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Co-substrate Fermentation of Jerusalem Artichoke Tubers and Crude Glycerol to Butanol with Integrated Product Recovery

Tahereh Sarchami
The University of Western Ontario

Supervisor
Dr. Lars Rehmann
The University of Western Ontario

Graduate Program in Chemical and Biochemical Engineering
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Abstract

Butanol has long been considered a potential advanced liquid biofuel, in addition to its current application as an industrial solvent. It can be produced biologically; however, the conventional ABE fermentation suffers from many limitations, including low butanol titer, high cost of traditional raw materials, end-product inhibition and high butanol recovery costs. Possible solutions are the use of renewable low-cost feedstocks, genetic manipulations of *Clostridia spp.* to improve the strains' butanol titer and tolerance, advanced fermentation techniques, and in-situ product recovery technologies.

In order to overcome some of these limitations, the overall goal of this thesis was to develop a process to produce butanol via fermentation using low-cost feedstocks and integrated product recovery. Jerusalem artichoke tubers and biodiesel-derived glycerol were investigated as potential feedstocks for fermentative butanol production. Pervaporation was evaluated as an online butanol recovery technique and was integrated into the butanol fermentation process.

In the first phase of this research the suitability of Jerusalem artichoke tubers as a renewable feedstock for butanol production was studied and statistical experimental design was used to optimize enzymatic and acid hydrolysis of the feedstock. Both enzymatic and sulfuric acid hydrolysate of Jerusalem artichoke tubers were fermented via solventogenic *Clostridia* to acetone- butanol- ethanol (ABE). An overall ABE productivity of 0.25 g L^{-1} hr^{-1} was obtained from both hydrolysates, indicating the suitability of this feedstock for fermentative butanol production.

In the second phase, the feasibility of butanol production from biodiesel-derived glycerol was investigated. The initial fermentation conditions for butanol production from glycerol were optimized via a central composite design. In the next phase, Jerusalem artichoke hydrolysate and crude glycerol were used as co-substrate for enhanced butanol production. A co-substrate system was characterized and optimized. The optimized conditions were then used for an integrated fed-batch fermentation including pervaporation for in situ butanol recovery. The integrated process achieved a butanol productivity of 0.6 g L^{-1} hr^{-1}.

Keywords

Jerusalem artichoke tubers, Crude glycerol, Fermentation, Optimization, Pervaporation.

Co-Authorship Statement

This thesis was completed under the supervision of Dr. Lars Rehmann. Six articles were written and coauthored, the extent of the collaboration of the co-authors is stated below.

Chapter 2.

Paper title: A review of process-design challenegs for industrial fermentation of butanol from crude glycerol by non-biphasic *Clostridium pasteurianum*

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Tahereh Sarchami: Coordinated the study and contributed to the writing, editing, and correction of the manuscript.

Garret Munch: Contributed to the writing, editing, and correction of the manuscript.

Erin Johnson: Contributed to the writing, editing, and correction of the manuscript.

Sascha Kießlich: Contributed to the writing, editing, and correction of the manuscript.

Lars Rehmann: Assisted in the editing and correction of the manuscript. He is also the corresponding author of this article.

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Chapter 5.

Paper title: Optimizing of fermentation condition favoring butanol production by *Clostridium pasteurianum* DSM 525

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Erin Johnson: Contributed to the editing, and correction of the manuscript.

Lars Rehmann: Assisted in the editing and correction of the manuscript. He is also the corresponding author of this article.

Chapter 6.

Paper title: Co-substrate fermentation of Jerusalem artichoke tubers and crude glycerol to butanol by *Clostridium pasteurianum* DSM 525

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Chapter 7.

Paper title: Enhanced butanol production in the integrated fed-batch fermentation process with pervaporation

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Erin Johnson: Carried out the desing and development of pervaporation unit. Assisted with key insights and contributed to the editing and correction of the manuscript.

Sascha Kießlich: Assisted with developing and training of the pervaporation running and cleaning protocole.

Lars Rehmann: Assisted in the editing and correction of the manuscript. He is also the corresponding author of this article.

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Dedication

To my parents, Karim Sarchami and Fatemeh Shamloo for their endless love, support and encouragement. To My son, Mohammad Sadeq Delavar! The love of my life!

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Acronyms list

ABE	Acetone-butanol-ethanol
ANOVA	Analysis of variance
CCD	Central composite design
FAME	Fatty acid methyl ester
FFA	Free fatty acid
HMF	Hydroxymethylfurfural
HPLC	High pressure liquid chromatography
JAH	Jerusalem artichoke hydrolysate
L	Liter
OD	Optical density
1,3-PDO	1,3-propanediol
PBE	1,3-propanediol, butanol, ethanol
PV	Pervaporation
RID	Refractive index detector
R^2	Regression coefficient
RSM	Response surface methodology
α	Selectivity coefficient
β	Separation factor
J	Partial flux
$\left[\frac{p_i}{l}\right]$	Permeance

Chapter 1

1 Introduction

1.1 Background

High global oil prices during the first decade on this century have stimulated substantial political, industrial and academic interest in the production of liquid transportation fuels from biomass. The interest remained high after volatile prices in the first half of the second decade and the low prices of second half, largely due to political interest in stable markets as well as environmental concerns such as greenhouse gas emissions, global warming and climate change (Song 2008; Huang et al. 2010; Ma et al. 2010). Biofuels have the potential to reduce and eventually replace the current needs of petroleum fuels with zero/near zero net emission of greenhouse gases (Demirbas 2009a; Demirbas 2009c; Hoekman 2017). Additional benefits of biofuels include energy security, potential foreign exchange savings, and development of rural areas (Demirbas 2009b; Hoekman 2017).

Compared to ethanol, butanol is considered a 'next generation' biofuel due to many advantages it offers, such as higher energy content and lower volatility, counteracted by a more challenging production process (Dürre 2007; Lee et al. 2008c; Nigam and Singh 2011). Butanol can be used directly or blended with gasoline and diesel as a fuel additive in current automobile engine without any modification or substitution. In addition, butanol is compatible with the current transportation pipelines for gasoline (Dürre 2007; Lee et al. 2008c).

Large scale biological production of butanol was first achieved during 1912–1914 via Acetone–Butanol–Ethanol (ABE) fermentation of molasses and cereal grains, and developed into a large industry, with acetone as the main commercial product. However, fermentative butanol production declined rapidly during the 1950's due to the rise of

cheaper petrochemical synthesis and increased cost of fermentation raw materials (Dürre 2007; Kumar and Gayen 2012).

Currently, three major difficulties are keeping butanol fermentation from becoming economically competitive; substrate costs, final butanol titer and butanol recovery.

The high cost of conventional substrates (molasses and cereal grains) is one of the main obstacles of fermentative butanol production. It has been estimated that substrate cost accounts for more than 50% of the total production cost in ABE fermentation (Dürre 2007). Hence, there is a constant demand to search available cost-effective raw materials.

The second difficulty is the low butanol concentration due to end-product inhibition. Butanol, the primary product of the fermentation, severely inhibits its further production at concentrations ranging from 10-20 g L^{-1} (Jones and Woods 1986a; Maddox 1989). This severe product inhibition leads to low volumetric productivity. To overcome the low butanol concentration and productivity, fed-batch and continuous fermentation techniques have been developed. Due to the accumulation of end product, which can cause inhibition on the cells, fed-batch fermentation is feasible only when coupled with online product recovery. Cell-recycle and cell immobilization have also been utilized to increase cell density and bioreactor productivity.

The third difficulty is the high cost of butanol recovery. The very low concentration of butanol, its high boiling point (118 °C), and the presence of other fermentation products in the broth make butanol recovery by distillation energy intensive (Ezeji et al. 2004; Qureshi et al. 2005; Abdehagh et al. 2014; Abdehagh et al. 2015). Therefore, significant energy savings can be achieved if the concentration of butanol in the fermentation broth is increased. In-situ product removal can be achieved through techniques such as gas stripping, vacuum stripping, pervaporation, liquid-liquid extraction, perstraction, and adsorption (Qureshi et al. 2005; Ha et al. 2010; Mariano et al. 2011; Mariano et al. 2012; Abdehagh et al. 2014; Errico et al. 2016). Among these techniques, pervaporation has been widely reported as an efficient butanol recovery technique that can be integrated with ABE fermentation for online butanol removal (Yen et al. 2012a; Yen et al. 2012b; Shin et al.

2015; Wu et al. 2015; Kong et al. 2016). Low energy consumption and no solvent requirements make pervaporation a green process which has no harmful effect on microorganisms.

In this study, glycerol as a by-product of biodiesel production, and Jerusalem artichoke tubers were evaluated as potential substrate for butanol production. Their abundance, availability, and cost competitiveness make both glycerol and Jerusalem artichoke tubers excellent substrates for butanol production. The feasibility of employing glycerol and Jerusalem artichoke tubers as co-substrate was also assessed. In addition, using aforementioned co-substrate strategy, a fed-batch culture was integrated with pervaporation as an online butanol recovery technique to overcome the low productivity, mitigate toxicity and to save on energy by distillation.

1.2 Research objectives and contributions

Towards the completion of this study, one overall objective and several sub-objectives were proposed.

1.2.1 General objective

The overall objective of this research was to develop a process to produce butanol via fermentation of Jerusalem artichoke tubers and biodiesel-derived glycerol and to integrate online product recovery with the fermentation process for enhanced butanol production, reactor productivity and substrate conversion.

1.2.2 Specific objectives

The following are specific sub-objectives or milestones of this study.

Objective 1: To study the effect of various variables on enzymatic hydrolysis of Jerusalem artichoke tubers.

Effects of temperature, pH, substrate concentration and enzyme loading on the enzymatic hydrolysis of Jerusalem artichoke-derived inulin were studied. Data obtained allowed to plot response surface graphics. Statistical data obtained from RSM led to the development

of an empirical model of inulin conversion as function of all four investigated factors. This model was numerically optimized to obtain the hydrolysis conditions that maximize inulin conversion to fermentable sugars.

Objective 2: To study the effect of various variables on acid hydrolysis of Jerusalem artichoke tubers using three different mineral acids.

Effects of temperature, pH, and time on the acid hydrolysis of Jerusalem artichoke-derived inulin using three different mineral acids (HCl, H_2SO_4, and H_3PO_4) were studied. Data obtained allowed to plot response surface graphics. Statistical data obtained from RSM led to the development of an empirical model of inulin conversion for each acid as function of all investigated factors. These models were numerically optimized to obtain the hydrolysis conditions that maximize inulin conversion to fermentable sugars. The influence of each acid on the formation of 5-hydroxymethylfurfural (HMF) was also investigated. Data obtained provided information on which acid is a better catalyst compared to two other acids for inulin hydrolysis.

Objective 3: To assess the feasibility of butanol production from the hydrolysate of Jerusalem artichoke's tuber.

The feasibility of butanol production from acid and enzymatic hydrolysate of Jerusalem artichoke's tuber by *Clostridium saccharobutylicum* DSM 13864 was studied. The results obtained indicated that hydrolysate of Jerusalem artichoke tubers is a reliable feedstock for butanol production.

Objective 4: To compare two most common media compositions for butanol production from glycerol.

The data obtained provided information on product profile of glycerol fermentation by *Clostridium pasteurianum* DSM 525 based on media composition.

Objective 5: To study the effect of butanol fermentation conditions on corresponding production yield using glycerol as substrate.

Effects of inoculum age, initial cell density, initial pH of medium and temperature on the butanol yield were studied. Data obtained allowed to plot response surface graphics. Statistical data obtained from RSM led to the development of an empirical model of butanol yield as function of all four investigated factors. This model was numerically optimized to obtain the fermentation conditions that maximize butanol yield.

Objective 6: To investigate butanol production from crude glycerol in a lab scale bioreactor at optimized conditions.

Based on the results obtained from objective 4, batch fermentations were performed at optimized conditions in a lab scale bioreactor using crude glycerol as substrates. The results indicated that biodiesel derived- glycerol is a reliable feedstock for butanol production.

Objective 7: To study the effect of adding acetate and butyrate on butanol production from glycerol.

The effect of acetic and butyric acid addition on butanol production was investigated in batch cultures. Data obtained confirmed that acetate and butyrate addition especially butyrate is beneficial to butanol production with *Clostridium pasteurianum* DSM 525 using pure glycerol as carbon source.

Objective 8: To assess the feasibility of using Jerusalem artichoke hydrolysate and crude glycerol as co-substrate for enhanced butanol production.

Using Jerusalem artichoke hydrolysate as a sugar source and glycerol as the main carbon source (co-substrate strategy) with a single culture of *Clostridium pasteurianum* was studied. The optimal co-substrate ratio was also investigated. Based on the estimated optimal conditions, Jerusalem artichoke hydrolysate and biodiesel-derived glycerol were used as carbon sources for the co-substrate based butanol production in a lab scale bioreactor. Data obtained indicated the feasibility of co-substrate strategy.

Objective 9: To evaluate pervaporation performance as butanol recovery technique using model solutions and fermentation broth.

The feasibility of pervaporative separation of butanol from binary butanol/water mixture and model solution (novenary mixture) using Pervap 4060 membrane was studied. Data obtained was used to simulate and develop the integration of a Pervap 4060 pervaporation unit into a fed-batch fermentation system.

Objective 10: To integrate PBE fermentation with online product recovery.

The integration of PBE fermentation with online pervaporation was demonstrated. The data obtained indicated that integrated fermentation-pervaporation system could mitigate butanol toxicity and increase productivity.

In addition to the objectives mentioned above, a comprehensive review of process-design challenges for industrial fermentation of butanol from crude glycerol by non-biphasic *Clostridium pasteurianum* was conducted. The objective of the review was to present recently published data on *Clostridium pasteurianum* as an alternative microbe for butanol production from crude glycerol and describe relevant challenges for its industrial fermentative conversion.

1.3 Research Structure

The first phase of research evaluated the feasibility of butanol production from Jerusalem artichoke tubers. A central composite design and response surface methodology were used to study the effect of various variables on both enzymatic and acid hydrolysis of Jerusalem artichoke tubers. Numerical optimization was used to maximize the sugar yield of Jerusalem artichoke tubers within the experimental range of each hydrolysis. The influence of acid hydrolysis on the formation of 5-hydroxymethylfurfural (HMF; a known by-product and inhibitor for fermentative organisms) was also investigated. Both acid and enzymatic hydrolysates were used for butanol production.

The second phase of the investigation evaluated the feasibility of butanol production from biodiesel-derived glycerol. The initial fermentation conditions for butanol production from pure glycerol by *Clostridium pasteurianum* DSM 525 were optimized via a central composite design. The effect of inoculum age, initial cell density, initial pH of medium and

temperature were quantified and a quadratic model was able to predict butanol yield as a function of all four investigated factors. Numerical optimization was used to maximize the butanol yield within the experimental range. Based on these results, batch fermentations in a 7 L bioreactor were performed using pure and crude (residue from biodiesel production) glycerol as substrates at optimized conditions.

For the third phase of the study, the effect of adding acetate and butyrate on butanol production from glycerol by C. *pasteurianum* DSM 525 was first investigated and confirmed. The product formation by the same strain using different mono-substrates was studied, followed by an optimization study of the co-substrate ratio. Based on the estimated optimal conditions, Jerusalem artichoke hydrolysate and crude glycerol (from biodiesel manufacturing waste) were used as low-cost carbon sources for the co-substrate based butanol production in a 5L laboratory bench bioreactor.

The final phase of this research evaluated pervaporation performance as a separation technique for in-situ butanol removal. First, a systematical investigation on butanol recovery from binary butanol/water solution was carried out to study the effectiveness of pervaporation of PDMS-based membrane, namely Pervap 4060, for selective separation and concentration of butanol. Next, a novenary mixture (consisted of all PBE fermentation substrates and products) was used to study the influence of coupling effect on the molecular transport during the pervaporation process. Based on the data obtained and analysis this membrane was brought into process intensification by integrating pervaporation with PBE fed-batch fermentation, to improve the butanol productivity via in-situ product removal.

1.4 Major contributions

The literature review conducted for the project was published and contributed to:

- Identifying and describing challenging aspects of butanol production from glycerol; the bottlenecks in the implementation of fermentative butanol production at industrial scale was clearly stated.

The study of acid and enzymatic hydrolysis of Jerusalem artichoke tubers contributed to:

- Being able to modulate the inulin conversion to fermentable sugars via acid and enzymatic hydrolysis by changing the hydrolysis condition using the empirical models obtained.

- Finding optimal points to maximize inulin conversion via hydrolysis

- Being able to compare two different methods of inulin hydrolysis in terms of operating condition and results

The study of Jerusalem artichoke tubers as low-cost substrate for butanol production contributed to:

- Indicating that Jerusalem artichoke tuber is a good feedstock for fermentative butanol production

The study of biodiesel-derived glycerol as low-cost and available substrate for butanol production contributed to:

- Identifying product profile of glycerol fermentation by *Clostridium pasteurianum* DSM 525 based on media composition.

- Being able to modulate butanol yield by changing inoculum age, initial cell density, initial pH of medium and temperature as fermentation variables.

- Finding optimal points to maximize butanol yield based on fermentation condition

- Indicating that biodiesel-derived glycerol is an excellent feedstock for fermentative butanol production.

The study of using Jerusalem artichoke hydrolysate and crude glycerol as co-substrate for enhanced butanol production:

- Being able to modulate co-substrate ratio by changing sugar and glycerol concentration.
- Finding optimal points to maximize butanol yield and productivity at the same time.

- Indicating the feasibility of co-substrate strategy.

The study of pervaporation performance using model solutions and fermentation broth contributed to:

- Indicating that Pervap 4060 is a reliable membrane for butanol separation from PBE fermentation.

- Indicating that integrated PBE fermentation with pervaporation system could mitigate butanol toxicity and increase productivity.

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Chapter 2

2 A review of process-design challenges for industrial fermentation of butanol from crude glycerol by non-biphasic *Clostridium pasteurianum*

Tahereh Sarchami, Garret Munch, Erin Johnson, Sascha Kießlich, and Lars Rehmann.

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is substantially as it appears in Fermentation Journal, June 2016, Vol 2, pages 1-33.

Abstract

Butanol, produced via traditional acetone-butanol-ethanol (ABE) fermentation, suffers from low yield and productivity. In this article, a non-ABE butanol production process is reviewed. *Clostridium pasteurianum* has a non-biphasic metabolism, alternatively producing 1,3-propanediol (PDO)-butanol-ethanol, referred to as PBE fermentation. This review discusses the advantages of PBE fermentation with an emphasis on applications using biodiesel-derived crude glycerol, currently an inexpensive and readily available feedstock. To address the process design challenges, various strategies have been employed and are examined and reviewed; genetic engineering and mutagenesis of *C. pasteurianum*, characterization and pretreatment of crude glycerol and various fermentation strategies such as bioreactor design and configuration, increasing cell density and *in-situ* product removal. Where research deficiencies exist for PBE fermentation, the process solutions as employed for ABE fermentation are reviewed and their suitability for PBE is discussed. Each of the obstacles against high butanol production has multiple solutions, which are reviewed with the end-goal of an integrated process for continuous high level butanol production and recovery using *C. pasteurianum* and biodiesel-derived crude glycerol.

2.1 Introduction

Continuous mass consumption of fossil fuels has led to high levels of greenhouse gas emissions (GHG), with little doubt in the scientific community on its dramatic impact on the world's climate (Malaviya et al. 2012; Jang et al. 2012). Thus, biofuels are considered an attractive option to break dependence on petroleum-based fuels, as mobility is a major part of the world's energy system. Biobutanol could be one of the most promising alternative biofuels due to its many advantages over ethanol (Mariano et al. 2013; Bankar et al. 2013b; Sarchami and Rehmann 2014). Compared to ethanol, butanol has lower solubility in water, higher energy content (27MJ L^{-1} vs. 19.6 MJ L^{-1}), lower volatility and is less corrosive (Lee et al. 2008a; Sarchami and Rehmann 2014) . As a result, butanol can replace up to a 100% of petroleum-based fuels without structural modifications of the current engine technologies (Lee et al. 2008b). Furthermore, butanol can be blended directly at the refinery and transported through existing pipeline infrastructure (Atsumi et al. 2008).

Currently, 11 biobutanol fermentation plants are in operation in China (plus an additional 2 under construction) (Ni and Sun 2009) and 1 in Brazil (Mariano et al. 2013). The current plants are all using either starch (corn, cassava, sweet potato) or sugars (molasses) as a carbon source. High and costly substrates that compete with human food (sugar, starch) is one of the main drawbacks of these fermentation plants. The butanol production cost and profitability of a plant largely depend on substrate cost and are extremely sensitive to any price fluctuation (Qureshi et al. 2008; Green 2011; García et al. 2011). Therefore, transition toward low-cost, non-edible, readily and reliably available feedstock at industrial scale is crucially important from a process economics perspective and can offer the biggest opportunity for cost reduction and improved sustainability (Sabra et al. 2014).

Glycerol as an alternative carbon source, produced as a major byproduct of the biodiesel industry, has recently been attracting much attention as a good substrate for bio-based butanol production (Dabrock et al. 1992; Yazdani and Gonzalez 2007; da Silva et al. 2009). As a consequence of the expanding biodiesel production, surplus quantities of biodiesel-derived glycerol (commonly referred to as 'crude' glycerol) are being produced (Rehman.

A, Wijesekara. S, Nomura. N, Sato. S 2008; da Silva et al. 2009). Disposal of crude glycerol has become a financial and environmental liability for the biodiesel industry, reducing the selling price of crude glycerol in the US to between 4 to 11 cents/kilogram in 2011 (Quispe et al. 2013). The impurities present in the crude glycerol are responsible for the greatly lowered price compared to pure glycerol (Szambelan and Nowak 2006; da Silva et al. 2009). Its abundance and cost competitiveness make glycerol an excellent alternative to other carbon substrates for butanol production (Khanna et al. 2013a; Sabra et al. 2014). Development of glycerol-based butanol production processes can add significant value to the biodiesel industry and presents excellent potential to establish industrial production of butanol near existing distribution infrastructure (Taconi et al. 2009; Khanna et al. 2013a; Gallardo et al. 2014; Venkataramanan et al. 2014).

Although butanol has many attractive properties, ABE fermentation suffers from low productivity and high operational and capital costs (Tashiro et al. 2013; Bankar et al. 2013b; Branduardi et al. 2014). Therefore, a number of researchers have tried to overcome these problems by means of genetic manipulations of *Clostridia spp.* to improve strains' butanol titer and tolerance (Dabrock et al. 1992; Malaviya et al. 2012; Jensen et al. 2012b), fermentative techniques to increase the cell density as well as butanol yield and productivity (Malaviya et al. 2012; Zheng et al. 2013; Khanna et al. 2014), and in-situ product recovery technologies to overcome the butanol toxicity to fermentative microorganism (Ha et al. 2010; Bankar et al. 2012; Abdehagh et al. 2013; Wiehn et al. 2014).

This review aims to present recently published data on *Clostridium pasteurianum* as an alternative microbe for biobutanol production from crude glycerol and relevant challenges for industrial fermentative conversion.

2.2 Characterization of biodiesel-derived crude glycerol

Because crude glycerol is itself a waste stream which has been highly processed, the concentration of the impurities varies between and within biodiesel production plants (Hansen et al. 2009; Hu et al. 2012). This is due to variation in feedstock, the type of

catalyst used, the transesterification efficiency, recovery efficiency of the biodiesel, and whether the methanol and catalysts were recovered. These impurities pose some of the greatest industrial challenges which need to be understood and addressed. Therefore, it is crucial to understand the chemical composition of crude glycerol before considering its fermentative conversion.

