godoy, P.,Ramos-González, M.I., Rojas, A., Terán, W., and Segura, A. 2002. “Mechanisms of Solvent Tolerance in Gram-negative Bacteria.” *Annual Review of Microbiology* 56 (1): 743–768.

Raynaud, C., Sarçabal, P., Meynial-Salles, I., Croux, C., and Soucaille, P. 2003. “Molecular Characterization of the 1, 3-propanediol (1, 3-PD) Operon of *Clostridium butyricum*.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (9): 5010.

Renn, S., Aubin-Horth, N., and Hofmann, H. 2004. “Biologically Meaningful Expression Profiling Across Species Using Heterologous Hybridization to a cDNA Microarray.” *BMC Genomics* 5 (1): 42.

Saint-Amans, S., Girbal, L., Andrade, J., Ahrens, K., and Soucaille, P. 2001. “Regulation of Carbon and Electron Flow in *Clostridium butyricum* VPI 3266 Grown on Glucose-glycerol Mixtures.” *Journal of Bacteriology* 183 (5): 1748.

Sardessai, Y., and Bhosle, S. 2002. "Toleranceof Bacteria to Organic Solvents." *Research in Microbiology*. 153 (5): 263-268.

Sauer, M., Branduardi, P., Gasser, B., Valli, M., Maurer, M., Porro, D., and Mattanovich, D. 2004. “Differential Gene Expression in Recombinant *Pichia pastoris* Analysed by Heterologous DNA Microarray Hybridisation.” *Microbial Cell Factories* 3 (1): 17.

Segura, A., Duque, E., Mosqueda, G., Ramos, J. L., and Junker, F.. 1999. “Multiple Responses of Gram-negative Bacteria to Organic Solvents.” *Environmental Microbiology* 1 (3): 191–198.

Sinensky, M. 1974. “Homeoviscous Adaptation—a Homeostatic Process That Regulates the Viscosity of Membrane Lipids in *Escherichia coli*.” *Proceedings of the National Academy of Sciences* 71 (2): 522.

Soni, B. K., Soucaille, P., and Goma, G. 1987. “Continuous Acetone-butanol Fermentation: Influence of Vitamins on the Metabolic Activity of *Clostridium acetobutylicum*.” *Applied Microbiology and Biotechnology* 27 (1): 1–5.

Sparling, M. L., Zidovetzki, R., Muller, L., and Chan, S. I. 1989. “Analysis of Membrane Lipids by 500 MHz 1H NMR.” *Analytical Biochemistry* 178 (1): 67–76.

Steen, E. J., Chan, R., Prasad, N., Myers, S., Petzold, C. J., Redding, A., Ouellet, M., and Keasling, J. D. 2008. “Metabolic Engineering of *Saccharomyces cerevisiae* for the Production of N-butanol.” *Microbial Cell Factories* 7 (1): 36.

Thompson, L., Cockayne, A., and Spiller, R. C. 1994. “Inhibitory Effect of Polyunsaturated Fatty Acids on the Growth of *Helicobacter pylori*: a Possible Explanation of the Effect of Diet on Peptic Ulceration.” *Gut* 35 (11): 1557.

Tomas, C. A., Alsaker, K. V., Bonarius, H.P.J., Hendriksen, W. T., Yang, H., Beamish, J. A., Paredes, C. J., and Papoutsakis, E. T. 2003. “DNA Array-based Transcriptional Analysis of Asporogenous, Nonsolventogenic *Clostridium acetobutylicum* Strains SKO1 and M5.” *Journal of Bacteriology* 185 (15): 4539.

Tomas, C. A., Beamish, J., and Papoutsakis, E. T. 2004. “Transcriptional Analysis of Butanol Stress and Tolerance in *Clostridium acetobutylicum*.” *Journal of Bacteriology* 186 (7): 2006.

Tomas, C. A., Welker, N. E., and Papoutsakis, E. T. 2003. “Overexpression of groESL in *Clostridium acetobutylicum* Results in Increased Solvent Production and Tolerance, Prolonged Metabolism, and Changes in the Cell’s Transcriptional Program.” *Applied and Environmental Microbiology* 69 (8): 4951.

Tummala, S. B., Junne, S. G., Paredes, C. J., and Papoutsakis, E. T. 2003. “Transcriptional Analysis of Product Concentration Driven Changes in Cellular Programs of Recombinant *Clostridium acetobutylicum* strains.” *Biotechnology and Bioengineering* 84 (7): 842–854.

Urbanchuk, J. M. 2011. “Economic Impact of Removing the Biodiesel Tax Credit for 2010 and Implementation of RFS2 Targets Through 2015. Prepared for the National Biodiesel Board. June 8. http://www.biodiesel.org/resources/reportsdatabase/reports/gen/20110608_GEN-425.pdf”. Prepared for National Biodiesel Board. http://www.biodiesel.org/resources/reportsdatabase/reports/gen/20110608_GEN-425.pdf.

Vasconcelos, I., Girbal, L., and Soucaille, P. 1994. “Regulation of Carbon and Electron Flow in *Clostridium acetobutylicum* Grown in Chemostat Culture at Neutral pH on Mixtures of Glucose and Glycerol.” *Journal of Bacteriology* 176 (5): 1443.

Vollherbst-Schneck, K., Sands, J. A., and Montenecourt, B. S. 1984. “Effect of Butanol on Lipid Composition and Fluidity of *Clostridium acetobutylicum* ATCC 824.” *Applied and Environmental Microbiology* 47 (1): 193–194.

Wang, F., Kashket, S., and Kashket, E. R. 2005. “Maintenance of {Delta} pH by a Butanol-tolerant Mutant of *Clostridium beijerinckii*.” *Microbiology* 151 (2): 607.

Willker, W., and Leibfritz, D. 1998. “Assignment of Mono- and Polyunsaturated Fatty Acids in Lipids of Tissues and Body Fluids.” *Magnetic Resonance in Chemistry* 36 (S1): S79–S84. doi:10.1002/(SICI)1097-458X(199806)36:133.0.CO;2-Z.

Winkler, J., Rehmann, M., and Kao, K. C. 2010. “Novel *Escherichia coli* Hybrids with Enhanced Butanol Tolerance.” *Biotechnology Letters*: 1–6.

Yazdani, S. S., and Gonzalez, R. 2007. “Anaerobic Fermentation of Glycerol: a Path to Economic Viability for the Biofuels Industry.” *Current Opinion in Biotechnology* 18 (3): 213–219.

Yokochi, T., Honda, D. Higashihara, T. and Nakahara, T. 1998. “Optimization of Docosahexaenoic Acid Production by *Schizochytrium limacinum* SR21.” *Applied Microbiology and Biotechnology* 49 (1): 72–76.

Yusof, S., Takriff, M. S., Amir, A., Kadhum, H., Mohammad, A. W., and Jahim, J. 2010. “The Effect of Initial Butyric Acid Addition on ABE Fermentation by *C. acetobutylicum* NCIMB 619.” *Journal of Applied Sciences* 10 (21): 2709–2712.