The crude glycerol impurities commonly are methanol, free fatty acids (FFAs), salts, moisture, ash, soap and methyl esters (Hansen et al. 2009). In one study, Rehman et al. (2008) reported that crude glycerol from the transesterification of sunflower oil as feedstock contained (w w^{-1} %): 30 glycerol, 50 methanol, 13 soap, 2 moisture, approximately 2-3 salts, and 2-3 other impurities. In another study, crude glycerol generated from biodiesel production using soybean oil contained 70% to 85% w w^{-1} glycerol (Mu et al. 2006). However, Thompson and He (2006) reported minimal variation between glycerol samples obtained from different feedstocks. Although it should be noted the crude glycerol was produced in a laboratory setting, rather than industrial in all aforementioned studies.

In the case of crude glycerol from an industrial biodiesel plant, De Carvalho et al. (2012) investigated the chemical composition of two types of crude glycerol generated from biodiesel production, using soybean oil and a mixture made of 80% animal fat and 20% soybean oil. Both samples were obtained from Biopar biofuel industry located in Brazil and contained about 55% glycerol and 4% ash. Soybean oil crude glycerol contained slightly higher amount of matter organic non-glycerol, methanol, and total fatty acids compared to crude glycerol generated from mixed substrate. Hansen et al. (2009) studied the chemical compositions of 11 crude glycerol samples collected from 7 Australian biodiesel producers and indicated that the glycerol content ranged between 38% and 96%, with some samples including about 14% methanol and 29% ash. In another study, the chemical composition of 5 crude glycerol samples from industry was investigated and described by eight components including: free glycerol, methanol, water, soap, fatty acid methyl esters, glycerides, free fatty acids and ash. The compositions of these four biodiesel-

derived crude glycerol samples varied significantly from each other; for example, free glycerol contents ranged from 22.9 to 63.0%.

In order to improve crude glycerol composition, heterogeneous catalysts such as solid and enzyme catalysts have been used as alternatives to homogenous alkaline catalysts. Bournay et al. (2005) reported 98% glycerol content in crude glycerol produced from biodiesel production using rapeseed oil as substrate with heterogeneous catalyst. Neither ash, nor inorganic compounds were detected in the crude glycerol produced with the major impurities being water, methanol and other 'matter organic non-glycerol' (MONG). Therefore, characterization of crude glycerol will need to be an ongoing part of quality assurance prior to bioconversion at industrial scale.

2.3 Microbial metabolism of glycerol

A number of microorganisms are able to grow anaerobically on glycerol as the sole carbon and energy source, such as *Citrobacter freundii* (Daniel et al. 1995; Seifert et al. 2001), *Klebsiella pneumoniae* (Biebl, H., Zeng, A. P., Menzel, K., Deckwer 1998; Németh et al. 2003), *Clostridium butyricum* (Colin et al. 2001; Malaoui and Marczak 2001), *Enterobacter agglomerans* (Barbirato et al. 1997; Barbirato and Bories 1997), *Enterobacter aerogenes* (Ito et al. 2005) and *Lactobacillus reuteri* (Talarico et al. 1990). However, most of them do not convert this substrate into butanol. The literature shows that the best studied organism to do so is *Clostridium pasteurianum*, a gram-positive, anaerobic and non-pathogenic bacteria (Taconi et al. 2009; Khanna et al. 2013a; Gallardo et al. 2014). The reported solvents produced by *C. pasteurianum* utilizing glycerol as substrate are: butanol, PDO and ethanol. By-products include acetic acid, butyric acid as well as CO_2 and H_2. In contrast to the ABE fermentation process, no acetone is produced. Therefore, fermentation of glycerol using *C. pasteurianum* could be referred to as a "PBE" process to reflect PDO production in lieu of acetone.

During anaerobic fermentation, the overall redox balance within the cell is maintained by shifting between metabolic pathways resulting in different products and reducing equivalents being formed. The highly reduced nature of glycerol results in the production

of twice the amount of reducing equivalents compared to the catabolism of lignocellulosic sugars such as glucose and xylose (Yazdani and Gonzalez 2007). These additional reducing equivalents provide glycerol with the natural advantage of higher theoretical product yield for reduced chemicals and fuels.

Moreover, compared to the characteristic growth pattern of acetogenesis and solventogenesis found in ABE fermentations, *C. pasteurianum* shows little biphasic behaviour when grown on glycerol (Venkataramanan et al. 2014; Johnson and Rehmann 2016). This is a result of the regulation of the metabolic pathway leading from glycerol to butanol. This pathway has a neutral redox balance and was reported to be energetically preferred (Biebl 2001). However, PDO plays an important role in maintaining glycerol fermentation of *C. pasteurianum*. In contrast to the *Clostridia spp.* used in ABE fermentation, *C. pasteurianum* has a reductive pathway for the production of PDO independent of glycolysis. The production of PDO enables *C. pasteurianum* to balance the cellular redox potential with reducing equivalents required when biomass is formed. Therefore, cellular energy can be produced in glycolysis, independent of acetic and butyric acid production, while butanol production can be maintained simultaneously to biomass formation (Venkataramanan et al. 2014; Johnson and Rehmann 2016). A simplified pathway showing the glycerol metabolism of *C. pasteurianum* with a focus on end-products is presented in Figure 2-1.

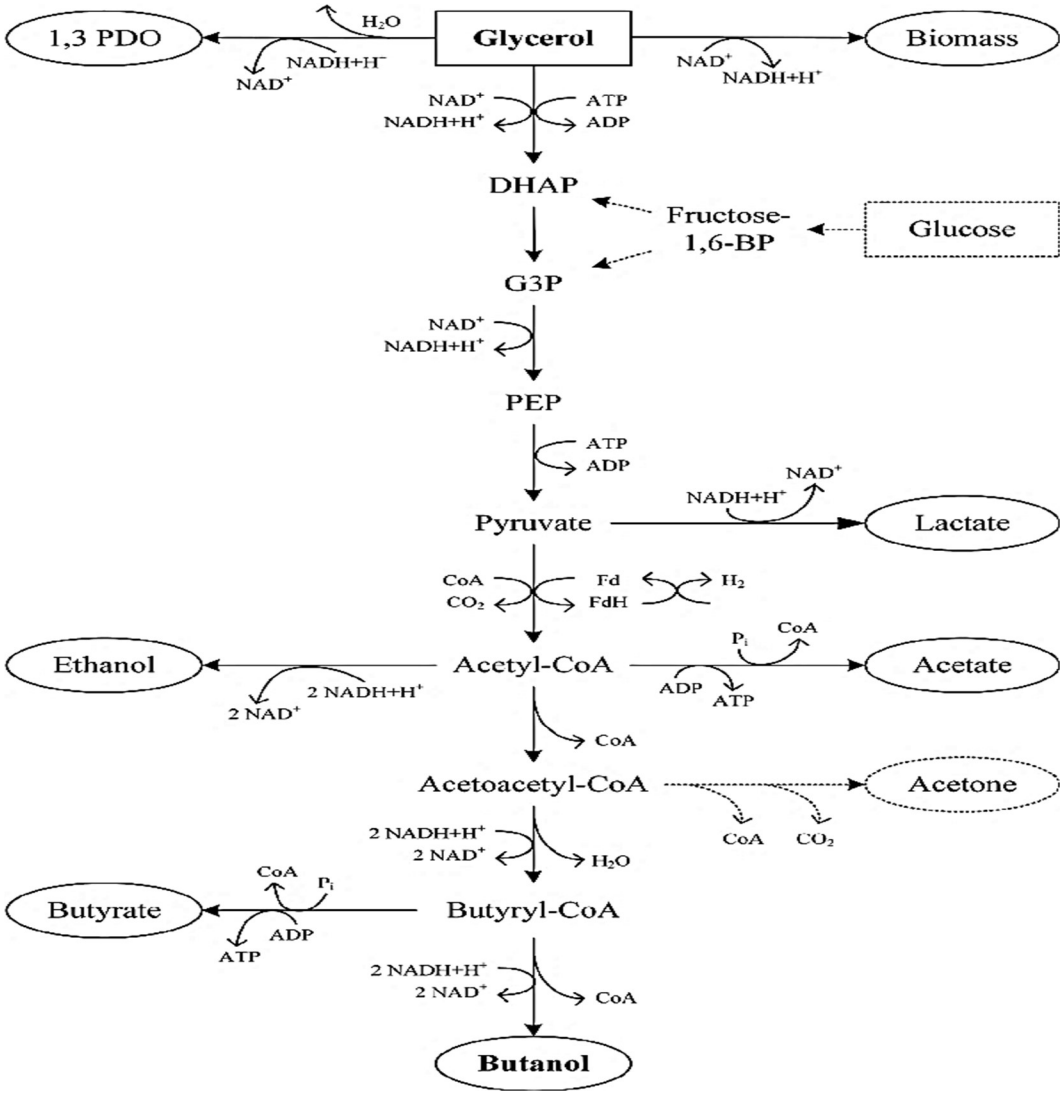

Figure 2-1 Possible metabolic pathway for glycerol fermentation by *C.pasteurianum*. Adapted from Biebl (2001), Venkataramanan et al. (2012), and Malaviya et al. (2012).

2.4 Biodiesel-derived crude glycerol pretreatment

The utilization of crude glycerol in fermentations may require pretreatments due to the impurities in crude glycerol composition acting as inhibitory agents, causing microbial growth inhibition, lengthening fermentation time and lowering butanol yield and productivity. However, reports investigating the individual effects of these impurities have shown that different compounds present in the crude glycerol can have a varying effect on *C. pasteurianum* (Venkataramanan et al. 2012). Venkataramanan et al. (2012) reported that

the addition of methanol and salt to the media did not affect the cell growth and butanol yield. However, free fatty acids (FFAs) present in vegetable oil and thus crude glycerol had inhibitory effect on both cell growth and butanol yield, particularly the unsaturated moieties such as oleic acid and linoleic acid. The authors found no reports on the effect of soap, glycerides or methyl esters on *C. pasteurianu*m growth or butanol yield.

For example, crude glycerol can be refined by the following steps: saponification using strong alkali material to transform fatty acid methyl esters (FAMEs) to soap and methanol and glycerides to soap and glycerol, acidification which converts all soap to free fatty acids (FFAs) and salts, phase separation into three layers (a top organic layer rich in FFAs, a middle layer rich in glycerol, and a bottom layer rich in inorganic salts), harvest of the glycerol rich portion, filtration, followed by neutralization. Water and salts can be removed by evaporation and centrifugation, respectively, as shown in Figure 2-2 (Hájek and Skopal 2009; Kongjao et al. 2010; Manosak et al. 2011).

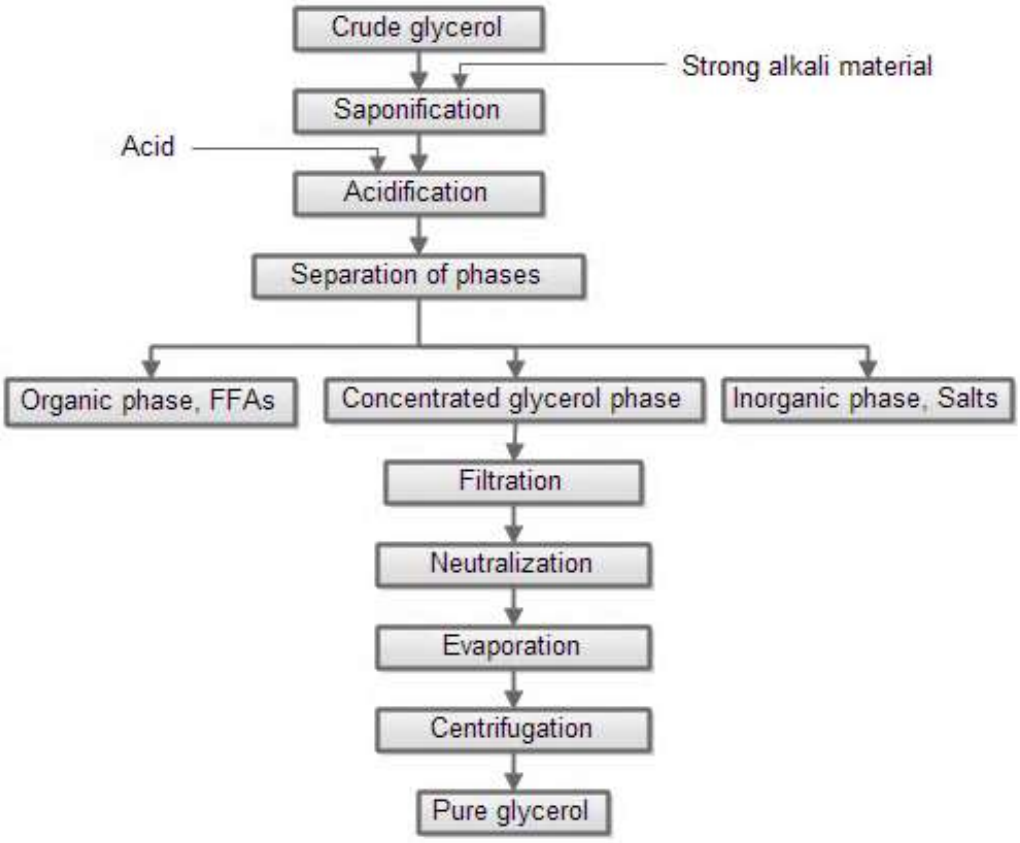

Figure 2-2 Diagram of possible steps of purification of crude glycerol

In one study, the crude glycerol was purified following the aforementioned steps excluding vacuum distillation and the results are presented in Table 2-1 (Hájek and Skopal 2009). Potassium hydroxide was used as a strong alkali catalyst in the saponification process with 1:1 molar ratio of potassium hydroxide to ester. In the next experiment the molar ratio of potassium hydroxide to ester was enhanced to 1.2:1. As shown in Table 2-1, a concentrated glycerol phase with 85% purity and an organic phase with 99.5% FFAs purity were obtained, indicating the efficiency of this purification process.

However, in most studies, *C. pasteurianum* has been shown to ferment biodiesel-derived crude glycerol, requiring minimal upgrading. For example, in one study, the crude glycerol was diluted with water and filtered through a 0.2 μm filter three times to remove solids and was used as sole carbon source for butanol production. It was reported that *C. pasteurianum* was capable of converting crude glycerol (50 g L^{-1}) to butanol with a maximum butanol

yield of 0.27 g g^{-1} after 35 hr, only slightly lower than the yield on pure glycerol (0.28 g g^{-1}, 30 hr) (Sarchami et al. 2016). In another study, Venkataramanan (2012) removed the FFAs via acid precipitation and phase separation from the crude glycerol prior to fermentation, resulting in butanol yields matching those found with pure (0.26 g g^{-1} for pure vs. 0.28 g g^{-1} with treated crude), with both fermentations taking 96 hours. When compared to untreated crude glycerol, the yield was 0.21 g g^{-1} over a two-week period. Some of the reported research on PBE fermentation using pure and crude glycerol as substrate is summarized in Table 2-2. As can be seen, volumetric productivity suffers when fermentation time is extended.

Table 2-1 The composition of crude glycerol (Average ± standard deviation), concentrated crude glycerol and organic acid after phase separation (Hájek and Skopal 2009).

Compound	Crude Glycerol wt %	Concentrated Glycerol Phase		Organic Phase	
		Molar Ratio of KOH: Esters		Molar Ratio of KOH: Esters	
		1:1	1.2:1	1:1	1.2:1
Glycerol	55.5 ± 3.9	84.7	85.1	Nt	Nt
Soap	18.6 ± 2.8	Nt	Nt	Nt	Nt
Salts	1.7 ± 0.28	2.39	2.87	Nt	Nt
Water	13.3 ± 1.37	12.1	11.1	Nt	Nt
Methanol	2.9 ± 1.48	0.46	0.37	Nt	Nt
Esters	8.1 ± 1.65	Nt	Nt	4.8	0
FFAs	Nt	Nt	Nt	95	99.5
Others	Nt	0.35	0.56	0.2	0.5

Nt: Not reported.

Table 2-2 Comparison of bioconversion of pure and crude glycerol to butanol under identical fermentation condition by *C. pasteurianum.*

Strains	Crude Glycerol Pretreatment/ (Fermentation Time)	Culture Condition	Max. Butanol Yield [a] g g^{-1} (mol mol^{-1})		Overall Butanol Productivity g L^{-1} hr^{-1}		Reference
			Pure Glycerol	Crude Glycerol	Pure Glycerol	Crude Glycerol	
C. pasteurianum (wild type; DSM 525)	Filtration (35 hr)	Batch, Free cells, Vol ~ 5 L	0.28 (0.35)	0.27 (0.34)	0.41	0.35	(Sarchami et al. 2016)
C. pasteurianum (wild type; ATCC 116)	None (120 hr)	Batch, Free cells, Vol < 1 L	0.18 (0.22)	0.13 (0.16)	<0.10	<0.10	(Khanna et al. 2014)
C. pasteurianum (wild type; ATCC 116)	None (120 hr)	Batch, Immobilized cells, Vol < 1 L	0.36 (0.45)	0.23 (0.29)	<0.10	<0.10	(Khanna et al. 2014)
C. pasteurianum (wild type; ATCC 6013)	None (14–24 days)	Batch, Free cells, Vol < 1 L	0.26 (0.32)	0.21 (0.26)	<0.1	<0.02	(Venkataramanan 2012)
C. pasteurianum (wild type; ATCC 6013)	Acid precipitation (4 days)	Batch, Free cells, Vol < 1 L	0.26 (0.32)	0.28 (0.35)	<0.1	<0.1	(Venkataramanan 2012)
C. pasteurianum (wild type; ATCC 6103)	None (25 days)	Batch, Free cells, Vol < 1 L	0.31 (0.39)	0.30 (0.37)	0.04	<0.02 [b,c]	(Taconi et al. 2009)

[a] yield calculated based on glycerol consumed; [b] data inferred from graphical representation;
[c] productivity calculated based on active fermentation (subtracted lag phase).

2.5 Media composition and fermentation condition

The product profile of glycerol fermentation by *C. pasteurianum* largely depends on media composition and fermentation parameters. Moon et al. (2011) reported that the optimal media composition for butanol production by *C. pasteurianum* was significantly different from media used for production of PDO. It was shown that iron and nitrogen limitations will favor PDO production. The influence of iron limitation matches with previous reports (Dabrock et al. 1992). The optimal yeast extract concentration for butanol production was also different from concentration used for PDO production (Moon et al. 2011).

When investigating different fermentation parameters, initial glycerol concentration, the inoculum age, initial cell concentration, initial pH of medium, temperature, and agitation rate were studied as major factors that influenced butanol yield and productivity (Malaviya et al. 2012; Khanna et al. 2013b; Sarchami et al. 2016). The highest butanol yield and productivity was reported to be 0.28 g g^{-1} and 0.41 g L^{-1} hr^{-1}, respectively, at optimal fermentation condition of inoculum age of 16 hr, initial cell density of 0.4 g L^{-1}_{DCW}, initial pH of 6.8, and temperature of 30°C (Sarchami et al. 2016). Sarchami et al. (2016) and Khanna et al. (2013b) reported that at optimal fermentation condition, the scale of operation had no effect on butanol yield and productivity. Some of the reported research on optimization of fermentation condition favoring butanol production by *C. pasteurianum* is summarized in Table 2-3.

Table 2-3 Summary of studies on optimization of 1,3-propanediol-butanol-ethanol (PBE) fermentation condition favoring butanol production by *C. pasteurianum*.

Strains	Culture Condition	Initial Glycerol Titer g L^{-1}	Inoculum Age hr	Initial Cell Density g L^{-1}_{DCW}	pH	Temperature °C	Agitation Rate rpm	Max. Butanol Titer g L^{-1}	Max. Butanol Yield [a] g g^{-1} (mol mol^{-1})	Overall Butanol Productivity g L^{-1} hr^{-1}	Ref.
C. pasteurianum (wild type; DSM 525)	Batch, Free cells, Vol < 1 L	Pure Non-Sig. 50	Sig. 16	Sig. 0.4	Sig 7.0	Sig. 30	Not-studied	12.3	0.28 (0.35)	0.41	(Sarchami et al. 2016)
C. pasteurianum (wild type; ATCC 6013)	Batch, Immobolized cells, Vol < 1 L	Pure Non-Sig. 25	Not-studied	Not-stdied	Sig 7.0	Non-Sig. 30	Non-Sig. 200	7.7	0.21 (0.26)	0.04	(Khanna et al. 2013b)
C. pasteurianum (wild type; ATCC 6013)	Batch, Immobolized cells, Vol < 1 L	Crude Sig. 25	Not-studied	Not-stdied	Sig 7.0	Non-Sig. 30	Non-Sig. 200	6.8	0.17 (0.21)	0.035	(Khanna et al. 2013b)
C. pasteurianum (wild type; ATCC6103)	Batch, Free cells, Iron limitation, Vol < 1 L	Pure Not-studied 86 [a]	Sig. 18	Sig 0.42	Sig 5.5–6.0	Not-studied 37	Not-studied	10.0	0.25 (0.31)	0.27	(Malaviya et al. 2012)

[a] yield calculated based on glycerol consumed; Sig: Significant effect on butanol production; Non-Sig: No-Significant effect on butanol production.

2.6 Metabolic engineering and mutagenesis

C. pasteurianum exhibits product inhibition at low levels (10-15 g L^{-1}). Therefore, mutagenesis can be applied to *C. pasteurianum* to create strains with improved product formation and tolerance (Dabrock et al. 1992; Malaviya et al. 2012; Jensen et al. 2012b). In one study, batch fermentations were performed on the wild type *C. pasteurianum* ATCC 6103 and its genetically modified strain MBEL_GLY2 (Malaviya et al. 2012). A maximum butanol yield and productivity of 0.30 g g^{-1} and 0.31 g L^{-1} hr^{-1} were achieved, respectively, using the MBEL_GLY2 strain. Under the same experimental condition, butanol yield and productivity of 0.25 g g^{-1} and 0.27 g L^{-1} hr^{-1} were obtained with the wild type *C. pasteurianum* ATCC 6103. Malaviya et al. (2012) demonstrated significantly increased production rates in a high cell density continuous bioreactor using the MBEL_GLY2 strain. In another study, the butanol yield and productivity of stored crude glycerol supplemented with activated stone carbon by *C. pasteurianum* DSM 525 and its mutants (MNO6) were investigated (Jensen et al. 2012b). The maximum stored crude glycerol utilization rate attained by MNO6 was 7.59 g L^{-1} hr^{-1}, whereas the wild type strain reached rates of 4.08 g L^{-1} hr^{-1}. This corresponds to an increased rate of 86% compared to the wild type. The butanol production rate was similarly increased by 38% compared to the wild type grown on stored crude glycerol. Some of reported studies on butanol production from glycerol by hyper producing mutants of *C. pasteurianum* are presented in Table 2-4.

Until recently, there was no information about the whole genome of *C. pasteurianum and* this restrained effort in applying metabolic engineering to this species of bacteria. Recently however, genomic information was revealed for these two wild-type strains *C. pasteurianum* DSM 525 and ATCC 6013 (Rotta et al. 2015; Science et al. 2015). Now,

further progresses in improving strains of *C. pasteurianum* by direct genetic engineering are likely to be seen in the future.

Table 2-4 Summary of studies on PBE fermentation by hyper producing mutants of *C. pasteurianum*.

Strains	Process Parameters	Glycerol Consumed g L^{-1}	Max. Bioreactor Butanol Titer g L^{-1}	Max. Butanol Yield [a] g g^{-1} (mol·mol^{-1})	Overall Butanol Productivity g L^{-1} hr^{-1}	Reference
C. pasteurianum (mutant MNO6; DSMZ 525)	Fed Batch, Free cells, *in-situ* butanol removal, Vol < 1 L	Crude 100–122	12.6	0.20 (0.25)	1.80 [c,d]	(Jensen et al. 2012b)
C. pasteurianum (mutant MBEL_GLY2; ATCC 6103)	Batch, Free cells, Vol < 1 L	Pure 86.0	13.7	0.30 (0.37)	0.31	(Malaviya et al. 2012)
C. pasteurianum (mutant MBEL_GLY2; ATCC 6103)	Batch, Free cells, Vol < 1 L, Optimized medium	Pure 79.3	17.3	0.30 (0.37)	0.33	(Malaviya et al. 2012)
C. pasteurianum (mutant MBEL_GLY2; ATCC 6103) High initial cell concentration	Batch, Free cells, Vol < 1 L, Optimized medium	Pure 82.0	17.8	0.30 (0.37)	0.43	(Malaviya et al. 2012)
C. pasteurianum (mutant MBEL_GLY2; ATCC 6103) High Cells/Cell Recycle	Continuous, Free cells, (D = 0.9 h^{-1}) Vol < 1 L, Optimized medium	Pure 35 [b]	8.6	0.25 (0.31) [b]	7.8	(Malaviya et al. 2012)
C. pasteurianum (spontaneous asporogenous mutant; DSM 525)	Continuous, Free cells, D = 0.05 h^{-1} V ~ 1 L	Pure 30.85	7.45	0.24 (0.30)	0.372	(Dabrock et al. 1992)

[a] yield calculated based on glycerol consumed; [b] data inferred from graphical representation;
[c] productivity calculated based on active fermentation (subtracted lag phase); [d] corrected for by accounting for butanol removed in gas stripping.

2.7 Advanced fermentative technologies for high productivity

Unfortunately, there is little reported on advanced fermentation technologies applied specifically to *C. pasteurianum*, however the ones used for ABE likely can be transferred. The process solutions as employed for ABE fermentation are reviewed and their suitability for PBE is discussed.

2.7.1 High cell density

Overcoming the low productivity and yield of butanol fermentation from crude glycerol sources is also a requirement in order to commercialize this process. While some fed-batch and continuous fermentations using free cells are capable of high levels of production over time, cell immobilization techniques are an option for even further increases in production, while simultaneously allowing for easier downstream recovery of products (Zhao et al. 2006; Khanna et al. 2013a).Various methods of cell immobilization have been successfully employed to enhance butanol productivity in the ABE and PBE processes, including adsorption of cells onto a solid surface, immobilization of cells within a porous matrix, encapsulation of cells within a permeable membrane, and cell recycling using ultrafiltration (Lee et al. 2008a; Zheng et al. 2013; Jang et al. 2013; Khanna et al. 2014).

The adsorption of cells onto a solid surface is relatively easy and inexpensive compared to other immobilization techniques, as it uses natural cellular adhesion and biofilm formation to attach to the support (Schlieker and Vorlop 2006; Gungormusler et al. 2011). Glutaraldehyde is commonly used as a linking agent to facilitate cellular adhesion, though other agents (metal oxides, aminosaline) can also be used (Khanna et al. 2013a). Research using *C. pasteurianum* cells immobilized on a silica support and using biodiesel-derived crude glycerol as the carbon source reported higher productivity of butanol, ethanol, and PDO than with suspended cells, though productivity was still higher when using immobilized cells and pure glycerol (Khanna et al. 2014). These results were similar when the same group immobilized *C. pasteurianum* cells on Amberlite, an ion-exchange resin. In this case, the bacteria was able to tolerate and metabolize waste glycerol to butanol and PDO at a higher production rate than suspended cells (Khanna et al. 2013a). Unfortunately, there was no comparison with pure glycerol to assess if immobilization on Amberlite helped cells overcome the inhibitory compounds present in crude glycerol. However, other groups have also reported that even with cell adsorption, the impurities cause delayed

growth of cells when using waste glycerol with *Clostridia spp*. (Taconi et al. 2009; Gungormusler et al. 2011).

Immobilizing cells within a matrix of materials is advantageous in that low-cost, environmentally friendly materials may be used to form the matrix, while cells are also protected from shear forces within the reactor. Survase et al. (2012) screened several lignocellulosic materials for their efficacy as a support matrix for repeated batch and continuous ABE fermentation using *C. acetobutylicum*, finding that immobilizing the cells within a matrix of wood pulp allowed for the highest increases in solvent titer (18.88 g L^{-1} total solvents produced, compared to 8.18 g L^{-1} when using suspended cells) (Survase et al. 2012). Using lignocellulosic materials, specifically corn stover, as a support matrix, *C. pasteurianum* was found to metabolize glycerol to butanol at a much higher rate in continuous cultures versus suspended cells, 4.2 g L^{-1} hr^{-1} butanol vs 0.1 g L^{-1} hr^{-1} butanol, respectively (Gallazzi et al. 2015).

Encapsulation of the cells has been shown to reduce susceptibility of cells to end-product inhibition and making them more tolerant to the inhibitory effects of crude glycerol (Zhao et al. 2006). Cells are typically grown to high densities in rich media, then mixed with the encapsulation material, completely separating the cells from the fermentation medium behind a semi-permeable membrane. This allows the substrate to enter the micro-bead while products (both desirable and inhibitory) are removed, allowing for higher substrate concentration to be tolerated and less end-product inhibition to be observed (Westman et al. 2012). However, encapsulation can affect the rate of transport into and out of the cell and impact rates of reaction. To the best of the authors' knowledge, no reports of encapsulated *Clostridium spp.* using crude glycerol have been reported. However, based on results with ABE fermentative organisms, this is an interesting area for future exploration with *C. pasteurianum*. Rathore et al. (2015) demonstrated that *C. acetobutylicum* encapsulated in gellan gum could be used in up to five cycles of fermentations, though the encapsulated cells produced less butanol than free cells in the first cycle (7.66 g L^{-1} vs. 9.79 g L^{-1}, respectively). However, the butanol yield from free cells in a second fermentation cycle was greatly diminished, down to 2.9 g L^{-1}, while encapsulated cells did not see a significant drop in butanol production until the fifth cycle (Rathore et al. 2015). Considering the success of microencapsulation techniques for ABE

fermentations, this technique could feasibly be successfully applied to PBE fermentations with *C. pasteurianum.*

Cell recycling is a technique used to simultaneously increase the concentration of cells and reaction rate in fermentation and separate the fermentation broth from the cells for collection. The fermentation broth is passed over a porous membrane through which cells cannot pass, separating the permeate from the cells. The cells can then be cycled back into the fermenter, while cell-free permeate can be collected and the desirable end-products recovered (Malaviya et al. 2012). By matching the substrate feed rate to the permeate outflow allows for high cell density continuous culture fermentations. This strategy has been successfully applied by groups to increase cell concentrations in fermentations using a variety of species and substrates, butanol fermentation using glycerol included (Chang et al. 2011; Malaviya et al. 2012). Using a *C. pasteurianum* mutant and pure glycerol, butanol productivity as high as 7.8 g L^{-1} hr^{-1} has been obtained, compared to 0.43 g L^{-1} hr^{-1} when grown without cell recycling (Malaviya et al. 2012). A recent study demonstrated the possibility of using this technology as a purification technique to remove cells and proteins prior to extraction of PDO with great success, however in this case, the cell retentate was discarded rather than reintroduced to the fermenter (Kaeding et al. 2015).

While the majority of the studies done using immobilization have been conducted on ABE fermentation processes, the technologies could be applied to fermentation processes producing primarily butanol using *C. pasteurianum*. The few studies using *C. pasteurianum* found that similar results could be expected; higher cell densities, productivity, and tolerance to inhibitors present in the fermentation medium. Table 2-5 demonstrates the effects of cell recycling and immobilization on cells in PBE fermentations, namely to increase the productivity of the cultures.

Table 2-5 Summary of studies for high cell density of PBE fermentation by *C. pasteurianum.*

Strains	Culture Condition	Carbon Source	Max. Butanol Yield [a] g g^{-1} (mol mol^{-1})		Butanol Productivity g L^{-1} hr^{-1}		Reference
Cell Immobilization			Free Cells	Cell Immobilization	Free cells	Cell Immobilization	
C. pasteurianum (wild type; DSM 525)	Continuous (D = 0.44 h^{-1} for immobilized cells and D = 0.01 h^{-1} for free cells) Vol ~ 400 mL	Pure glycerol	0.4 (0.50)	0.33 (0.41)	0.1	4.2	(Gallazzi et al. 2015)
C. pasteurianum (wild type; MTCC 116)	Batch Vol < 1 L	Pure glycerol	0.18 (0.22)	0.36 (0.45)	<0.10	<0.10	(Khanna et al. 2014)
C. pasteurianum (wild type; MTCC 116)	Batch Vol < 1 L	Crude glycerol	0.13 (0.16)	0.23 (0.29)	<0.10	<0.10	(Khanna et al. 2014)
C. pasteurianum (wild type; MTCC 116)	Batch Vol < 1 L	Crude glycerol	Nt	0.35 (0.43)	Nt	<0.10	(Khanna et al. 2013a)
C. pasteurianum (wild type; MTCC 6013)	Batch Vol < 1 L	Pure glycerol	Nt	0.21 (0.26)	Nt	0.04	(Khanna et al. 2013b)
C. pasteurianum (wild type; MTCC 6013)	Batch Vol < 1 L	Crude glycerol	Nt	0.17 (0.21)	Nt	0.035	(Khanna et al. 2013b)
Cell Recycling			Free cells	Cell Recycling	Free cells	Cell Recycling	
C. pasteurianum (mutant MBEL_GLY2; ATCC 6103)	Continuous (D = 0.9 h^{-1}), Vol < 1 L Optimized medium	Pure glycerol	0.3 (0.37) [b]	0.25 (0.31)	0.43 [b]	7.8	(Malaviya et al. 2012)

[a] yield calculated based on glycerol consumed; [b] Batch Vol < 1 L; Nt: Not reported.

2.7.2 Continuous bioreactors for high productivity

The high cell density continuous bioreactor creates a static metabolic state for a stable production culture, eliminating the unproductive phases of cell proliferation (i.e. lag or growth phase) and downtime to clean and restart, with its associated extra costs (labour, water, chemicals, etc.) (Kumar and Gayen 2012; Kadic and Heindel. 2014). Continuous reactors allow controlling the product concentration by manipulating the feed concentration and dilution rate so that product inhibition is avoided. Thus, continuous fermentation would appear to be the best choice for scale-up of butanol production at industrial scale for these reasons. Continuous industrial bioreactors for the bioethanol industry have been as large as 2 million litres or more and typically are simple fluidized tanks, mixed by external recirculation loops aided by large eductors (jet pumps) (GreenField Specialty Alcohols Inc. Chatham, Ontario, Canada, 2010, verbal communication). At industrial scale cell recycle is common in continuous systems and is typically accomplished using large centrifuges (Iogen Corp., Ottawa, Ontario, Canada, 2005, verbal communication). On the other hand, in the lab or pilot plant the bioreactor design and configuration may look different, however it is intended to mimic the ideal design for industrial scale. For continuous regime, the bioreactor is initiated in a batch regime, inoculated from seed cultures typically 5-10 v v^{-1}%. When the cell growth reaches a desired phase of exponential growth, the bioreactor is continuously fed with medium, while the product stream is withdrawn to keep constant volume in the reactor. For cell recycle in the lab or pilot plant, ultrafiltration units can be used such as that shown in Figure 2-3, where an internal loop for recirculation (feed and bleed mode) can be used to achieve

high velocity in the UF membrane and reduce fouling.

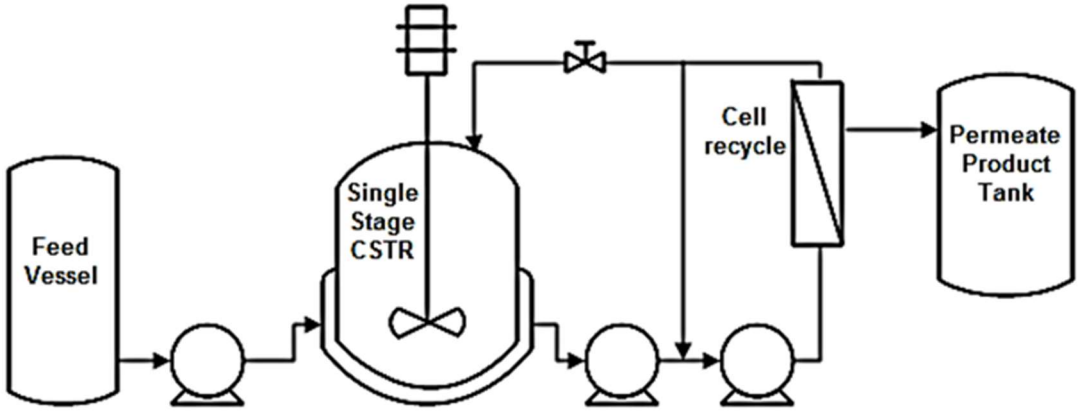

Figure 2-3 Continuous single stage continually stirred tank reactor (CSTR) with cell recycle

For ABE fermentation, research has focused on multi-staged types of bioreactors that can accommodate the physical separation of the environments required for biphasic metabolism, the sequential phases of acetogenesis followed by solventogenesis, typically requiring different pH and residence times. These multistage bioreactors typically have individual stage-wise parameter controls for pH, temperature, feeding, cell recycle, temperature, etc. An example of a three-stage continually stirred Tank Reactor (CSTR) in series is shown in Figure 2-4.

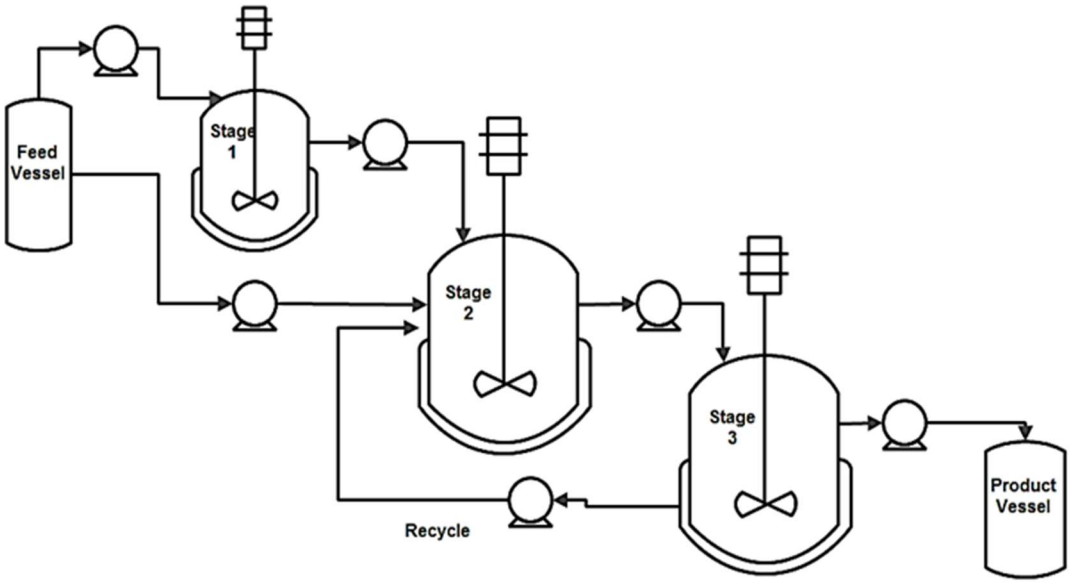

Figure 2-4 Continuous multi-staged CSTRs in Series

Tank volume can be used to alter the residence time without interrupting flow. Series stirred tanks and plug flow packed bed bioreactors have dominated in recent years. One disadvantage of continuous systems is that cell degeneration can occur especially with lower pH, requiring re-seeding with inoculum at various stages (Chang et al. 2016). The design and configuration of multi-staged systems are usually more complex and more difficult to control as can be seen by non-steady state data. Two popular biofilm reactors are the packed bed bioreactor (PBB) and the fibrous bed bioreactor (FBB) with the trickle bed bioreactor (TBB) being less popular in recent years (Figure 2-5).

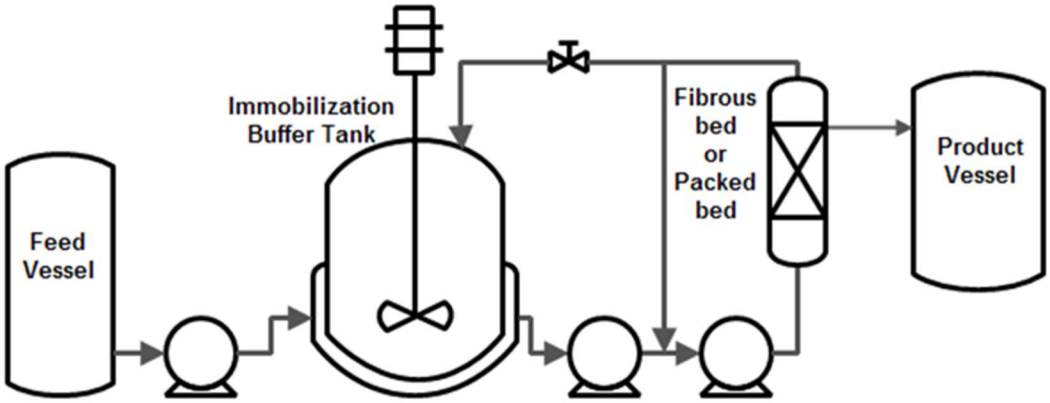

Figure 2-5 Continuous Biofilm Reactor

The PBB and FBB are vessels in which the immobilization support material remains in the tank and liquid flows through, usually co-currently to the gas phase. The biofilm bioreactors always require a pre-production growth phase where medium and inoculum recirculate until biofilm is formed on the support material. Packed beds are generally operated in a plug flow regime in order to achieve the separate physical environments, where the first zone has a higher pH and lower volume for acetogenesis, followed by solventogenesis. The PBB tends to suffer from head loss because of excessive cell growth. The main difference between the FBB and the PBB is that the FBB is packed with spiral wound highly porous fibrous material for support of biofilm (usually hydrophilic) such that the majority of cells in the bioreactor are present in the void space between the fibrous matrix and as such they can continually be sloughing off and renewed. Also, there is a gap between the sheets of the fibrous material, allowing liquid and solids to flow and gas to be released and thus reducing the risk of plugging. The FBB has been used for cell immobilization, often in multi-staged systems to achieve very high cell densities (up to 100 g L^{-1}), and in applications of extractive fermentation. The FBB can be operated as a trickle bed, a packed bed, or as an air lift where gas is sparged through the hollow core area and liquid circulates through the matrix. TBBs are fed at the top of the reactor thus obtaining product at the bottom. Stagnant pockets can form in the TBB and may affect the efficiency of the reactor. Table 2-6 summarizes the more successful examples of uses in research for

these bioreactors while Table 2-7 summarizes the advantages and disadvantages of these bioreactors.

Very high productivities have been reached in either case (CSTR with cell recycle versus biofilm bioreactors), however the long term operation of biofilm bioreactors has been plagued by plugging and multi-staged bioreactors with degeneration and lack of control and product consistency. With regard to biobutanol production from crude glycerol, there is an advantage to using the simple mixed tank (CSTR in lab-scale) with cell recycle design, as is the case in the bioethanol industry, which could be conceivable if the metabolism is non-biphasic.

Table 2-6 Types of continuous high cell density bioreactor used in research

Bioreactor Type	Fermentation Mode	Cell Configuration/Support	Bacteria	Dilution Rate hr^{-1}	Hours of Operation hr	Substrate	Max Production $g\ L^{-1}\ hr^{-1}$	Ref.
			CSTR with cell recycle					
3-stage CSTR (600 mL)	Continuous single pass	Immobilized on corn stover	*C. acetobutylicum* ABE 1201	0.04 overall	~400	Corn stover juice	0.45	(Chang et al. 2016)
Single stage CSTR (400 mL)	Continuous single pass	Cell recycle with ultrafiltration	*C. saccharoper-butylacetonicum* N1-4 ATCC 13564 (DCW = 18.0 g/L)	0.78	~100	Xylose	3.32	(Zheng et al. 2013)
Single stage CSTR (400 mL)	Continuous single pass	Cell recycle with ultrafiltration	*C. pasteurianum* ATCC 6013	0.9	~50	Glycerol	7.8	(Malaviya et al. 2012)
			Packed Bed Bioreactor					
Single stage PBB (200 mL)	Continuous single pass	Immobilized on corn cob residue	*C.pasteurianum* NRRL B-598	0.12	~700	Glucose	0.48	(Lipovsky et al. 2016)
Single stage PBB (180 mL)	Continuous single pass	Immobilized on corn stover pieces (1 cm^3)	*C. pasteurianum* DSM 525	0.44	~300	Glycerol	4.2	(Gallazzi et al. 2015)
Single stage PBB (250 mL)	Continuous single pass	Tygon ring carriers (ID = 3.2 mm)	*C. acetobutylicum* DSM 792 (DCW = 74 g/L)	0.97	~750	Lactose	4.4	(Napoli et al. 2010)

Single stage PBB (100 mL)	Continuous single pass	Immobilized on corn stover (5–8 mm)	*C.beijerinckii* ATCC 55025 on corn stock	1.00	~480	Glucose	5.06	(Zhang et al. 2009)
			Fibrous Bed Bioreactor					
Two-stage FBB (2 L)	Continuous single pass	Immobilized on spiral wound fibrous material	Co-culture *C. tyrobutyricum* ATCC 25755 *C. beijerinckii* ATCC 55025	0.144	~100	cassava starch	0.96	(Li et al. 2013)
Single stage FBB (150 mL)	Continuous single pass	Immobilized on spiral wound fibrous cotton sheets	*C.beijerinckii* ATCC 55025 DCW = 100 g/L, 70% viable	1.88	~350	glucose/butyric acid	17.29	(Chang 2010)
Single stage FBB (200 mL)	Continuous single pass	Immobilized on spiral wound fibrous sheets	*C. acetobutylicum* ATCC 55025	0.90	~1100	glucose/butyric acid	4.6	(Huang et al. 2004)

Table 2-7 Advantages and disadvantages of common types of bioreactors for continuous high cell density fermentation

	Advantages	Disadvantages
(a) CSTR w/cell recycle	• high cell density • high reaction rate • well mixed, therefore no gradients in pH, temperature or pressure • easy to operate, model, sample • no risk of plugging • easy to scale up • simple mechanically	• increased heat production • physical zone separation not possible • Higher viscosity of liquid
(b) Biofilm Reactors	• high cell density • high reaction rate • plug flow regime/physical zone separation • simple mechanically	• increased heat production • pH, temperature, pressure gradients (mixing problems) • low substrate utilization on single pass • difficult to sample for biomass quantification/viability • immobilization of biofilm growth phase required • lack of control of biofilm overgrowth issues with plugging • gas hold up pockets, channeling • PBB has higher risk of plugging • TBB has poor solid-liquid-gas contact, lower substrate utilization and mass transfer, pH gradients, sporulation and difficult to achieve plug flow regime

PBB: Packed Bed Bioreactor; TBB: Trickle Bed Bioreactor.

2.8 One stage in-situ butanol recovery technologies

Distillation remains the standard industrial method of recovery and concentration of butanol from dilute aqueous solutions, due to advantages such as its ease of scale-up, high recovery efficiency, and high concentration factors. However, the very low concentration of butanol, its high boiling point (118°C), and the presence of other fermentation products in the broth make butanol recovery by distillation energy intensive (Ezeji et al. 2004; Qureshi et al. 2005; Abdehagh et al. 2014; Abdehagh et al. 2015). There are many studies in the literature where modeling has been used to evaluate and optimize energy usage in distillation; however, there is a great deal of discrepancies (Vane 2008; Xue et al. 2013b; Abdehagh et al. 2014). At a concentration of 10 g L^{-1} butanol, distillation requires 1.5 times the energy contained in the resulting butanol (36 MJ kg^{-1}butanol). If fermentation could result in a butanol concentration of 40 g L^{-1} this ratio would decrease to 0.25 (Ezeji et al. 2004). Therefore, it is crucial to develop techniques for simultaneous butanol fermentation and in-situ product recovery to mitigate toxicity and enhance productivity. The high concentration of butanol resulting from in-situ product removal would potentially and substantially lower the energy consumption in distillation, which would follow in a commercial process (Kraemer et al. 2011; Errico et al. 2016).

Over the years many relatively economic and feasible techniques have been developed for in-situ butanol removal including gas stripping, vacuum stripping, pervaporation, liquid-liquid extraction, perstraction, and adsorption (Qureshi et al. 2005; Ha et al. 2010; Mariano et al. 2011; Mariano et al. 2012; Abdehagh et al. 2014; Errico et al. 2016). Table 2-8 summarizes the principles, advantages, and disadvantages of these techniques.

Table 2-8 Alternative separation techniques for butanol recovery from fermentation broth.

Method	Principle	Advantages	Disadvantages
Gas stripping	• Volatile solvents being stripped out by oxygen-free nitrogen or fermentation gases (H_2 and CO_2) and then condensed • Stripping gas can be recycled back into the process • Can be integrated with fermentation in the bioreactor, or performed in an individual stripping column	• Easy to operate • No harm to the culture • Strips only the volatiles • Ability to operate under fermentation temperature	• Low selectivity • Low efficiency • Requires high gas flow rate
Vacuum stripping	• Volatile solvents being stripped out by vacuum and then condensed • Can be integrated with fermentation in the bioreactor, or performed in an individual stripping column	• Easy to operate • No harm to the culture • Strips only the volatiles • Ability to operate under fermentation temperature • No need for extra volume in the fermentation tank for gases compared to gas stripping	• Low selectivity
Pervaporation	• Using membrane to selectively let the vaporous solvents pass through, driven by a chemical potential gradient • Vacuum pervaporation: Permeate side is under vacuum • Thermal pervaporation: the permeate is condensed on a cold wall at atmospheric pressure • Can be selective due to differences in membrane properties affecting sorption and diffusion • Diffusion is governed by the molecule size, shape, molecular weight, and inter/intra molecular free space in the membrane	• Low operating temperature • Low operating cost • No harm to the culture • Reduced energy demand • No loss of substrate or nutrients from fermentation broth • High selectivity	• Membrane fouling • Require high liquid flow rates • Redundancy for batch wise cleaning
Liquid-liquid extraction	• Using the soluble differences of solvents in fermentation broth and water-insoluble organic extractant for separation • Extractant can be recycled back into the process • Can be integrated with fermentation in the bioreactor, or performed in an individual extractor column	• High selectivity, efficient	• Forming emulsion • Toxic to the culture • High extractant recovery cost and loss
Perstraction	• Membrane-based extraction, separating the fermentation broth from the extractive solvents • Extractant can be recycled back into the process	• High selectivity • Low toxicity to the culture compared to liquid-liquid extraction	• Forming emulsion • Membrane fouling
Adsorption	• Adsorption of solvents onto the surface of adsorbent • Adsorbent can be regenerated for reuse • Can be integrated with fermentation in the bioreactor, or performed in an individual adsorption column	• Low energy requirement • Fully immiscible and unsusceptible to emulsification	• Adsorbent regeneration

Due to high butanol productivity and less labor and maintenance cost of continuous fermentation, the main emphasis of this section is being placed on the review of in-situ butanol recovery integrated with continuous fermentation. To the authors' knowledge there are very limited reports on integrated PBE fermentation with in-situ product recovery, none of which used a continuous mode. Therefore, we first report on those few studies found in the literature on integrated PBE fermentation with the aforementioned in-situ recovery techniques. Next, we report on those for continuous ABE fermentation coupled with in-situ recovery and their potential for application to PBE fermentation. Table 2-9 summarizes studies found on integrated PBE fermentation with in-situ butanol recovery.

Integrated PBE fermentation with in-situ butanol recovery

Gas Stripping

In recent years, gas stripping has been attracting much attention as an alternative for butanol removal from fermentation broth (Lu et al. 2012; Xue et al. 2012; Ezeji et al. 2013; Xue et al. 2013a). The studies found in the literature on integrated PBE fermentation with gas stripping used only fed-batch mode (Jensen et al. 2012b; Jensen et al. 2012c). In one study, Jensen et al. (2012b) evaluated butanol production from biodiesel-derived crude glycerol using *C. pasteurianum* DSMZ 525 in a fed-batch pH-controlled fermentor integrated with gas stripping. The crude glycerol was pretreated using a combination of addition of activated stone carbon and storage of the crude glycerol for 10 months at 20^{o} C. Using pretreated glycerol resulted in a productivity of 1.3 g L^{-1} hr^{-1}, whereas using technical grade glycerol without gas stripping resulted in the productivity of 1.21 g L^{-1} hr^{-1}. It should be noted that the productivities were calculated based on 'active fermentation time' by eliminating the lag phase time from calculations. In the next study, Jensen et al. (2012a) repeated the same experiment but using the mutant *C. pasteurianum* (MNO6). Under the same experimental conditions as the first study higher butanol productivity of 1.8 g L^{-1} hr^{-1} was achieved (Table 2-9). From the results of these two studies it is clear that the application of gas stripping resulted in reduced butanol inhibition and enhanced productivity, however a considerable lag phase still existed.

Liquid-liquid extraction

Liquid-liquid extraction (LLE) is another separation technique that can be applied for butanol in-situ recovery during fermentation or as a separate step after fermentation. The authors could find only one study in the literature on integrated PBE fermentation with LLE which used batch mode (Zhang. J, Gao. M,Hua. D, Li. Y, Xu. H, Liang. X, Zhao. Y, Jin. F, Chen. L, Meng. G, Si. H, Zhang. X 2013). Zhang et al (2013) investigates the capability of the *C. pasteurianum* SE-5 to produce butanol using crude glycerol as the sole carbon source and biodiesel as the extractant. This resulted in 89.1 g L^{-1} of crude glycerol consumption and 24.6 g L^{-1} of butanol production with more than 50% of the butanol extracted into the biodiesel phase. A butanol yield of 0.3 g g^{-1} and productivity of 0.34 g L^{-1} hr^{-1} were obtained, whereas using pure glycerol as substrate without extraction resulted in a butanol yield and a productivity of 0.29 g g^{-1} and 0.27 g L^{-1} hr^{-1}, respectively. The results suggested that the application of LLE resulted in reduced butanol inhibition, thereby improving butanol productivity and yield in a fermentation process directly in biodiesel solution.

Table 2-9 Summary of studies on integrated PBE fermentation with *in-situ* butanol removal.

Bacteria	Fermentation Mode	Substrate	Max Butanol Yield g g^{-1}	Overall Butanol Productivity g L^{-1} hr^{-1}	Hours of Operation hr	Comment	Reference
			Gas Stripping				
C. pasteurianum (mutant MNO6; DSMZ 525)	Fed Batch, Single-stage, Free cells, Vol < 1 L	Crude glycerol	0.20	1.8 (1.2)	~96–120	H_2 and CO_2, Stripping temperature 37 °C, Condensation temperature 0 °C	(Jensen et al. 2012a)
C. pasteurianum (wild type; DSMZ 525)	Fed Batch, Single-stage, Free cells, Vol < 1 L	Crude glycerol	0.225	1.3 (1.2)	~96–120	H_2 and CO_2, Stripping temperature 37 °C, Condensation temperature 0 °C	(Jensen et al. 2012b)
			Liquid-liquid extraction				
C. pasteurianum SE-5	Batch, Single-stage, Free cells, Vol = 1 L	Crude glycerol	0.30 (0.29)	0.34 (0.27)	~72	Biodiesel was used as extractant	(Zhang *et al.* 2013)

Integrated fed-batch and continuous ABE fermentation with in-situ butanol recovery

Gas Stripping

Ezeji et al. (2013) studied a single- stage fermentation integrated with gas stripping using *C. beijerinckii* BA101. A concentrated glucose solution (250-500 g L^{-1}) was fed as substrate to the bioreactor and a continuous bleed of bioreactor contents to reduce. The bioreactor produced 461.3 g L^{-1} ABE from 1,125.0 g total glucose as compared to a control batch process in which 18.4 g L^{-1} ABE was produced from 47.3 g glucose. This resulted in an ABE productivity of 0.92 g L^{-1} hr^{-1} with no change in yield. These results demonstrated that in-situ butanol removal improved the ABE fermentation; however there remained some inhibitory by-products that had to be bled from the reactor for stable operation and producing very noisy product data. In another study, Qureshi and Maddox (1990) investigated continuous ABE fermentation with gas stripping using immobilized cells of *C. acetobutylicum*. A single-stage fluidized bed bioreactor was used for butanol production from whey permeate. The integrated system was operated for 380 hours and was improved over the non-integrated system for ABE yield and productivity, however if the bioreactor substrate concentration (lactose) fell below a critical level, the reactions reverted to an acetogensis phase leading to a loss of substrate. (Table 2-10). Figure 2-6 shows a schematic diagram of a typical gas stripping process integrated with fermentation. Table 2-10 summarizes gas-stripping coupled with fed-batch and continuous ABE fermentation.

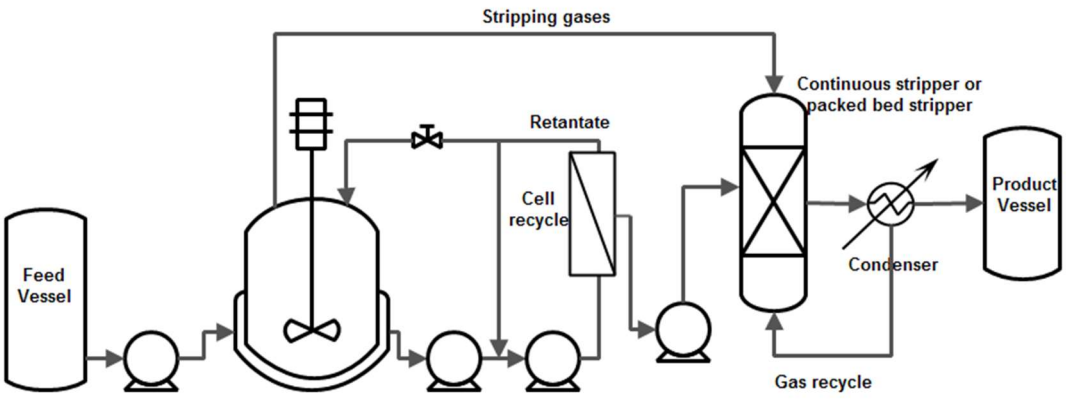

Figure 2-6 Continuous butanol fermentation integrated with gas stripping.

Table 2-10 Summary of studies on integrated continuous acetone-butanol-ethanol (ABE) fermentation with gas stripping.

Bacteria	Fermentation Mode	Substrate	ABE Yield g g^{-1}	ABE Productivity g L^{-1} hr^{-1}	Hours of Operation hr	Comment	Ref.
C. beijerinckii BA101	Continuous, Single-stage, Free cells	glucose	0.41 (0.39)	0.92 (0.29)	~504	H_2 & CO_2, Stripping temperature 35 °C, Condensation temperature 1 °C	(Ezeji et al. 2013)
C. acetobutylicum P262	Continuous, Single-stage, Immobilized cells in a fluidized bed reactor	Whey permeate.	0.4 (0.33)	5.1 (1.66)	~380	N_2, Stripping temperature 65–67 °C, Condensation temperature 3–4 °C	(Qureshi and Maddox 1990)
Clostridium sp. DSM 2152	Continuous, Single-stage, Free cells	Glucose	0.34 (0.37)	0.18 (0.17)	~300	N_2, 10 $L{\cdot}L^{-1}$ min, Stripping temperature 30 °C, Condensation temperature −5 to −40 °C	(W. J. Groot, R. G. J. M. van der Lans 1989)
C.acetobutylicum P262	Continuous, Single-stage, Free cells	Whey permeate	0.35 (0.32)	0.62 (0.15)	~52	N_2, 2.0 $L{\cdot}min^{-1}$, Stripping temperature 34 °C, Condensation temperature 4 °C	(Ennis et al. 1986)

Values in parenthesis were from the control experiments or fermentation without integrated product removal

Vacuum stripping

To the authors' knowledge there are no studies in the literature on PBE or continuous ABE fermentation coupled with vacuum stripping. Therefore, we report on an integrated batch ABE fermentation with vacuum stripping. This technique is in the early stages of its development but seems to be a promising method for butanol in-situ removal.

Mariano et al. (2011) investigated simultaneous ABE fermentation and in-situ product recovery using a vacuum process. Vacuum was applied continuously or intermittently with 1.5 hr vacuum sessions separated by 4, 6, and 8 hr intervals. Fermentation coupled with in-situ recovery by both continuous and intermittent vacuum modes resulted in a decrease in fermentation time, complete utilization of glucose, greater cell growth, and more concentrated product stream. The fermentation under continuous vacuum resulted in ABE yield and productivity of 0.22 g g^{-1} and 0.28 g L^{-1} hr^{-1}, whereas solvent yield and productivity of 0.35 g g^{-1} and 0.26 g L^{-1} hr^{-1} were achieved from a control experiment without in-situ recovery. Operation of the vacuum in intermittent mode with vacuum sessions of 1.5 h at intervals of 4 hr resulted in the shortest fermentation time and highest ABE productivity (0.34 g L^{-1} hr^{-1}) compared to control experiment, continuous vacuum, and 6 and 8 hr intervals.

The high level of productivity achieved by vacuum stripping is an important factor that can turn this process into a promising technology for the fermentative butanol production. Figure 2-7 shows a schematic diagram of a vacuum stripping process coupled with continuous fermentation. Table 2-11 summarizes vacuum stripping coupled with fed-batch and continuous ABE fermentation.

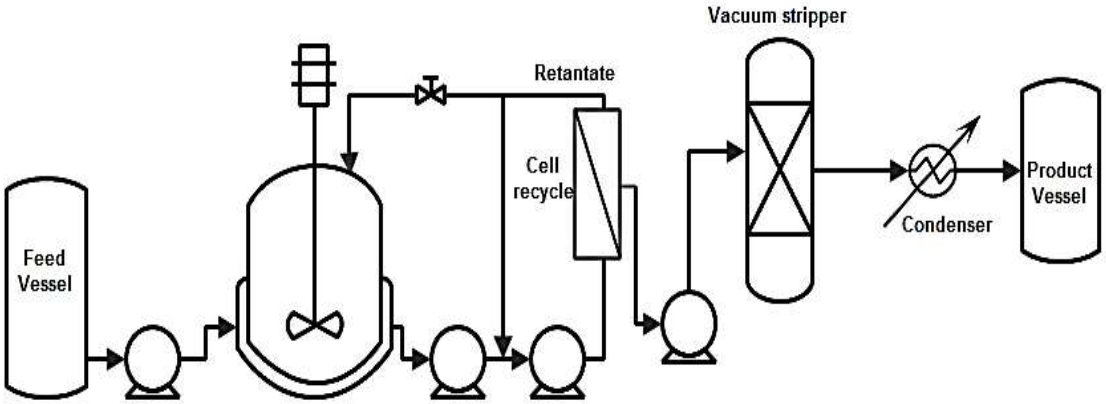

Figure 2-7 Continuous butanol fermentation integrated with vacuum stripping.

Table 2-11 Summary of studies on integrated continuous acetone-butanol-ethanol (ABE) fermentation with vacuum stripping.

Bacteria	Fermentation Mode	Substrate	ABE Yield g g^{-1}	ABE Productivity g L^{-1} hr^{-1}	Hours of Operation hr	Comment	Ref.
C. beijerinckii 8052	Batch [a], 7 L fermentation volume, Free cells	Glucose	0.29	0.43	~44	Continuous vacuum	(Mariano et al. 2012)
C. beijerinckii P260	Batch [a], 14 L Bioreactor (7 L fermentation volume), Free cells	Glucose	0.22	0.28	~48	Continuous vacuum	(Mariano et al. 2011)
C. beijerinckii P260	Batch [a], 14 L Bioreactor (7 L fermentation volume), Free cells	Glucose	0.26	0.34	~63	Intermitten vacuum, 1.5 h vacuum sessions were separated by 4 h time periods	(Mariano et al. 2011)

[a] Authors could not find any continuous study on butanol fermentation integrated with vacuum stripping.

Pervaporation

There were no reports on PBE fermentation integrated with pervaporation. Most of the studies found in the literature on integrated continuous ABE fermentation-pervaporation lacked stable fermentation operation, likely due to the biphasic nature of the *Clostridia spp*. used (Matsumura et al. 1992; Izák et al. 2008). Figure 2-8 shows a schematic diagram of a pervaporation process coupled with continuous fermentation.

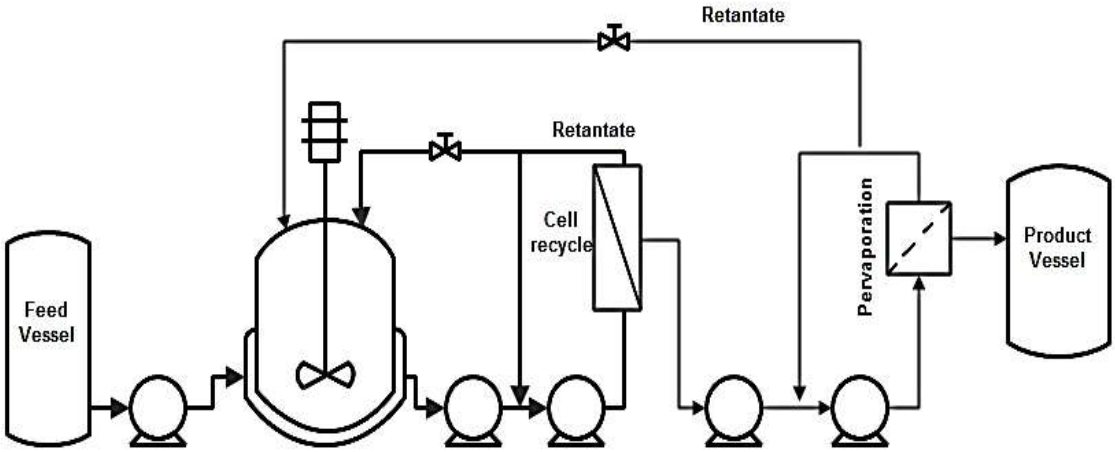

Figure 2-8 Continuous butanol fermentation integrated with pervaporation.

In one study, Van Hecke et al. (2013) ran a continuous 2-stage CSTR fermentation integrated with pervaporation in the second fermentor using freely suspended cells (*C. acetobutylicum*) and PDMS composite membrane for a duration of 825 hours, however this time was broken into 5 phases with different operating parameters. In the phase with the highest stable operation, they achieved an overall productivity of 0.88 g L^{-1} hr^{-1} with an average total ABE flux of 621 g m^{-2} hr^{-1} and a permeate enriched to 202 g L^{-1} total solvents. In another study, Li et al. (2014) obtained steady state conditions at a very low dilution rate (0.0117 hr^{-1}) in an ABE fermentation integrated with pervaporation for 268 hours and achieved an ABE productivity of 0.97 g L^{-1} hr^{-1}. This resulted in a total flux of 486 g m^{-2} hr^{-1} and a separation factor of 31.6. More recently, Van Hecke et al. (2016) prepared a Chemcad simulation for a conceptual plant design which resulted in a 50% energy savings when pervaporation was integrated. The process involved a two-stage continuous ABE

fermentation (dilution rate 0.109 h^{-1}, lignocellulosic hydrolysate as substrate). The productivity of 0.65 g L^{-1} hr^{-1} and 185 g kg^{-1} solvent in the permeate resulted (Van Hecke et al. 2016). The details of the studies on fed-batch and continuous ABE fermentation integrated with pervaporation can be found in Table 2-12. Also a recent review of the literature on butanol removal using pervaporation can be found in Kujawska et al. (2015).

Table 2-12 Summary of studies on integrated continuous acetone-butanol-ethanol (ABE) fermentation with pervaporation.

Bacteria	Fermentation Mode	Substrate	ABE Yield g g^{-1}	ABE Productivity g L^{-1} hr^{-1}	Hours of Operation hr	Comment	Ref.
C. acetbutylicum (CICC 8012)	Continuous, Single-stage, Free cells	Glucose	0.24	0.23 [a]	~192	PDMS (800 cm^2)	(Yao et al. 2016)
C. acetobutylicum DP 217	Continuous, Single-stage	Glucose	0.37	0.97	~268	PDMS (240 cm^2), $\alpha_{butanol}$ = 31.6	(Li et al. 2014)
C. acetobutylicum ATCC 824	Continuous 2 stage, Free cells	Glucose	0.28	0.88	~475	PDMS (180–270 cm^2), $\alpha_{butanol}$ = 17.67–19.81	(Van Hecke et al. 2013)
C. isopropylicum	Continuous, Single-stage, Immobilized cells	Molasses	0.29 [a]	Nt	~370	Liquid (1500 cm^2), Butanol flux of 3.3 $g \cdot m^{-2} \cdot h^{-1}$, $\alpha_{butanol}$ = 66	(Matsumura et al. 1992)

[a] butanol yield or productivity;

Nt:Not-reported.

Liquid-liquid extraction (LLE)

Bankar et al. (2012) studied a two-stage immobilized column bioreactor system integrated with LLE using immobilized *C. acetobutylicum* B 5313. The extraction module and the settling tank consisted of two glass jacketed bioreactors with a total volume of 1 L. Glucose was used as a substrate for continuous ABE production. The integrated system was operated for 720 hr without any technical problems. This resulted in ABE productivity of 2.5 g L^{-1} hr^{-1} and yield of 0.35 g g^{-1} at a dilution rate of 0.2 hr^{-1}, whereas solvent productivity and yield of 2.12 and 0.25 were achieved from a single stage system without in-situ recovery at a dilution rate of 0.6 hr^{-1}. Maximum total ABE solvent concentration of 25.32 g L^{-1} was achieved at a dilution rate of 0.05 hr^{-1}. Bankar et al. (2013) went on to study the sugar mixture (glucose, mannose, galactose, arabinose, and xylose) representative to the lignocellulose hydrolysates as a substrate for continuous ABE production. The experiments were carried out using the same system as the first study however the cells were immobilized on wood pulp (Table 2-13) and the ABE productivity of 10.85 g L^{-1} hr^{-1} and yield of 0.38 g g^{-1} were achieved. The integrated system was operated for 1152 hr (48 days) at 7 different dilution rates and maximum total ABE solvent concentration of 20.30 g L^{-1} was achieved at a dilution rate of 0.2 hr^{-1}. Figure 2-9 shows a schematic diagram of an integrated continuous fermentation with LLE. The details of the studies on fed-batch and continuous ABE fermentation integrated with LLE can be found in Table 2-13.

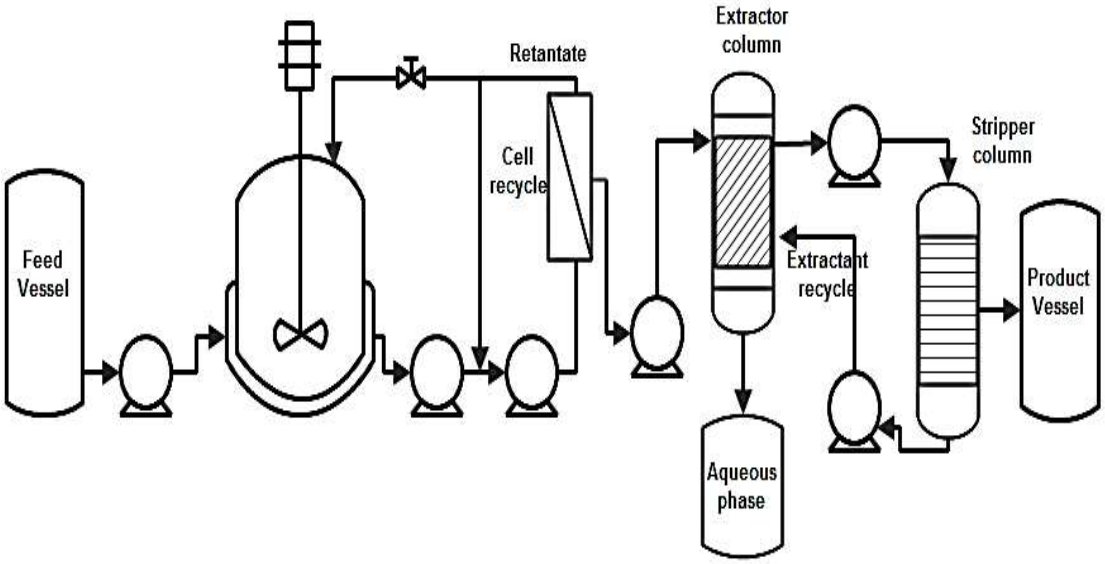

Figure 2-9 Continuous butanol fermentation integrated with liquid-liquid extraction (LLE).

Table 2-13 Summary of studies on integrated continuous ABE fermentation with liquid-liquid extraction.

Bacteria	Fermentation Mode	Substrate	ABE Yield g g^{-1}	ABE Productivity g L^{-1} hr^{-1}	Hours of Operation hr	Comment	Ref.
C. acetobutylicum DSM 792	Continuous, 2 stage immobilized column reactor, Free cells, D = 1.0 h^{-1}	Sugar mixture	0.38 (0.33)	10.85 (12.14)	~1152	oleyl alcohol and decanol (4:1)	(Bankar et al. 2013a)
C. acetobutylicum B5313	Continuous, two stage, Free cells, chemostat system, D = 0.05 h^{-1}	glucose	0.35 (0.25)	2.5 (2.12)	~720	oleyl alcohol and decanol (4:1)	(Bankar et al. 2012)
C. acetobutylicum P262	Continuous, Single-stage, Immobilized cells	Whey permeate	0.23 (0.36) 0.39 (0.36) 0.36 (0.35)	1.5 (3.5) 1.9 (3.6) 1.9 (3.0)	Nt	Dibutyl phthalate Benzyl benzoate Oleyl alcohol	(Qureshi and Maddox 1995)
C. acetobutylicum P262	Continuous, Single-stage, Free cells	Whey permeate	0.35 (0.32)	0.14 (0.07)	~170	Oleyl alcohol	(Qureshi et al. 1992)

Perstraction

Perstraction is a membrane based LLE technique that was developed to overcome problems associated with LLE. There are very limited reports in the literature on perstraction coupled with ABE fermentation.

Qureshi et al. (1992) studied ABE fermentation in an integrated continuous one-stage fermentation and perstraction product recovery system using a silicone membrane and oleyl alcohol as the perstraction solvent. The continuous system was operated for about 290 hr and the bioreactor produced 57.8 g L^{-1} ABE with a maximum concentration 9.8 g $L^{-1}$of ABE in the oleyl alcohol. This resulted in an ABE productivity of 0.24 g L^{-1} hr^{-1} and a yield of 0.37 g g^{-1}, whereas an ABE productivity of 0.07 g L^{-1} hr^{-1} and yield of 0.32 g g^{-1} were obtained from batch fermentation without product recovery.

Adsorption

To the authors' knowledge there are no studies in the literature on continuous ABE fermentation integrated with adsorption. The studies found in the literature on integrated ABE fermentation with adsorption used fed-batch fermentation, some used batch mode. The details of the fed-batch studies on ABE fermentation integrated with adsorption can be found in Table 2-14. Figure 2-10 shows a schematic diagram of an adsorption process coupled with continuous fermentation.

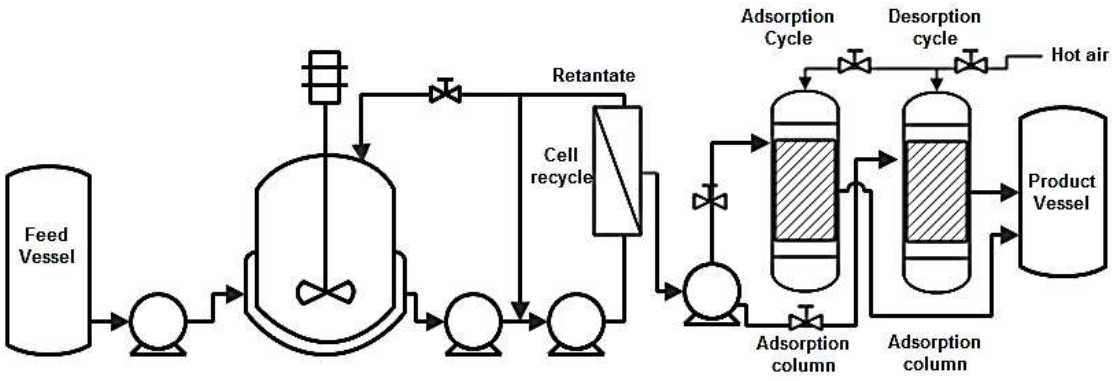

Figure 2-10 Continuous butanol fermentation integrated with adsorption.

Of note, Wiehn et al. (2014) investigated the application of expanded bed adsorption on butanol productivity. In this case, the expanded bed consisted of 0.17 L glass column containing 75 g of Dowex® Optipore L-493. This resulted in ~55% free head space (column volume unoccupied) in the column for bed expansion. The contents of the1 L culture were continuously re-circulated between the bioreactor and adsorption bed at a rate of about 100 mL min^{-1}. This integrated system was operated for 38.5 hours with maximum butanol and total solvent production of 27.2 g L^{-1} and 40.7 g L^{-1}, respectively. The butanol concentration in the cold trap reached as high as 85.8 g L^{-1} and an average 81% butanol recovery was obtained via adsorbent regeneration. Yang and Tsaot (1995) studied integrated repeated fed-batch fermentation with adsorption and cell recyle. Glucose was fermented by *C. acetobutylicum* and Polyvinylpyridine were used as adsorbent. This integrated system was operated for about 250 hours and 47.2 g L^{-1} of ABE was produced. Also, ABE yield of 0.32 g g^{-1} and productivity of 1.69 g $L^{-1}hr^{-1}$ were obtained compared to ABE yield of 30.9 g g^{-1} and productivity of 0.4 g $L^{-1}hr^{-1}$ in conventional batch fermentation. (Table 2-14).

Table 2-14. Summary of studies on integrated continuous ABE fermentation with adsorption.

Bacteria	Fermentation Mode	Substrate	ABE Yield g g^{-1}	ABE Productivity g L^{-1} hr^{-1}	Hours of Operation hr	Comment	Ref.
C. acetobutylicum ATCC 824	Fed-batch [a], Free cells, 1 L culture, expanded bed adsorption	Glucose	0.28 (0.17)	0.72 (0.63)	~38.5	hydrophobic polymer resin Dowex Optipore L-493	(Wiehn et al. 2014)
C.acetobutylicum	Repeated Fed-batch [a], Free cells, Cell recycle	Glucose	0.32 (30.9)	1.69 (0.4)	~250	Polyvinylpyridine	(Yang and Tsaot 1995)
C.acetobutylicum	Fed-batch [a], Free cells	Glucose	0.32 (30.9)	1.33 (0.4)	~250	Polyvinylpyridine	(Yang and Tsaot 1995)

Values in parenthesis were from the control experiments or fermentation without integrated product removal. [a] Authors could not find any continuous study on butanol fermentation integrated with adsorption.

Transferring in-situ recovery techniques from ABE fermentation to PBE fermentation

From the results reported by various authors it is clear that the application of in-situ recovery techniques resulted in reduced butanol inhibition, thereby improving butanol productivity. Also, due to simultaneous product removal, the microorganism can utilize concentrated substrates in an integrated fermentation process, which would otherwise cause substrate inhibition. However, most of these studies have short operation periods, especially if using batch or fed batch fermentation. Many did not provide sufficient experimental details to compare or did not analyze the in-situ recovery technique fully, likely due to a lack of steady state operation. It would appear that steady state operations were rarely achieved as can been seen from some of the data, thus there appears to be some issues with unstable operation of integrated ABE systems and it is not clear if this stems from unstable fermentation or unstable in-situ removal or both. It is also noted that with continuous ABE fermentation of biphasic *Clostridia spp*. with free cells, dilution rates have been very low for single stage bioreactors, whereas two-stage bioreactors have been able to achieve higher dilution rates. It is suggested that by using *C. pasteurianum* in a continuous PBE fermentation, unstable fermentation due to biphasic behavior would at least be eliminated from the other challenges, as reported by Johnson and Rehmann (2016), however that has yet to be demonstrated.

It should also be noted that no acetone (boiling point 56°C) is produced in PBE fermentation, but instead PDO whose boiling point is between 211 and 217°C. Thus, PDO is much less volatile than butanol and will likely remain in the fermentation broth versus be removed by in-situ recovery processes, accumulating if the dilution rate is not greater than or equal to the production rate. However, there is a lack of information in the literature on the toxicity of PDO to *C. pasteurianum*. Therefore, more research is needed to investigation the effect of the by-products in PBE fermentation broth, more specifically the effect of glycerol and PDO on the performance and efficiency of in-situ recovery techniques.

Table 2-15 summarizes a brief assessment of the technologies. It should be noted that pervaporation and perstraction, both involving membranes, will likely require batch-wise switching and cleaning and adsorption, involving ion exchange resin would require batch-wise switching and regeneration.

Therefore, vacuum stripping, pervaporation and adsorption appear to be promising technologies for in-situ butanol removal for PBE fermentation.

Table 2-15. Summary of brief assessment of different *in-situ* recovery technologies.

Technology	Green	Energy Demand	Efficiency
Gas stripping	Yes	High	High
Vacuum stripping	Yes	Low	High
Pervaporation	Yes	Low	High
Liquid-liquid extraction	No	Low	Low
Perstraction	No	Low	High
Adsorption	Yes	Low	High

2.9 Hybrid *in-Situ* Butanol Recovery Processes

In order to remove butanol toxicity from the fermenter, *in-situ* butanol removal is necessary, however, a single stage of *in-situ* butanol recovery is not efficient enough. A hybrid process therefore is needed to compliment technologies for the purpose of energy savings as well as to increase fermenter titers and productivity for commercialization. Single separation technologies have their inherent weaknesses but when coupled they can enhance each other. As well there is an advantage to run the fermenter at the highest butanol concentration possible. Also, it is noted that there have been very little reports of the implementation of hybrid *in-situ* butanol removal with demonstrated successful steady-state continuous operation.

As already mentioned, due to the much higher boiling point of PDO compared to acetone, PDO will remain in the fermentation broth. Thus, downstream purification will be different in PBE compared to ABE fermentation.

Unfortunately, the authors could not find any reports on hybrid *in-situ* butanol recovery coupled to PBE fermentation, however we report on those for ABE fermentation using glucose as substrate and their potential for application to PBE fermentation.

2.9.1 Two-Stage Gas Stripping

Xue *et al.* (2014) tested a two-stage gas stripping *in-situ* removal process coupled with ABE fed-batch fermentation (*C. acetobutylicum*) in a fibrous bed bioreactor (Xue et al. 2014). The first stage removed ABE *in-situ* from the fermenter and the second stage concentrated the aqueous portion of the condensate from the first stage. After process optimization, overall effective 48.5 g L^{-1} butanol (73.3 g L^{-1} ABE) was produced from the coupled hybrid process from 270.8 g glucose in 201 hours, as a result of reduced butanol inhibition on cells. The resultant butanol yield and productivity was 0.27 g g^{-1} and 0.24 g L^{-1} hr^{-1} respectively. The first-stage condensate contained 147.2 g L^{-1} butanol (199.0 g L^{-1} ABE), while the second stage condensate contained 515.3 g L^{-1} butanol (671.1 g L^{-1} ABE).

2.9.2 Gas Stripping-Pervaporation

One advantage of using gas stripping *in-situ* prior to pervaporation (GS-PV) is that the condensate from stripping will be void of salts, cell debris, residual sugars and other fermentation media components and remediate membrane fouling. A fed-batch fermentation with immobilized *C. acetobutylicum*, coupled to *in-situ* gas stripping (stripper external to fermenter) followed by pervaporation (GS-PV) relieved inhibition in the fermenter and producing a permeate from pervaporation with high concentration of ABE (706.68 g L^{-1}) and butanol (482.55 g L^{-1}). The high concentration of butanol (98.8 $w\,v^{-1}$%) would potentially lower the energy consumed in distillation, which would follow in a commercial process (Cai et al. 2016). Unfortunately, the pervaporation stage was only operational for 11 hr.

A similar lab scale hybrid system was studied by (Xue et al. 2016) using fed batch fermentation with *C. acetobutylicum* in a fibrous bed bioreactor for cell immobilization for 224 hr coupled to *in-situ* gas stripping and a second stage of pervaporation. Fermentation resulted in a butanol yield and productivity of 0.24 g g^{-1} and 0.34 g L^{-1} hr^{-1} respectively. In this study, the condensate from gas stripping was separated into an organic and an aqueous phase, where only the aqueous phase (85.6 g L^{-1} butanol) went to pervaporation. The organic phase from gas stripping was reunited with the permeate from pervaporation. The process resulted in a butanol selectivity of 97.8 and a final product concentration of 521.3 g L^{-1} butanol after combination. Gas stripping was relatively stable, however the pervaporation (second stage) was operated batch-wise in unsteady state for only 28 hr. Fed-batch fermentation is not a steady state operation and has limits of operation, unlike continuous fermentation.

2.9.3 Gas Stripping—Gas Permeation

Vane and Alvarez (2013) studied an experimental hybrid *in-situ* butanol removal process including vapor stripping, vapor compression, and a vapor permeation membrane separation in series, referred to as 'membrane assisted vapor stripping' (MAVS); however, the process was not coupled to fermentation, rather processed batch-wise (Vane and Alvarez 2013). The separation of solvents from ABE fermentation was benchmarked by a conventional distillation-decanter process. In the MAVS, feed liquid containing a solvent was fed into the top of a vapor stripping column. Solvent was stripped from the water in the column and the overhead vapor leaving the column was enriched in solvent, relative to the feed liquid, owing to favorable vapor liquid equilibria (VLE). The overhead vapor was compressed and the resulting higher pressure vapor was fed to a vapor permeation membrane module with a water-selective (hydrophilic) membrane. Pilot unit demonstrations were carried out on actual bacterial ABE fermentation broth (1.3 wt % butanol) produced in an 80 L batch fermention using *C. acetobutylicum*. The fermentation proceeded in a biphasic behavior taking 96 hours to finish followed by cell separation by centrifugation and down-stream batch-processing in the MAVS system. A product of 95 wt % butanol resulted using approximately 54% less energy compared to a distillation-decanter system.

2.9.4 Extraction-Gas Stripping

Lu and Li (2014) investigateßd an integrated *in-situ* extraction-gas stripping butanol removal process coupled with batch fermentation (*C. acetobutylicum*) in a 500 mL serum bottle (Lu and Li 2014). The non-volatile solvent oleyl alcohol acted as the extraction solvent and nitrogen was used for gas stripping. At first butanol was extracted by oleyl alcohol during ABE fermentation and gas stripping was initiated after 48 hr of fermentation in the oleyl alcohol phase. The butanol yield and productivity of 0.226 g g^{-1}, 0.28 g L^{-1} hr^{-1} was obtained respectively, after 96 hr of fermentation. 121 g L^{-1} glucose was consumed during fermentation and butanol concentration of 93–113 g L^{-1} was achieved in the condensate.

In summary, more research is needed to study hybrid *in-situ* butanol removal for PBE fermentations, more specifically the effect of having PDO and glycerol but not acetone and glucose in the fermentation broth. This is required for achieving higher productivity in fermentation by implementing high cell density and high gravity feeds, all of which require *in-situ* butanol removal to keep the fermenter butanol titer below toxic levels. Finally, it would appear that this technology has many benefits that should be transferrable from ABE to PBE.

2.10 Conclusions

The production of butanol using *C. pasteurianum* is an attractive option, given the possible use of crude glycerol as the feedstock and the non-biphasic nature of *C. pasteurianum* allowing for a single-stage continuous fermentation process. However, several obstacles still must be addressed before economic large scale butanol production can be implemented.

Most of the reports on butanol production in the literature implement ABE fermentation. As mentioned earlier, these *Clostridia* spp. cannot utilize glycerol as sole carbon and energy source. Therefore a considerable portion of this review, especially concerning technologies for *in-situ* butanol removal and high cell density come from studies from ABE

fermetnation. These technologies can be transferred to fermentation with *C. pasteurianum* and glycerol as substrate.

However, impurities and the variable nature of the crude glycerol must be overcome. Therefore, a consistent, inexpensive and broad-reaching pretreatment method to allow for efficient use of the crude glycerol from any source is required. *C. pasteurianum* is then able to convert crude glycerol into butanol by what appears to be non-biphasic fermentation. However, the fermentation can still be improved in terms of yield and productivity and needs to be validated at larger scale. Successful applications of mutagenesis and metabolic engineering towards improved butanol production with *C. pasteurianum* were demonstrated and suggest even further advances are in the near future, while higher reaction rates have been achieved using high cell density via cell recyling or immobilization in CSTRs, packed bed and fibrous bed bioreactors in single or multi-staged. Most important is that the process design and configuration be scaled to industrial size and perform with long term stable operation without plugging from biofilm overgrowth, while for a non-biphasic production host such as *C. pasteurianum* a multi-staged bioreactor design may not be necessary and the bioethanol industry could be used as the standard, with large mixed submerged culture tanks easy to control and operate. Multi-staged bioreactor design tends to be more difficult to control with product variability and cellular metabolic inconsistency.

To date, most research has been performed on very small volume systems. More research is needed at a larger scale and for longer duration at constant operating parameters, with additional focus on the downstream. Distillation systems are energy-intensive due to the low solvent titers in the fermentation broth. Various *in-situ* butanol removal technologies can alleviate butanol inhibition, improve productivity and mitigate energy consumption of the butanol purification system, where the lack of acetone will allow for simplified design and lowered costs, both operational and capital, involved in downstream distillation. More research is needed to study *in-situ* butanol removal (one-stage and hybrid) for PBE fermentations, more specifically the effect of having PDO and glycerol in the fermentation broth (Anand and Saxena 2012).

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Chapter 3

3 Optimizing enzymatic hydrolysis of Jerusalem artichoke tubers for fermentative butanol production

Tahereh Sarchami, Lars Rehmann.

Preface

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is substantially as it appears in Biomass and Bioenergy, July 2014, Vol 69, pages 175-182.

Jerusalem artichokes (*Helianthus tuberosus L.)* is considered a suitable feedstock for biofuel production due to many attractive characteristics, which include high biomass yield with low requirement on fertilizers, resistance to frost and plant diseases, native to temperate North America, and not competing with grain crops for arable land (Szambelan et al. 2005; Matías et al. 2011). The principal storage carbohydrate of Jerusalem artichoke tubers is inulin which cannot be directly fermented by most microorganisms. Therefore, inulin first needs to be hydrolyzed into sugar monomers. One way to do so is by enzymatic hydrolysis. Among fungi one of the best inulinase yields can be obtained from *Aspergillus niger* (75 Unit ml^{-1}) (Ricca et al. 2007), therefore many studies have been conducted using inulinase from this fungus for enzymatic hydrolysis of inulin (Ohta et al. 1993; Sirisansaneeyakul et al. 2006). However, information on the optimal condition of hydrolysis using inulinase from *Aspergillus niger* is limited in the literature.

Therefore, in this study, the effects of temperature, pH, substrate concentration and enzyme loading on the enzymatic hydrolysis of Jerusalem artichoke-derived inulin were studied. Statistical data obtained from RSM led to the development of an empirical model of inulin conversion as function of all four investigated factors. This model was numerically optimized to obtain the hydrolysis conditions that maximize inulin conversion to fermentable sugars. Finally, enzymatic hydrolysate of Jerusalem artichoke tubers was subjected to butanol fermentation.

The results of this chapter provided suitable conditions for inulin hydrolysis and further showed that the obtained hydrolysate was a good raw material for butanol production.

Abstract

In this study, a central composite design and response surface methodology were used to study the effect of various enzymatic hydrolysis variables (temperature, pH, substrate concentration and enzyme loading) on the enzymatic hydrolysis of Jerusalem artichoke-derived inulin. It was found that a quadratic model was able to predict inulin conversion as a function of all four investigated factors. The model was confirmed through additional experiments and via analysis of variance (ANOVA). Subsequently, numerical optimization was used to maximize the inulin conversion (94.5%) of Jerusalem artichoke powder within the experimental range (temperature of 48°C, pH of 4.8, substrate concentration of 60 g L^{-1}, and enzyme loading of 10 units g-1substrate for 24 hours). The enzymatic hydrolysate of Jerusalem artichoke was fermented via solventogenic clostridia to acetone- butanol-ethanol (ABE). An ABE yield of 0.33 gSolvent g-1sugar and an overall fermentation productivity of 0.25 g L^{-1} hr^{-1} were obtained indicating the suitability of this feedstock for fermentative ABE production.

3.1 Introduction

In the current decade interest in research on the conversion of agricultural biomass into automotive fuels and chemicals has increased substantially, with a strong focus on ethanol (Sánchez and Cardona 2008; Alvira et al. 2010). Butanol contains two more methyl-groups as compared to ethanol, rendering it more hydrophobic, less volatile, higher in its energy density, and it is fully miscible with gasoline. Therefore, the fermentative production of butanol has received renewed attention in recent years (Atsumi et al. 2008).

One of the major obstacles to commercial acetone-butanol-ethanol (ABE) fermentation is the high cost and availability concerns of conventional substrates (corn, molasses) (Jones and Woods 1986). Substrate cost constitutes at least 50% of the total production cost during the ABE fermentation, and the process economics and feasibility largely depends on the

availability of cost-effective raw materials (Dürre 2007; Qureshi et al. 2008; García et al. 2011; Schwab et al. 2013; Luque et al. 2014; Gao and Rehmann 2014). To overcome this limitation lignocellulosic biomass such as corncob (García et al. 2011) and wastewater streams such as cheese whey (Raganati et al. 2013), have been investigated and identified as alternative substrates for butanol production via ABE fermentation. Jerusalem artichokes (*Helianthus tuberosus L.)* as an alternative carbon source have potential as a renewable feedstock for solvent production when fermented by suitable microorganisms (Ge and Zhang 2005). It is a low requirement crop with a high sugar production usually grown for its tubers. This plant is not only very resistant to frost and plant diseases but also can grow on poor land (Szambelan et al. 2005). It has one of the highest carbohydrate yields ranging from 5 to 14 tons per hectare (Matías et al. 2011) and therefore had been considered for butanol production in the past (Marchal et al. 1985; Chen et al. 2010). Jerusalem artichoke can be grown in various climate zones in North America, although the plant is better adapted to cooler climates (Baltacıo 2013). It can potentially be grown in Ontario on lands traditionally used for Tabaco production. Demand for Tabaco is decreasing and the land requirements for the two crops are similar. Replacing Tabaco fields with Jerusalem artichoke fields does not interfere with the current food production practices. Jerusalem artichoke tubers typically comprise about 80% water, 15-20% carbohydrates, 1-2% protein and virtually no fat (Matías et al. 2011). The principal storage carbohydrate of Jerusalem artichoke is inulin; however, monomeric sucrose, glucose and fructose are also present. Inulin consists of linear chains of β (2→1) linked D-fructose units. Each chain is terminated by a D-glucose residue linked to fructose by α (1→2) bond (Szambelan et al. 2005). Most organisms cannot directly ferment inulin, therefore inulin first needs to be hydrolyzed into fructose and glucose monomers. Hydrolysis can be achieved via an acid catalyst or enzymatically. Acid hydrolysis can lead to fermentation-inhibiting by-products, while enzymatic hydrolysis is dependent on potentially expensive enzymes, and therefore should be optimized. Among fungi one of the best inulinase yields can be obtained from *Aspergillus niger* (75 Unit ml^{-1}) (Ricca et al. 2007), therefore many studies have been conducted using inulinase from this fungus for enzymatic hydrolysis of inulin (Ohta et al. 1993; Sirisansaneeyakul et al. 2006). However, information on the

optimal condition of hydrolysis using inulinase from *Aspergillus niger* is limited in the literature.

The objective of this study is therefore twofold, the optimization of enzymatic hydrolysis of inulin to maximize its conversion to fermentable sugars, and the subsequent fermentation of hydrolysate to butanol, an advance biofuel.

3.2 Materials and Methods

3.2.1 Enzymatic hydrolysis

3.2.1.1 Preparation of Jerusalem artichoke flour

Jerusalem artichoke tubers, white flesh, were obtained from the Institute for Chemicals and Fuels from Alternative Resources (ICFAR), University of Western Ontario. The entire Jerusalem artichoke tubers were washed and sliced to approximately 2 cm cubes. The obtained slices of were transferred directly to a drying oven and dried at 105ºC for 72 hours, then ground to fine particles using a coffee grinder and passed through a 250 µm mesh. The prepared sample with approximately 3% moisture content was stored in a dry container at 4ºC for further use.

3.2.1.2 Inulin extraction

Inulin extraction was performed based on a method by Bekers et al (2007). Extracts were obtained by adding 100 ml of water to 5 g of Jerusalem artichoke powder. The slurry was put into a water bath at 25ºC and agitated using a magnetic stirrer at 300 rpm for 1 hour. The samples were then centrifuged for 20 minutes at 12,000 xg (Bekers et al. 2007). The supernatant contained the extractable carbohydrate fraction of Jerusalem artichoke tubers including 0.52 g g^{-1} inulin, 0.16 g g^{-1} fructose, 0.1 g g^{-1} glucose and 0.05 g g^{-1} sucrose (Table 4.1). The precipitate contained the non-extractable fraction of Jerusalem artichoke tubers including 0.03 g g^{-1} cellulose and 0.02 g g^{-1} hemicellulose (Dao et al. 2013). The cellulose and hemicellulose fraction in Jerusalem artichoke tubers is relatively small; therefore, only the supernatant was removed for HPLC analysis and acid hydrolysis.

3.2.1.3 Enzymes

Inulinase from *Aspergillus niger* was purchased from Sigma-Aldrich with 286 units g^{-1} activity.

3.2.1.4 Experimental Design

A central composite design (CCD) with four factors was selected to evaluate the response pattern and to determine the optimal combination of temperature, pH, substrate concentration and enzyme loading for maximizing inulin conversion to fermentable sugars (an initial full factorial design had shown significant curvature and confirmed the significance of all four parameters, data not shown). The un-coded values for each parameter were as follows [low star point, low central point, center point, high central point, high star point]: Temperature in ºC [35.9, 40, 50, 60, 64.1], pH [3.6, 4, 5, 6, 6.4], substrate concentration in g L^{-1} [11.7, 20, 40, 60, 68.3], and enzyme loading in units g^{-1} [0.34, 2, 6, 10, 11.66]. The experimental design was developed using Design Expert 8.0.7.1 (Statease, Inc., Minneapolis, MS, USA) and resulted in 26 conditions. All conditions were tested in triplicated, including 3 center points. The resulting 87 conditions (16 * 3 factorial + 10 * 3 augmented +3 * 3 center points) were fully randomized.

3.2.1.5 Enzymatic hydrolysis of inulin

Batch enzyme reactions were performed for fructose production employing the selected experimental conditions. Enzymatic hydrolysis of extracted inulin was performed in 20 ml glass scintillation vials filled with a 10 ml working volume containing inulinase from *Aspergillus niger*. Each vial contained 5 ml of Jerusalem artichoke extract and 5 ml of 0.05 M sodium acetate buffer at the desired pH. Inulinase was mixed with inulin in the aforementioned buffer. All contents of the vials were at desired temperature prior to enzyme addition. The vials were hermetically covered with Parafilm and aluminum foil to avoid evaporative losses, and the mixture was incubated at the desired temperature for 24 hours while shaking at 250 rpm.

3.2.1.6 Statistical analysis

Linear regression analysis was used to fit the experimental data with a second-order model as given in equation (3-1):

$$Y= \beta_0+\sum_{i=1}^{4} \beta_i x_i+\sum_{i=1}^{4} \beta_{ii} x_i^2+\sum_{1\leq i\leq j}^{4} \beta_{ij} x_i x_j+\varepsilon \quad (3\text{-}1)$$

The experimental data was analyzed using Design Expert 8.0.7.1. The significance of each term was verified via analysis of the variance (ANOVA). The significance of each parameter, the interaction and quadratic effects were determined based on an α of 0.05 using the F test. The fitted model was evaluated by normal probability plots, R^2 and adjusted R^2 and lack of fit coefficient for determining the adequacy. Numerical optimization via Design Expert 8.0.7.1 determined the optimal conditions for maximizing inulin conversion. The model and optimization results were validated by performing experiments closely around the predicted optimum.

3.2.1.7 Analytical methods

Concentration of sugars in Jerusalem artichoke juice as well as hydrolysate was determined by high performance liquid chromatography on an Agilent 1260 infinity (Agilent USA, Santa Clara) using an Agilent Hi-plex H (7.7 × 300 mm) column and Cation H^+ guard column (Agilent USA, Santa Clara) operating at 60°C. A refractive index detector (RID) was used for compound detection. Water was used as the isocratic mobile phase at a constant flow rate of 0.6 ml $min^{-1.}$ Before injection, samples were diluted to appropriate concentration with deionized water and filtered through a 0.2 µm membrane filter. Total carbohydrates were analyzed using pure inulin (Sigma Aldrich Co.) fructose, glucose and sucrose (VWR Co.) as standards.

The inulin conversion was evaluated based on fructose and glucose production. The average glucose to fructose ratio after complete conversion was 4±0.45 (Table 3-1). Full conversion resulted in 0.15 g g^{-1} and 0.60 g g^{-1} glucose and fructose (per g dry matter), respectively.

3.2.2 Fermentation

3.2.2.1 Chemicals

Yeast extract and peptone were obtained from BD- Becton, Dickinson and company (New Jersey, USA). Soluble starch was purchased from Alfa Aesar (Massachusetts, USA). Glucose was from Amresco (Ohio, USA) and $MgSO_4$ was from EMD Millipore (Massachusetts, USA). Ammonium acetate, KH_2PO_4, and K_2HPO_4 were purchased from Caledon (Ontario, Canada). $FeSO_4$ and NaCl were obtained from BDH (Georgia, USA).

3.2.2.2 General microbiological conditions

All microbiological work was performed in an aseptic anaerobic chamber (Model 855-ACB, Plas Labs, Lansing, MI).

3.2.2.3 Strain and maintenance

Clostridium saccharobutylicum DSM 13864 was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Cultures of this strain were routinely maintained as spore suspensions in seed medium containing (per liter) 3 g of yeast extract, 5 g of peptone, 5 g of soluble starch, 5 g of glucose, 2 g of ammonium acetate, 2 g of NaCl, 3 g of $MgSO_4$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.1 g of $FeSO_4$, pH 6.0 at 4°C. Spores in the seed medium were heat shocked for 2 minutes at 90°C and transferred to fresh seed medium. Three ml of actively growing cells were inoculated into 50 ml of inoculum development P2 medium, prepared in a 100 ml screw-capped bottle. The P2 medium contained 30 g L^{-1} glucose, 1 g L^{-1} yeast extract, and stock solutions (minerals, buffer, and vitamins) (Qureshi et al. 2008). The solution containing glucose and yeast extract was sterilized at 121ºC for 20 minutes and 0.5 ml of each of the filter-sterilized stock solutions were added to 50 ml glucose-yeast extract solution. Then the bottles were placed in an anaerobic chamber for 24 hours. The culture (inoculum) was allowed to grow for approximately 10 hours at 37ºC when it was ready to be inoculated into the ABE production medium.

3.2.2.4 ABE fermentation

All fermentation studies were conducted in 150 ml flasks containing 100 ml of fermentation medium. Control fermentation medium contained 60 g L^{-1} glucose, fructose or mixed sugars (fructose and glucose in the ratio of 3:1), 1 g L^{-1} yeast extract, and stock solutions (minerals, buffer, and vitamins) (Qureshi et al. 2008). The solution containing glucose and yeast extract was sterilized at 121°C for 20 minutes and 1 ml of each of the filter-sterilized stock solutions were added to 100 ml glucose-yeast extract solution. 10 ml of actively growing cells were inoculated into 100 ml of fermentation medium. Then the flasks were kept in an anaerobic chamber and placed on shaker at 200 rpm for 72hours.

For the hydrolysate fermentation, the pH was adjusted to 6.0 using 1M NaOH solution. To the bottle, 1 ml of sterile 1 g L^{-1} yeast extract solution and 1 ml of each stock solution were added to reach the same nutrient concentration level as in P2 medium. Subsequently the bottles were inoculated with 10 ml of actively growing culture followed by incubation at 37°C. Following this the bottles were kept in an anaerobic chamber and placed on shaker running at 200 rpm for 72 hours. Samples were taken intermittently and filtered using 0.2 µm grade filters. Clear liquid was stored at -20°C for ABE and sugar analysis (HPLC, see section 3.2.2.5 for conditions).

3.2.2.5 Analytical methods

Bacterial growth was monitored by measuring the optical density (OD) at 600 nm using a 200 pro infinite series microplate reader (Tecan, Switzerland) using 96 well microplates at 200 µl per well. Concentrations of solvents produced in the fermentation were determined by high performance liquid chromatography on an Agilent 1260 infinity (Agilent USA, Santa Clara) using an Agilent Hi-plex H (7.7 × 300 mm) column (Agilent USA, Santa Clara) at 15°C. A refractive index detector (RID) was used for compound detection. Water was used as the isocratic mobile phase at a constant flow rate of 0.6 ml $min^{-1.}$ Before injection, samples were diluted to appropriate concentration with deionized water and filtered through a 0.2 µm membrane filter. Total solvents were quantified using pure

butanol (Sigma-Aldrich, St. Louis, MO), acetone (Sigma-Aldrich, St. Louis, MO), and ethanol (Sigma-Aldrich, St. Louis, MO) as standards.

Productivity was calculated as the maximum ABE concentration achieved (g L^{-1}) divided by the fermentation time at a fixed time of 60 hours and is expressed as g L^{-1} hr^{-1}. Product yield was calculated as the total amount of solvents produced, divided by the amount of fermentable sugar utilized and is expressed as $g_{Solvent}$ g^{-1}_{sugar}. At least three parallel samples were used in all analytical determinations, and data are presented as the mean of three replicates.

3.3 Results and Discussion

The percent total solid content of Jerusalem artichoke tuber used in this study was about 30% of the fresh weight. Inulin, fructose, glucose and sucrose composition of the material are shown in Table 3-1. The small standard deviation indicates a homogenous carbohydrate composition within the tested Jerusalem artichoke tubers. The measured values are in agreement with values typically found for Jerusalem artichoke tubers (Matías et al. 2011).

Table 3-1 Jerusalem artichoke carbohydrate composition (original composition of raw material and composition of hydrolysate). Data represents the average of triplicates ± standard deviation.

Compound	g Sugar/g Jerusalem artichoke (DW)	g Sugar/g Jerusalem artichoke (DW) - Fully hydrolyzed
Inulin	0.52±0.05	0.003±0.002
Fructose	0.16±0.02	0.60±0.06
Glucose	0.10±0.01	0.15±0.03
Sucrose	0.05±0.008	0.09±0.002

3.3.1 Enzymatic hydrolysis

Experimental conditions were chosen based on a central composite design and the actual values of the independent variables and the measured responses are shown in Table 3-2.

Table 3-2 Enzymatic inulin conversion (average of triplicates ± standard deviation) under conditions determined for CCD.

Temperature (°C)	pH	Substrate Concentration (g L^{-1})	Enzyme Loading (unit $g^{-1}_{substrate}$)	Inulin Conversion (%)
35.9	5	40	6	73.6±1.3
40	4	20	2	68.9±1.6
40	4	20	10	81.1±0.4
40	4	60	2	79.2±0.5
40	4	60	10	87.2±0.6
40	6	20	2	55.7±1.3
40	6	20	10	69.3±1.2
40	6	60	2	67.2±1.1
40	6	60	10	83.1±0.3
50	3.6	40	6	72.9±1.1
50	5	11.7	6	65.7±0.8
50	5	40	0.34	59.1±2.1
50	5	40	6	84.1±0.4
50	5	40	6	83.8±0.4
50	5	40	6	83.6±0.4
50	5	40	6	83.3±0.4
50	5	40	6	82.9±0.4
50	5	40	11.66	88.3±0.5
50	5	68.3	6	93.9±1.4
50	6.4	40	6	58.1±0.6
60	4	20	2	56.7±1.4

60	4	20	10	71.7±0.9
60	4	60	2	73.2±0.6
60	4	60	10	81.9±0.8
60	6	20	2	35.9±1.5
60	6	20	10	51.7±0.7
60	6	60	2	65.1±0.9
60	6	60	10	78.5±0.5
64.1	5	40	6	62.1±1.3

3.3.2 Response surface model validation

As observed from the experimental results in Table 3-2, the enzymatic hydrolysis using inulinase from *Aspergillus niger* was successful in converting inulin to monomeric sugars within the ranges of the input variables.

The complete dataset could be fitted with a quadratic model as describe in equation (3-1). The resulting model parameters are shown in Table 3-3. The *F* value of the model is 40.55 which is very high compared to the critical value, indicating that the model is highly significant. The significance of each parameter coefficient was determined by *P* values, the smaller the *P* values the more significance of the coefficient. In this case, all factors have great effect on enzymatic reaction. The quadratic effects of temperature as well as pH, and interaction effect of temperature-substrate concentration, as well as pH-substrate concentration have also significant effects on inulin conversion. The goodness of fit of the model was confirmed by the coefficient of determination R^2=0.98 and adjusted determination coefficient Adj. R^2=0.95. A ratio of 26.44 of the adequate precision indicates an adequate signal to noise ratio for navigating the design space.

Based on the selected significant variables, the quadratic model for the inulin conversion in terms of actual factors is shown as follows:

Inulin Conversion = -177.60 + 5.11 * Temperature + 61.03 * pH – 0.82 * Substrate Concentration +2.69 * Enzyme Loading+ 0.01* Temperature * Substrate Concentration + 0.12 * pH * Substrate Concentration - 0.06 * Temperature^2 – 6.94 * pH^2 (3-2)

The residuals can be judged as normally distributed based on a normal probability (data not shown).

Table 3-3 Analysis of variance of fitted model

Source	**Remark**	**Sum of squares**	**Degrees of freedom**	**Mean square**	***F* value**	***P* value Prob>*F***
Model	Significant	4602.3	14	328.74	40.55	<0.0001
Temperature (A)	Significant	434.90	1	434.90	53.64	<0.0001
pH (B)	Significant	653.57	1	653.57	80.61	<0.0001
Substrate Concentration (C)	Significant	1349.4	1	1349.4	166.42	<0.0001
Enzyme Concentration (D)	Significant	1019.1	1	1019.1	125.70	<0.0001
AC	Significant	105.06	1	105.06	12.95	0.0032
BC	Significant	91.20	1	91.20	11.25	0.0052
A^2	Significant	288.85	1	288.85	35.63	<0.0001
B^2	Significant	418.56	1	418.56	51.63	<0.0001
R-Squared						0.98
Adj-Squared						0.95
Pre R-Square						0.86
Adeq Precisior						26.44

3.3.3 Combined effect of Temperature, pH, substrate concentration and enzyme loading

Response surface methodology was used to study the interaction effects of the four factors. The three dimensional surface plots of the combined effect of temperature and substrate concentration on inulin conversion at a constant pH of 5.0 and enzyme loading of 6 unit $g^{-1}_{substrate}$ are shown in Fig 3-1A. The inulin conversion is a function of both the temperature and substrate concentration. Fig. 3-1B shows the three-dimensional surface plots of the combined effect of pH and substrate concentration on inulin conversion at a constant temperature of 50ºC and enzyme loading of 6 units $g^{-1}_{substrate}$. The inulin conversion is also a function of both the pH and substrate concentration. The plots clearly indicate that an optimum exists within the observed design space with respect to pH and temperature, increasing the substrate concentration (at the same substrate to enzyme ratio) appears to increase inulin conversion over the observed design space, likely due to a simple increase in the reactant concentrations.

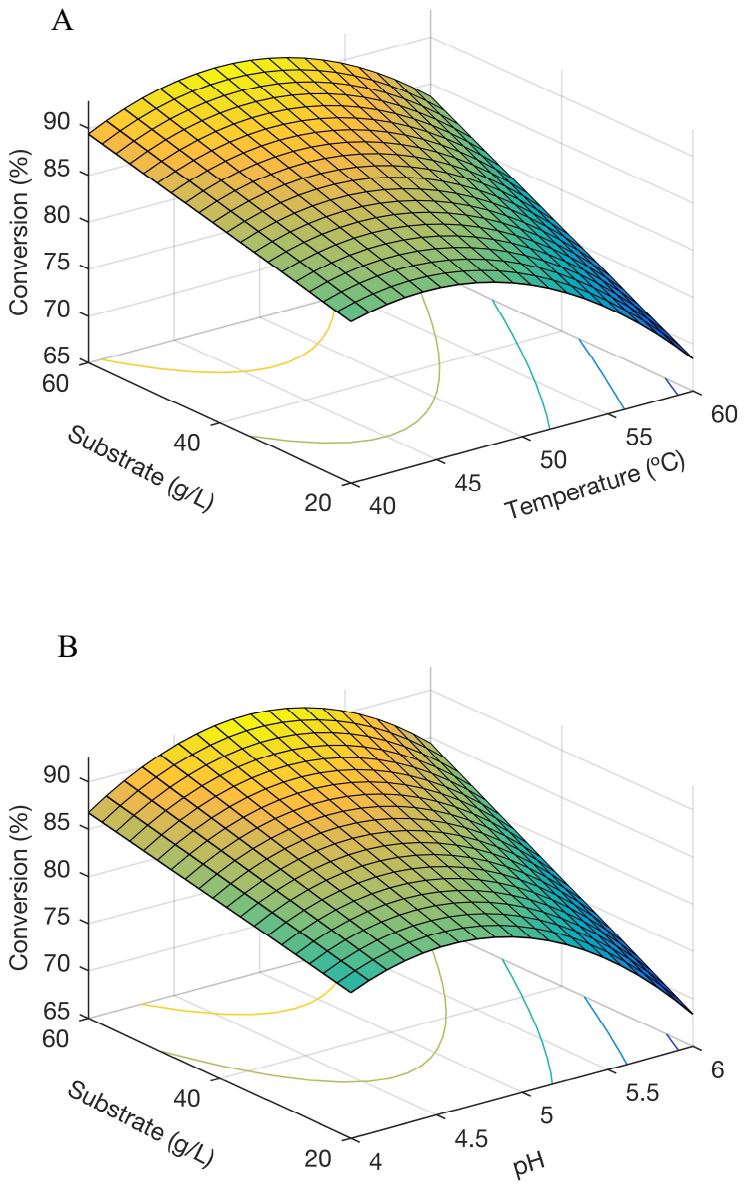

Figure 3-1 Surface plots of combined effect of process variables on inulin conversion. A) Temperature and substrate concentration, B) pH and substrate concentration.

3.3.4 Response optimization and model validation

Based on the model, numerical optimization was used to determine the optimal combination of process parameters for maximum inulin conversion. The optimal conditions for inulin conversion were a temperature of 48°C, pH of 4.8, substrate concentration of 60 g L^{-1}, and enzyme loading of 10 units $g^{-1}_{substrate}$. To the best knowledge

of the authors, this is the first attempt to optimize the conditions of enzymatic hydrolysis of Jerusalem artichoke using *Aspergillus niger*-derived enzyme. In a closely related study, the hydrolysis of Jerusalem artichoke was carried out using inulinase from *Aspergillus tamarii* at the optimal temperature of 45ºC, pH of 5.2, and 30 units of inulinase. 71.6% hydrolysis of inulin was reported after 120 minutes but enzyme activity reduced to 89% after only 90 minutes of exposure and continued decreasing further (Saber and El-Naggar 2009). In another study, Nakamura et al. (1997) reported 70% of inulin hydrolysis in 72 hours using inulinase from *Penicillium sp.* TN-88. It was also reported that *Aspergillus niger* mutant 817 inulinase hydrolyzed 50% of inulin in 24 hours and remained constant thereafter (Nakamura et al. 1994). *Aspergillus niger* A42, *Kluyveromyces. marxianus* NCYC 587 and a mixed culture of the two strains were used for enzymatic hydrolysis of Jerusalem artichoke meal (Ongen-Baysal and Sukan 1996). The experiments were carried out at 50 ºC resulted in the hydrolysis of inulin at 34.7, 62.6 and 87.9 %, respectively. Sirisansaneeyakul et al. (Sirisansaneeyakul et al. 2006) also reported that mixed inulinases from *Aspergillus niger* TISTR 3570 and *Candida guilliermondii* TISTR 5844 proved superior to individual crude inulinases in hydrolysing inulin to fructose. By comparison, the inulinase from *Aspergillus niger* in this study can hydrolyze up to 94.5% of inulin under optimal conditions after 24 hours. Such a high inulin conversion is usually achieved when mixed cultures are used which contain adequate quantities of both inulinase and invertase. Therefore, *Aspergillus niger* inuliase seems to have industrial potential for inulin conversion.

To validate the applicability of this RSM model, some confirming experiments were carried out around the estimated optimal conditions. The measured and predicted inulin conversions of three conditions around the optimum are listed in Table 3-4. The predicted results were compared with the actual values obtained experimentally. *T* test at 95% confidence showed no significant difference between the predicted and actual values. In summary, the proposed RSM model could be a useful model for the prediction of maximum inulin conversion.

Table 3-4 Predicted and measured enzymatic inulin conversion around estimated optimal conditions

Temperature (°C)	pH	Substrate concentration (g L^{-1})	Enzyme loading (unit $g^{-1}_{substrate}$)	Inulin Conversion (%)	
				Predicted	Experimental
48.3	4.8	60	10	94.5±1.7	94.1±0.9
52.8	4.7	60	10	93.7±1.7	93.9±0.8
50.9	5.0	60	10	94.1±1.7	94.2±0.3

3.3.5 ABE fermentation from mixed sugars and enzymatic hydrolysate of Jerusaelm artichoke by *Clostridium saccharobutylicum* DSM 13864

Prior to carrying out butanol fermentation on Jerusalem Artichoke-derived carbohydrates, control experiments with synthetic media simulating the hydrolysate were carried out with *Clostridium saccharobutylicum* DSM 13864. The initial total sugar level was 55 g L^{-1}, including 14 g L^{-1} glucose and 41 g L^{-1} fructose. As was shown in Fig. 3-2A, the culture started to use glucose and fructose directly after the inoculation. Almost all the glucose was utilized by the culture within 24 hours. In contrast, 79.6 % fructose was consumed at 60 hours, leaving behind 8.4 g L^{-1} unused fructose in the medium. It was anticipated that the glucose utilization rate was greater than the fructose rate, as glucose is the preferred carbon source (Gao et al. 2012). In a closely related study, batch fermentation of the glucose/fructose mixture by *Clostridium acetobutylicum* L7 on a complex medium showed that this bacterium metabolizes glucose first and rapidly before utilizing fructose for ABE production (Chen et al., 2010). Results presented in Fig. 3-2A, suggest that the *Clostridium saccharobutylicum* DSM 13864 was able to utilize glucose and fructose simultaneously. A solvent concentration of 15.1 g L^{-1} was achieved after 60 hours fermentation with 3.1 g L^{-1} acetone, 2.3 g L^{-1} ethanol and 9.7 g L^{-1} butanol. Yield and productivity of the solvent were 0.32±0.008 $g_{Solvent}$ g^{-1}_{sugar} and 0.25±0.002 g L^{-1} hr^{-1}, respectively. The yields obtained in

this work are of similar values reported for ABE fermentation with *Clostridium* (0.25-0.37 $g_{Solvent}\ g^{-1}_{sugar}$) (Shaheen et al. 2000) .

Enzymatic hydrolysate of Jerusalem artichoke was subsequently used for ABE fermentation by *Clostridium saccharobutylicum* DSM 13864 under similar condition as in the control experiment. For the hydrolysate fermentation, the pH was adjusted to 6.0 using 1M NaOH solution. At the beginning, 55.8 g L^{-1} sugars were present of which glucose and fructose were 15.3 and 40.5 g L^{-1}, respectively. After 24 hours of fermentation, glucose was completely utilized, as was shown in Fig. 3-2B. When the fermentation stopped at 60 hours, only 74.8% fructose was used, leaving behind 10.2 g L^{-1} fructose unused, compared to 8.4 g L^{-1} fructose when mixed sugar was used. At the end of the fermentation, the culture produced 14.9 g L^{-1} ABE, resulting in a productivity of 0.25±0.005 g L^{-1} hr^{-1}. The individual levels of solvents were acetone 3.1 g L^{-1}, ethanol 2.2 g L^{-1}, and butanol 9.6 g L^{-1} (Fig. 3-2B). The culture used 45.6 g L^{-1} sugar to produce 14.9 g L^{-1} ABE, thus resulting in a yield of 0.33±0.003 $g_{Solvent}\ g^{-1}_{sugar}$. Based on the amount of sugars present in the medium, the maximum theoretical yield is 0.39±0.009 $g_{Solvent}\ g^{-1}_{sugar}$ (Yerushalmi et al. 1983), the current data therefore represents 85% of the theoretical yield. In a comparable study, *Clostridium acetobutylicum* L7 was used for hydrolysate fermentation of Jerusalem artichoke with 62.9 g L^{-1} sugars, resulting in an solvent concentration of 17.2 g L^{-1}, corresponding to a yield of 0.29 $g_{Solvent}\ g^{-1}_{sugar}$ (Chen et al. 2010), which appears to be lower than the results obtained in this study, however a larger amount of sugars could be converted. It has been reported raising the initial carbohydrate concentration in the medium above 60 g L^{-1}, as was the case for the work of Chen et al. (2010), will reduce the fermentation efficiency (Shaheen et al. 2000). Additional deviation can be potentially explained by strain characteristics of the *Clostridia* (L7 vs. DSM 13864).

Fermentation with Jerusalem artichoke showed identical yields within error for that of ideal fermentations with pure glucose and fructose which indicates enzymatic hydrolysate of Jerusalem artichoke is a reliable feedstock for ABE production. Despite the inefficiency of fructose utilization by the culture, from the identical ABE yield obtained in hydrolysate fermentation it can be speculated that small amount of sucrose and protein (amino acids

and peptides) in Jerusalem artichoke hydrolysate might have stimulatory effect on ABE production. At this stage, inefficiency of fructose utilization by the culture is still unknown.

The experimental work in the study largely focuses on the enzymatic hydrolysis of Jerusalem artichoke as a potential feedstock for butanol production. The fermentation process was not optimized and the setup used in this study is not intended to represent a potential industrial process. More advanced fermentation process design, possibly including continuous fermentation and/or in-situ product removal would likely have to be used in an industrial process, as evaluated for different feedstocks elsewhere (Lee et al. 2008; Napoli et al. 2011)

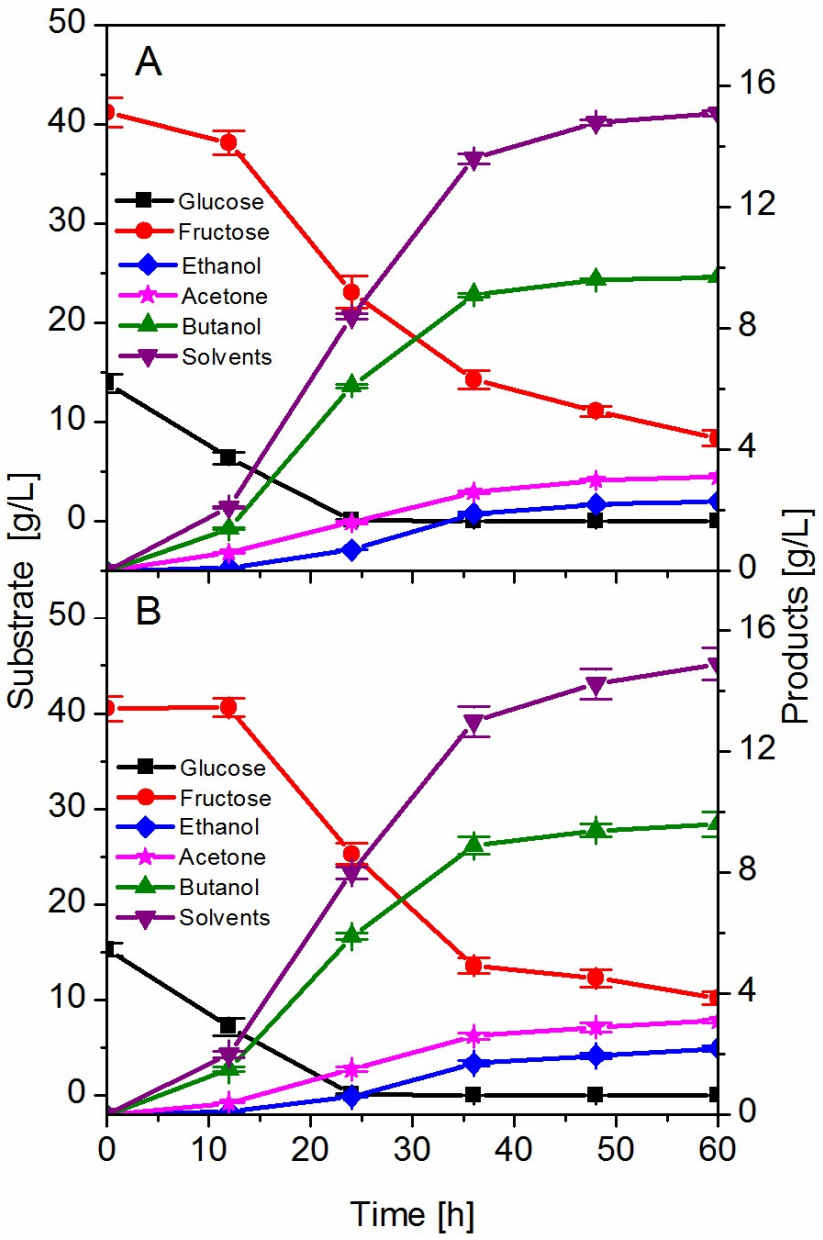

Figure 3-2 Profiles of solvents production and sugar utilization in a) mixed sugar b) hydrolysate of Jerusalem artichoke by *Clostridium saccharobutylicum* DSM 13864; A biomass increased was observed during the fermentation through an increase of turbidity from 0.185 to 1.654 OD units.

3.4 Conclusions

The objective of this study was to find the optimal conditions of enzymatic hydrolysis of Jerusalem artichoke to maximize inulin conversion. The optimal conditions for inulin conversion were a temperature of 48°C, pH of 4.8, substrate concentration of 60 g L^{-1}, and enzyme loading of 10 unit $g^{-1}_{substrate}$.

Clostridium saccharobutylicum DSM 13864 was able to ferment enzymatic hydrolysate of Jerusalem artichoke. The culture used 45.6 g L^{-1} sugar to produce 9.6 g L^{-1} butanol, resulting in a yield of 0.33 $g_{Solvent}$ g^{-1}_{sugar}, corresponding to 0.21 $g_{Solvent}$ $g^{-1}_{raw\ material}$. Therefore, the inulin and tuber of Jerusalem artichoke were found to be good raw materials for butanol production.

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Chapter 4

4 Optimizing acid hydrolysis of Jerusalem artichoke-derived inulin for fermentative butanol production

Tahereh Sarchami, Lars Rehmann.

Preface

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is substantially as it appears in Bioenergy Research, December 2014, Vol 8, pages 1148-1157.

Chapter 3 focused on enzymatic hydrolysis of Jerusalem artichoke tubers and the subsequent fermentation of this hydrolysate to butanol. However, acid hydrolysis as a simple pretreatment for inulin feedstock has a number of important advantages including a low-cost easily available acid catalyst and a short hydrolysis time (Tasić et al. 2009), and was therefore investigated next. Acidic pretreatment strategies are also known for the irreversible production of growth and fermentation inhibitors, such as hydroxymethylfurfural (HMF) (Pedersen et al. 2010). At high concentrations, these inhibitors can substantially affect the fermenting organism (Almeida et al. 2007; Schwab et al. 2013). The available information about potentially fermentation-inhibiting hydrolysis by-product (HMF) during inulin hydrolysis is limited in the current literature. Also, the available literature provides little information about the optimum condition and yield of inulin acid hydrolysis, nor a controlled comparison of different acids (Jain and Baratti 1985; Kim and Hamdy 1986; Tasić et al. 2009; Razmovski et al. 2011; Razmovski et al. 2013).

Therefore, this study was undertaken to investigate the effects of various hydrolysis variables (temperature, pH, and time) on the acid hydrolysis of Jerusalem artichoke-derived inulin using three different mineral acids (HCl, H_2SO_4, and H_3PO_4). Statistical data obtained from RSM led to the development of an empirical model of inulin conversion for each acid as function of all investigated factors. These models were numerically optimized

to obtain the hydrolysis conditions that maximize inulin conversion to fermentable sugars. The influence of each acid on the formation of 5-hydroxymethylfurfural (HMF) was also investigated. Also, the feasibility of butanol production from acid hydrolysate of Jerusalem artichoke's tubers was studied.

The results of this chapter show that the highest HMF concentration in this study (6.4 mg $_{HMF}$ $g^{-1}_{Reducing\ Sugar}$) was noticeably lower than the HMF concentration typically considered inhibitory to the growth and fermentation. Within the current design space, phosphoric acid produced the highest HMF concentration, followed by hydrochloric acid, and sulfuric acid. It is clear that the nature of the acid can influence the HMF formation. Taking into consideration the efficiency of inulin hydrolysis, expressed as maximum yield of fermentable sugars (fructose and glucose) and non-inhibiting HMF concentration, it was concluded that H_2SO_4 seems to have a better potential as a catalyst for inulin hydrolysis compare to two other acids (HCl and H_3PO_4). Also, robust butanol yield comparable to control fermentation with glucose and fructose as substrates was obtained from acid hydrolysate of Jerusalem artichoke tubers, indicating that this feedstock is suitable substrate for butanol fermentation.

By comparison, sulphuric acid in this study can hydrolyze up to 98.5% of inulin within 35 minutes with non-inhibiting HMF concentrations, while according to the results obtained in Chapter 3 the same Jerusalem artichoke extract required 24 hours to achieve similar numbers enzymatically. The shorter reaction times and lower catalyst costs would imply acid hydrolysis to be favorable over enzymatic conversion if conducted as separate process steps.

Abstract

In this study, a central composite design and response surface methodology were used to study the effect of various hydrolysis variables (temperature, pH, and time) on the acid hydrolysis of Jerusalem artichoke-derived inulin using three different mineral acids (HCl, H_2SO_4, and H_3PO_4). Numerical optimization was used to maximize the sugar yield of Jerusalem artichoke powder within the experimental range for each of the mentioned acid.

The influence of each acid on the formation of 5-hydroxymethylfurfural (HMF; a known by-product and inhibitor for fermentative organisms) was also investigated. H_2SO_4 was found to have a better potential for sugar yields compare to two other acids (HCl and H_3PO_4) since it can hydrolyze the highest amount of inulin (98.5%) under optimal conditions (temperature of 97°C, pH of 2.0, and time period of 35 minutes) without producing inhibiting HMF concentrations. The sulfuric hydrolysate of Jerusalem artichoke was fermented via solventogenic clostridia to acetone- butanol- ethanol (ABE). An ABE yield of 0.31 g g^{-1} and an overall fermentation productivity of 0.25 g L^{-1} hr^{-1} were obtained, indicating the suitability of this feedstock for fermentative ABE production.

4.1 Introduction

In recent years, the academic and industrial biofuel sectors are increasingly investigating options beyond grain-based ethanol. Alternative biofuels, such as biomass-derived long-chain alcohols, are of growing importance (Sánchez and Cardona 2008; Alvira et al. 2010; Schiel-bengelsdorf et al. 2016). Butanol (n-butanol) is a very promising biofuel exhibiting several advantages over ethanol and represents also an important bulk chemical for industrial purposes. It is more hydrophobic than ethanol (due to its two additional methyl-groups), possesses less volatility, has a higher energy density, and is fully miscibility with gasoline (Sarchami and Rehmann 2014).

One of the major obstacles to commercial acetone-butanol-ethanol (ABE) fermentation is the high cost and availability concerns of conventional substrates (corn, molasses) (Jones and Woods 1986). Substrate cost contributes over 50% of the total production costs; therefore, it is crucially important, from a process economics perspective, to identify inexpensive biomass feedstocks that can be fermented by *Clostridium* species (Dürre 2007; Qureshi et al. 2008; García et al. 2011; Sarchami and Rehmann 2014). While a number of low-cost fermentation substrates have previously been evaluated (Raganati et al. 2013; Gao and Rehmann 2014), Jerusalem artichokes (*Helianthus tuberosus L.)* as an alternative carbon source have a good potential to be fermented to butanol. Jerusalem artichoke can grow well in non-fertile land and is resistant to plant diseases, not competing with grain crops for arable land (Szambelan et al. 2005; Sarchami and Rehmann 2014). Unlike typical

crops that use starch, a glucose polymer, as energy storage, Jerusalem artichoke (as all member of the *Asteraceae* family) stored excess carbon as inulin, linear chains of β (2→1) linked D-fructose units terminated by a D-glucose linked to fructose by α (1→2) bond (Szambelan et al. 2005). Though the principal storage carbohydrate of Jerusalem artichoke is inulin (15 to 20%), monomeric sucrose, glucose and fructose are also present (Matías et al. 2011).

Most microorganisms cannot directly ferment inulin, therefore inulin first needs to be hydrolyzed into fructose and glucose monomers. Hydrolysis can be achieved via an acid catalyst or enzymes. Acid hydrolysis as a simple pretreatment for inulin feedstock has a number of important advantages including a low-cost easily available acid catalyst and a short hydrolysis time (Tasić et al. 2009). However, acidic pretreatment strategies are also known for the irreversible production of growth and fermentation inhibitors, such as HMF (Pedersen et al. 2010). At high concentrations, these inhibitors can substantially affect the fermenting organism (Almeida et al. 2007; Schwab et al. 2013).

Various acids can be used as catalyst for inulin hydrolysis, but mineral acids were shown to be more effective compare to organic acids. Among mineral acids, hydrochloric acid, sulphuric acid, and phosphoric acid have been used in many studies for inulin hydrolysis; however, the available literature provides little information about the optimum condition and yield of inulin hydrolysis, nor a controlled comparison of different acids (Jain and Baratti 1985; Kim and Hamdy 1986; Tasić et al. 2009; Razmovski et al. 2011; Razmovski et al. 2013). Also the information about potentially fermentation-inhibiting hydrolysis by-product (HMF) is limited in the current literature.

The purpose of this study is therefore threefold, 1) to optimize acid hydrolysis of inulin to maximize its corresponding fermentable sugar yield using three different mineral acids (HCl, H_2SO_4, and H_3PO_4), 2) to examine the influence of each acid (HCl, H_2SO_4, and H_3PO_4) on HMF formation, and 3) to study the feasibility of butanol production from the hydrolysate of Jerusalem artichoke's tuber.

4.2 Materials and Methods

4.2.1 Acid hydrolysis

4.2.1.1 Preparation of Jerusalem artichoke flour

Jerusalem artichoke flour was prepared following the protocol described in Chapter 3. Section 2.1.1

4.2.1.2 Inulin extraction

Inulin extraction was performed following the protocol described in Chapter 3. Section 2.1.2

4.2.1.3 Chemicals

Hydrochloric acid (12.2 M), sulphuric acid (18.0 M), and phosphoric acid (14.8 M) were obtained from Caledon (Ontario, Canada).

4.2.1.4 Experimental Design

A central composite design (CCD) with three factors was selected to evaluate the response pattern and to determine the optimal combination of temperature, pH, and time for maximizing inulin hydrolysis to fermentable sugars using three different mineral acids (HCl, H_2SO_4, and H_3PO_4). An initial full factorial design had shown significant curvature and confirmed the significance of all three parameters (data not shown), and the design was expanded to a CCD. The un-coded values for each parameter were as follows [low star point, low central point, center point, high central point, high star point]: temperature in ºC [77.31, 80, 88.5, 97, 99.69], pH [1.84, 2.0, 2.5, 3.0, 3.16], and time in minutes [1.78, 7.0, 23.5, 40, 45.22]. The experimental design was developed using Design Expert 8.0.7.1 (Statease, Inc., Minneapolis, MS, USA) and resulted in 14 conditions for each acid. All conditions were tested in triplicates, including 3 center points. The resulting 51 conditions (8×3 factorial + 6×3 augmented + 3×3 center points) were fully randomized.

4.2.1.5 Acid hydrolysis of inulin

Batch acid hydrolysis was performed in 20 ml scintillation vials using the above selected experimental conditions for each of the mentioned acids (hydrochloric acid, sulphuric acid, and phosphoric acid). Each vial contained 10 ml of water-extracted inulin from Jerusalem artichoke tubers obtained in section 4.2.1.2, and the pH was adjusted using the respective acid. The concentration of acid added to adjust the pH of extract to [2.0, 2.5, and 3.0] were as follows: HCl [4.4 µl L^{-1}, 3.2 µl L^{-1}, and 2.5 µl L^{-1}], H_2SO_4 [1.7 µl L^{-1}, 1.2 µl L^{-1}, and 0.8 µl L^{-1}], and H_3PO_4 [4.3 µl L^{-1}, 2.5 µl L^{-1}, and 1.6 µl L^{-1}]. The vials were hermetically covered with Parafilm and aluminum foil to avoid evaporative loss, and the mixture was heated at the required temperature for the selected reaction time while shaking at 300 rpm. All hydrolysis assays were conducted in triplicate.

4.2.1.6 Statistical analysis

Linear regression analysis was used to fit the experimental data with a second-order model as given in equation (4-1):

$$Y= \beta_0+\sum_{i=1}^{3} \beta_i x_i+\sum_{i=1}^{3} \beta_{ii} x_i^2+\sum_{1\leq i\leq j}^{3} \beta_{ij} x_i x_j+\varepsilon \quad (4\text{-}1)$$

The experimental data was analyzed using Design Expert 8.0.7.1 as discussed in Chapter 3. Section 2.1.6

4.2.1.7 Analytical methods

Concentration of sugars and HMF in Jerusalem artichoke juice, as well as hydrolysate, was determined via high performance liquid chromatography (HPLC) on an Agilent 1260 infinity (Agilent USA, Santa Clara) using an Agilent Hi-plex H (7.7 × 300 mm) column and Cation H^+ guard column (Agilent USA, Santa Clara) operating at 60°C. A refractive index detector (RID) was used for sugar detection. Also a diode array detector (DAD) was used for HMF detection with spectral absorbance at 276 nm. Water was used as the isocratic mobile phase at a constant flow rate of 0.6 ml $min^{-1.}$ Before injection, samples were diluted to the appropriate concentration with deionized water and filtered through a

0.2 µm membrane filter. The analytes were quantified using pure inulin, HMF (Sigma Aldrich Co.) fructose, glucose and sucrose (VWR Co.) as standards.

The total sugar yield was evaluated based on fructose and glucose production. The fructose to glucose ratio after complete conversion was 4±0.45 (Table 4.1). Complete hydrolysis resulted in 0.15 g g^{-1} $_{\text{Jerusalem artichoke}}$ and 0.60 g $g^{-1}{}_{\text{Jerusalem artichoke}}$ glucose and fructose, respectively. This was achieved by acid hydrolysis developed for analytical purposes at a temperature of 100°C, pH of 2.0, and 60 minutes reaction time using H_2SO_4 (Szambelan and Nowak 2006).

4.2.2 Fermentation

4.2.2.1 Chemicals

See Chapter 3. Section 2.2.1

4.2.2.2 General microbiological conditions

All microbiological work was performed in an aseptic anaerobic chamber (Model 855-ACB, Plas Labs, Lansing, MI).

4.2.2.3 Strain and maintenance

Microorganism and cell culture condition previously described in Chapter 3. Section 2.2.3

4.2.2.4 ABE fermentation

All fermentation studies were conducted following the protocol in Chapter 3. Section 2.2.4

4.2.2.5 Analytical methods

Bacterial growth was monitored following the protocol in Chapter 3. Section 2.2.5 Concentration of solvents produced in the fermentation was determined via HPLC as described in section 4.2.1.7. Total solvents were quantified using pure butanol, acetone, and ethanol (Caledon, Ontario, Canada) as standards.

Productivity was calculated as the maximum ABE concentration achieved (g L^{-1}) divided by the fermentation time at a fixed time of 60 hours and is expressed as gram per liter per hour. Product yield was calculated as the total amount of solvents produced divided by the amount of fermentable sugar utilized and is expressed as $g_{Solvent}$ g^{-1}_{sugar}. At least three parallel samples were used in all analytical determinations, and data are presented as the means of three replicates.

4.3 Results and Discussion

The total solid content of Jerusalem artichoke tuber used in this study was about 30% of the fresh weight. Inulin, fructose, glucose, and sucrose composition of the material are shown in Table 4-1. Samples for analysis were randomly taken from the available material and the small standard deviation indicates the compositional homogeneity of the tubers. The measured values are in agreement with values typically found for Jerusalem artichoke (Böhm et al. 2004; Matías et al. 2011).

Table 4-1 Jerusalem artichoke carbohydrate composition (average of triplicates±standard deviation) of raw material and of water extract after analytical acid hydrolysis based on reference method (Szambelan and Nowak 2006)

Compound	**g_{sugar} $g^{-1}_{Jerusalem\ artichoke\ (DW)}$**	
	Raw material	**Hydrolysate**
Inulin	0.52±0.05	0.003±0.002
Fructose	0.16±0.02	0.60±0.06
Glucose	0.10±0.01	0.15±0.03

4.3.1 Acid hydrolysis

Experimental conditions were chosen based on a central composite design, and the actual values of the independent variables and the measured responses are shown in Table 4-2. Acid hydrolysis was performed on water-extracted inulin. Hydrolyzing the complete tuber

could increase the total sugar yield, as the selected condition would also favor hydrolysis of the cellulose faction, releasing additional glucose monomers. However, the typical cellulose content in Jerusalem Artichoke tuber is <0.03 g g^{-1} while also 0.02 g g^{-1} hemicellulose is present (Dao et al. 2013). The potential increase in fermentable sugar is negligible considering 0.83 g g^{-1} of extractable carbohydrates (Table 4-1), and the hemicellulose fraction would likely result in the formation of fermentation-inhibiting by-products under the employed acid hydrolysis conditions.

The sugar yield was defined as the amount of measured glucose and fructose as the percentage of the two sugars after analytical acid hydrolysis of the same based on reference method (Szambelan and Nowak 2006)

Table 4-2 Sugar yield and HMF selectivity after acid hydrolysis (average of triplicates±standard deviation) under conditions determined for CCD (ND: not detected)

Temperature (°C)	pH	Time (min)	Sugar Yield (%)			HMF Selectivity (mg $_{HMF}$ $g^{-1}_{reducing\ sugar}$)		
			HCl	H_2SO_4	H_3PO_4	HCl	H_2SO_4	H_3PO_4
77.31	2.5	23.5	74.3±0.8	65.8±1.4	69.4±1.6	ND	ND	ND
80	2	7	68.4±1.1	70.8±0.3	63.0±0.5	ND	ND	ND
80	2	40	88.5±0.8	87.8±0.6	88.4±0.7	ND	ND	ND
80	3	7	26.9±0.7	36.1±0.9	25.8±0.4	ND	ND	ND
80	3	40	67.4±0.8	64.4±0.5	70.9±0.7	ND	ND	ND
88.5	1.84	23.5	88.4±0.3	90.7±0.2	88.9±0.5	0.7	0.5	0.8
88.5	2.5	1.78	34.2±1.4	54.7±1.1	38.2±1.2	ND	ND	ND
88.5	2.5	23.5	76.0±0.25	76.5±0.2	68.2±0.5	0.5	0.3	0.6
88.5	2.5	23.5	76.3±0.25	76.9±0.2	69.1±0.5	0.4	0.3	0.6
88.5	2.5	23.5	75.8±0.25	76.8±0.2	68.3±0.5	0.4	0.3	0.6
88.5	2.5	45.22	84.3±0.5	84.3±0.4	84±0.5	1.6	1.3	1.9
88.5	3.16	23.5	60.3±1.1	59.2±1.5	71.9±1.3	0.3	0.2	0.5
97	2	7	84.4±1.3	87.6±0.9	72.6±0.8	1	0.7	1.2
97	2	40	95.5±0.2	98.5±0.3	94.0±0.2	5.4	4	6.4
97	3	7	38.2±0.5	58.8±0.9	36.2±0.9	0.9	0.5	1.1
97	3	40	79.9±1.2	85.3±0.6	80.0±1.3	4.8	3.6	5.8
99.69	2.5	23.5	77.8±0.9	84.8±0.5	85.7±0.2	2.6	1.7	2.9

4.3.2 Response surface model validation

The total sugar yield was chosen as the only response factor when evaluating the results of the CCD experiments. The HMF selectivity was not a suitable response as it was below the detection limit for multiple experimental conditions. Other factors such as rate constants or pseudo-rate constants were also not suitable, as the goal of the CCD was to establish a simple empirical correlation between the final sugar yield and multiple parameters, including the hydrolysis time. The hydrolysis would not affect the rate constant, unless hydrolysis follows a more complex mechanism as in the case of hemicellulose (Zhu et al. 2012; Zhang et al. 2014). A detailed mechanistic study of acid hydrolysis was not the aim of this study, hence the choice of a CCD followed by response surface methodology.

As can be seen from the experimental results in Table 4-2, the hydrolysis using any of the three mentioned acids (HCl, H_2SO_4, and H_3PO_4) was successful in converting inulin to monomeric sugars within the ranges of the input variables.

The complete dataset could be fitted with a quadratic model as describe in equation (4-1) for each acid. The resulting model parameters are shown in Table 4-3. The *F* values of the models are 73.4, 304.2, and 49.7 for HCl, H_2SO_4, and H_3PO_4, respectively. The *F* values are very high compared to the critical values, indicating that all three models are highly significant. The significance of each parameter coefficient was determined by *P* values. In this case, all factors have great effect on acid hydrolysis using any of the three acids. The quadratic effects of pH, time, as well as temperature, and interaction effect of every two variables on sugar yields for each acid are shown in Table 4-3. The goodness of fit of each model was confirmed by the coefficient of determination R^2 and adjusted determination coefficient Adj. R^2 (Table 4-3).

Based on the selected significant variables, the quadratic model for the sugar yield in terms of actual factors is shown as follows:

HCl

$$\text{Sugar Yield} = 105.4 - 46.3 \times \text{pH} + 0.49 \times \text{Time} + 0.5 \times \text{Temperature} + 0.8 \times \text{pH} \times \text{Time} - 0.03 \times \text{Time}^2 \quad (4\text{-}2)$$

H_2SO_4

$$\text{Sugar Yield} = 157.4 - 76.2 \times \text{pH} + 0.21 \times \text{Time} - 0.197 \times \text{Temperature} + 0.4 \times \text{pH} \times \text{Time} + 0.47 \times \text{pH} \times \text{Temperature} - 0.012 \times \text{Time}^2 \quad (4\text{-}3)$$

H_3PO_4

$$\text{Sugar Yield} = 185.1 - 125.4 \times \text{pH} + 0.6 \times \text{Time} + 0.58 \times \text{Temperature} + 0.64 \times \text{pH} \times \text{Time} + 17.64 \times \text{pH}^2 - 0.024 \times \text{Time}^2 \quad (4\text{-}4)$$

The residuals can be judged as normally distributed based on a normal probability (data not shown).

Table 4-3 Analysis of variance of fitted model

Source	Acid	Model	pH (A)	Time (B)	Temperature (C)	AB	AC	A^2	B^2
Remark	HCl	Sig.	Sig.	Sig.	Sig.	Sig.	Not-Sig	Not-Sig	Sig.
	H_2SO_4	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Not-Sig	Sig.
	H_3PO_4	Sig.	Sig.	Sig.	Sig.	Sig.	Not-Sig	Sig.	Sig.
F value	HCl	73.4	136.3	168.3	13.9	19.5			29.33
	H_2SO_4	304.2	788.5	582.4	363.5	40.8	14.62		35.3
	H_3PO_4	49.7	74.7	176.6	14.5	11.7		6.8	15.8
P value	HCl	<0.0001	<0.0001	<0.0001	0.0029	0.0008			0.0002
	H_2SO_4	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0028		<0.0001
	H_3PO_4	<0.0001	<0.0001	<0.0001	0.0029	0.0057		0.0245	0.0022
R-Squared	HCl	0.97							
	H_2SO_4	0.99							
	H_3PO_4	0.96							
Adj-Squared	HCl	0.96							
	H_2SO_4	0.99							
	H_3PO_4	0.95							
Adeq Precisior	HCl	27.6							
	H_2SO_4	64.88							
	H_3PO_4	23.7							

4.3.3 Combined effect of Temperature, pH, and Time

Response surface methodology was used to study the interaction effects of the three factors using any of the mentioned mineral acids. Surface plots of the combined effects of pH and time on sugar yield using HCl, H_2SO_4, and H_3PO_4 at a constant temperature of 88.5 are shown in Fig 4-1a, b, and c, respectively. The sugar yield is a function of both the pH and time using any of the mentioned mineral acids. The plots clearly indicate that an optimum exists within the observed design space with respect to pH and time, and increasing the temperature appears to increase the sugar yield over the observed design space. The sugar yield is also a function of both the pH and temperature only when H_2SO_4 was used as catalyst.

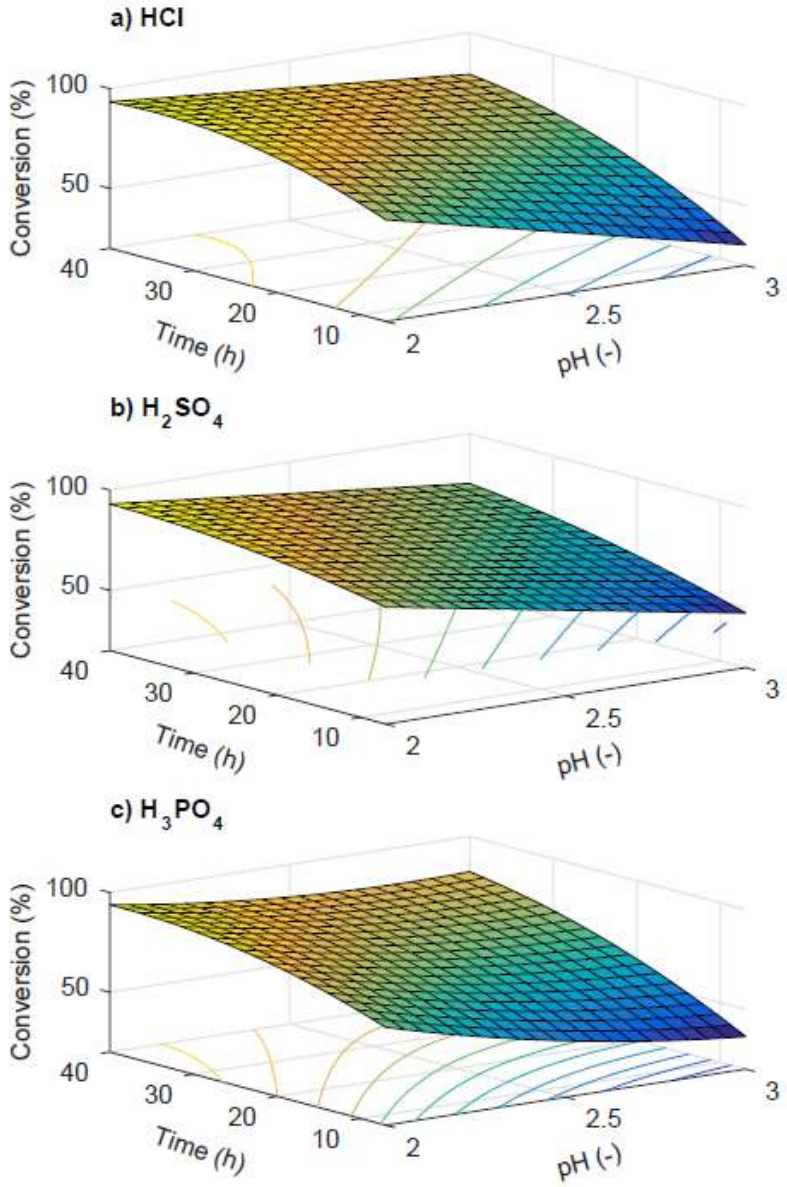

Figure 4-1 Surface plots of combined effect of process variables on the sugar yield. a) pH and time using HCl as catalyst, b) pH and time using H_2SO_4 as catalyst, c) pH and time using H_3PO_4 as catalyst.

4.3.4 Response optimization and model validation

Based on the model, numerical optimization was used to determine the optimal combination of process parameters for the maximum sugar yield. The optimal conditions

were a temperature of 96°C, pH of 2.0, and time period of 36 minutes using HCl; temperature of 97°C, pH of 2.0, and time period of 35 minutes using H_2SO_4; and temperature of 95°C, pH of 2.0, and 39 minutes time period using H_3PO_4. The best result was observed using H_2SO_4 which can hydrolyze up to 98.5% of inulin under optimal conditions compared to 95.5% and 94% using HCl and H_3PO_4, respectively. To the best knowledge of the authors, this is the first attempt to optimize the conditions of acid hydrolysis of water-extracted inulin from Jerusalem artichoke tubers using different mineral acids and to compare the effectiveness of each acid on hydrolysis at optimal conditions.

In a closely related study, the hydrolysis of Jerusalem artichoke tubers was carried out using HCl at a temperature of 120ºC, pH of 2.5, for 60 minutes resulting in 68% hydrolysis of inulin (Razmovski et al. 2013), which is lower than the results obtained in this study. It has been reported that the sugar yield, evaluated as fructose and glucose production, is very heat sensitive in acidic conditions as the products easily degrade at temperature higher than 97ºC (Kim and Hamdy 1986), as was the case for the work of Razmovski et al. (2013) , resulting in a lower hydrolysis yield. Szambelan and Nowak (2006b) reported complete inulin hydrolysis in 1 hour using H_2SO_4 at temperature of 100ºC, and pH of 2.0, which is close to the results obtained in this study in terms of temperature and pH. The longer hydrolysis time can be potentially explained by different methods of preparation of Jerusalem artichoke tubers for hydrolysis. The Jerusalem artichoke powder used in this study appears to have positive effects on hydrolysis time compare to mashed tubers used by Szambelan and Nowak (2006). In another study, H_3PO_4 was used to hydrolyze 90% of inulin in 7 hours at temperature of 80ºC, and pH of 2.0 (Jain and Baratti 1985), which is close to the result of this study at temperature of 80ºC, pH of 2.0, and time period of only 40 minutes.

To validate the applicability of this RSM model, some confirming experiments were carried out around the estimated optimal conditions. The measured and predicted sugar yields of three conditions around the optimum are listed in Table 4-4. The predicted results were compared with the actual values obtained experimentally. *T* test at 95% confidence

showed no significant difference between the predicted and actual values. In summary, the proposed RSM model could be a useful model for the prediction of maximum sugar yield.

Table 4-4 Predicted and measured sugar yields around estimated optimal conditions

Acid	Temperature (°C)	pH	Time (min)	Sugar yield (%)	
				Predicted	Experimental
HCl	96	2	36	95.5±0.7	93.9±0.9
HCl	96	2	35	94.9±0.7	94.2±0.7
H_2SO_4	97	2	35	98.5±0.5	98.0±0.8
H_2SO_4	94	2	32	96.6±06	97.8±0.9
H_3PO_4	95	2	39	94.0±0.4	93.4±0.3
H_3PO_4	96	2	37	93.3±0.7	93.5±0.4

4.3.5 HMF contents of Jerusalem artichoke hydrolysate

The data on HMF formed during acid hydrolysis of Jerusalem artichoke using three different mineral acids (HCl, H_2SO_4, and H_3PO_4) are shown in Table 4-2. An increase in temperature, decrease in the pH, and increase in hydrolysis time are expected to result in an increase of HMF during dilute acid hydrolysis (Razmovski et al. 2011; Razmovski et al. 2013) which is also the result of this study. However, the use of less intense conditions, temperature of 80°C to 97°C, and time of 7 to 40 minutes resulted in a low amount of HMF (0.0-6.4 $mg_{HMF}\ g^{-1}_{Reducing\ Sugar}$). Accordingly, the highest HMF concentration in a batch fermentation of the Jerusalem artichoke hydrolysate with an initial sugar concentration of 50 g L^{-1} would be 0.32 g L^{-1}. This worst-case value was achieved in the Jerusalem artichoke hydrolysate using H_3PO_4 as catalyst at temperature of 97°C, pH of 2.0, and time period of 40 minutes (Table 4-2). In a related study, the effect of HMF on *Clostridium beijerinckii* growth and fermentation was assessed which shows no negative effect on growth and butanol fermentation up to 2 g L^{-1} HMF concentration (Ezeji et al. 2007) . A similar result was obtained from the work of Zhang et al. (2012) and Qureshi et al. (2012). By comparison, the highest HMF concentration in this study (6.4 mg $_{HMF}\ g^{-1}_{Reducing\ Sugar}$) is

noticeably lower than the HMF concentration typically considered inhibitory to the growth and fermentation.

Within the current design space, phosphoric acid produced the highest HMF concentration, followed by hydrochloric acid, and sulfuric acid. It is clear that the nature of the acid can influence the HMF formation. Taking into consideration the efficiency of inulin hydrolysis, expressed as maximum yield of fermentable sugars (fructose and glucose) and non-inhibiting HMF concentration, it can be concluded that H_2SO_4 seems to have a better potential as a catalyst for inulin hydrolysis compare to two other acids (HCl and H_3PO_4).

4.3.6 Comparison of acid and enzymatic hydrolysis of water-extracted inulin from Jerusalem artichoke tubers

Hydrolysis of water-extracted inulin from Jerusalem artichoke tubers can be achieved via an acid catalyst or enzymes. Acid hydrolysis can lead to fermentation-inhibiting by-products, while enzymatic hydrolysis is dependent on potentially high-cost enzymes and has a longer hydrolysis time. Results typically achieved through enzymatic hydrolysis are summarized in in Table 4-5 with hydrolysis times varying between 2 hours and 72 hours and yields between 70% and 95 %. By comparison, sulphuric acid in this study can hydrolyze up to 98.5% of inulin within 35 minutes with a non-inhibiting HMF concentrations, while the same Jerusalem artichoke extract required 24 hours to achieve similar numbers enzymatically (Sarchami and Rehmann 2014). The shorter reaction times and lower catalyst costs would imply acid hydrolysis to be favorable over enzymatic conversion if conducted as separate process steps.

Alternatively, consolidated bioprocessing can be used allowing enzymatic hydrolysis to occur simultaneously with the fermentation step. In such cases simultaneous saccharification and fermentation (SSF) can be performed by adding commercial enzyme during the fermentation stage (Dao et al. 2013), which might also be possible for the butanol process due to the relatively low pH value during the butanol fermentation. Yeast strains expressing high levels of inulases have been developed and successfully used for ethanol production from Jerusalem artichoke (Guo et al. 2013).

Table 4-5 Reference data on enzymatic hydrolysis of extracted inulin from Jerusalem artichoke tubers

Enzyme	Temperature	pH	Time	Yield	Reference
Inulinase from *Aspergillus niger*	48ºC	4.8	24 hr	94.5 %	(Sarchami and Rehmann 2014)
Inulinase from *Penicillium sp.* TN-88	50ºC	5.2	72 hr	70%	(Zhang et al. 2012)
Inulinase from *Aspergillus tamari*	45ºC	5.2	2 hr	71.6%	(Qureshi et al. 2012)

4.3.7 ABE fermentation from mixed sugars and acid hydrolysate of Jerusaelm artichoke by *Clostridium saccharobutylicum* DSM 13864

Prior to carrying out butanol fermentation on Jerusalem artichoke-derived carbohydrates, control experiments with synthetic media simulating the hydrolysate were carried out with *Clostridium saccharobutylicum* DSM 13864. The initial total sugar level was 55 g L^{-1}, including 14 g L^{-1} glucose and 41 g L^{-1} fructose. As was shown in Fig. 4-2a, the culture started to use glucose and fructose directly after the inoculation. Almost all the glucose was utilized by the culture within 24 hours. In contrast, 79.6 % fructose was consumed at 60 hours, leaving behind 8.4 g L^{-1} unused fructose in the medium. It was anticipated that the glucose utilization rate was greater than the fructose rate, as glucose is the preferred carbon source (Gao et al. 2012). In a closely related study, batch fermentation of the glucose/fructose mixture by *Clostridium acetobutylicum* L7 on a complex medium showed that this bacterium metabolizes glucose first and rapidly before utilizing fructose for ABE production (Chen et al. 2010). Results presented in Fig. 4-2a, suggest that the *Clostridium saccharobutylicum* DSM 13864 was able to utilize glucose and fructose simultaneously. A

solvent concentration of 15.1 g L^{-1} was achieved after 60 hours fermentation with 3.1 g L^{-1} acetone, 2.3 g L^{-1} ethanol and 9.7 g L^{-1} butanol. Yield and productivity of the solvent were 0.32±0.008 $g_{Solvent}$ g^{-1}_{sugar} and 0.25±0.002 g L^{-1} hr^{-1}, respectively. The yields obtained in this work are of similar values reported for ABE fermentation with *Clostridium* (0.25-0.37 $g_{Solvent}$ g^{-1}_{sugar}) (Shaheen et al. 2000).

Hydrolysate of Jerulsaem artichoke was obtained using H_2SO_4 under optimal conditions. It was subsequently used for ABE fermentation by *Clostridium saccharobutylicum* DSM 13864 under similar condition as in the control experiment. For the hydrolysate fermentation, the pH was adjusted to 6.0 using 1M NaOH solution. At the beginning, 59.4 g L^{-1} sugars were present, of which glucose and fructose were 16.8 and 42.6 g L^{-1}, respectively. After 24 hours of fermentation, glucose was completely utilized, as was shown in Fig. 4-2b. When the fermentation stopped at 60 hours, 73.6% fructose was used, leaving behind 11.2 g L^{-1} fructose unused, compared to 8.4 g L^{-1} fructose when mixed sugar was used. At the end of the fermentation, the culture produced 15.1 g L^{-1} ABE, resulting in a productivity of 0.25±0.008 g L^{-1} hr^{-1}. The individual levels of solvents were acetone 3.0 g L^{-1}, ethanol 2.3 g L^{-1}, and butanol 9.8 g L^{-1} (Fig. 4-2b). The culture used 48.2 g L^{-1} sugar to produce 15.1 g L^{-1} ABE, thus resulting in a yield of 0.31±0.004 $g_{Solvent}$ g^{-1}_{sugar}. Based on the amount of sugars present in the medium, the maximum theoretical yield is 0.39 $g_{Solvent}$ g^{-1}_{sugar} (Yerushalmi et al. 1983) corresponding to a percent yield of 80% . In a comparable study, *Clostridium acetobutylicum* L7 was used for hydrolysate fermentation of Jerusalem artichoke with 62.9 g L^{-1} sugars, resulting in an solvent concentration of 17.2 g L^{-1}, corresponding to a yield of 0.29 $g_{Solvent}$ g^{-1}_{sugar} (Chen et al. 2010), which appears to be lower than the results obtained in this study, however, a larger amount of sugars could be converted. It has been reported that raising the initial carbohydrate concentration above 60 g L^{-1}, as was the case for the work of Chen et al. (2010), will reduce the fermentation efficiency (Shaheen et al. 2000). Additional deviation can be potentially explained by strain characteristics of the *Clostridia* (L7 vs. DSM 13864).

Fermentation with water-extracted inulin from Jerusalem artichoke tubers showed identical yields (within error) with mixture of glucose and fructose as a control fermentation. This

indicates acid hydrolysate of Jerusalem artichoke is a reliable feedstock for ABE production.

The experimental work in the study largely focuses on the acid hydrolysis of water-extracted inulin from Jerusalem artichoke tubers as a potential feedstock for butanol production. The fermentation process was not optimized and the setup used in this study is not intended to represent a potential industrial process. More advanced fermentation process design, possibly including continuous fermentation and/or in-situ product removal would likely have to be used in an industrial process, as evaluated for different feedstocks elsewhere (Lee et al. 2008; Napoli et al. 2011).

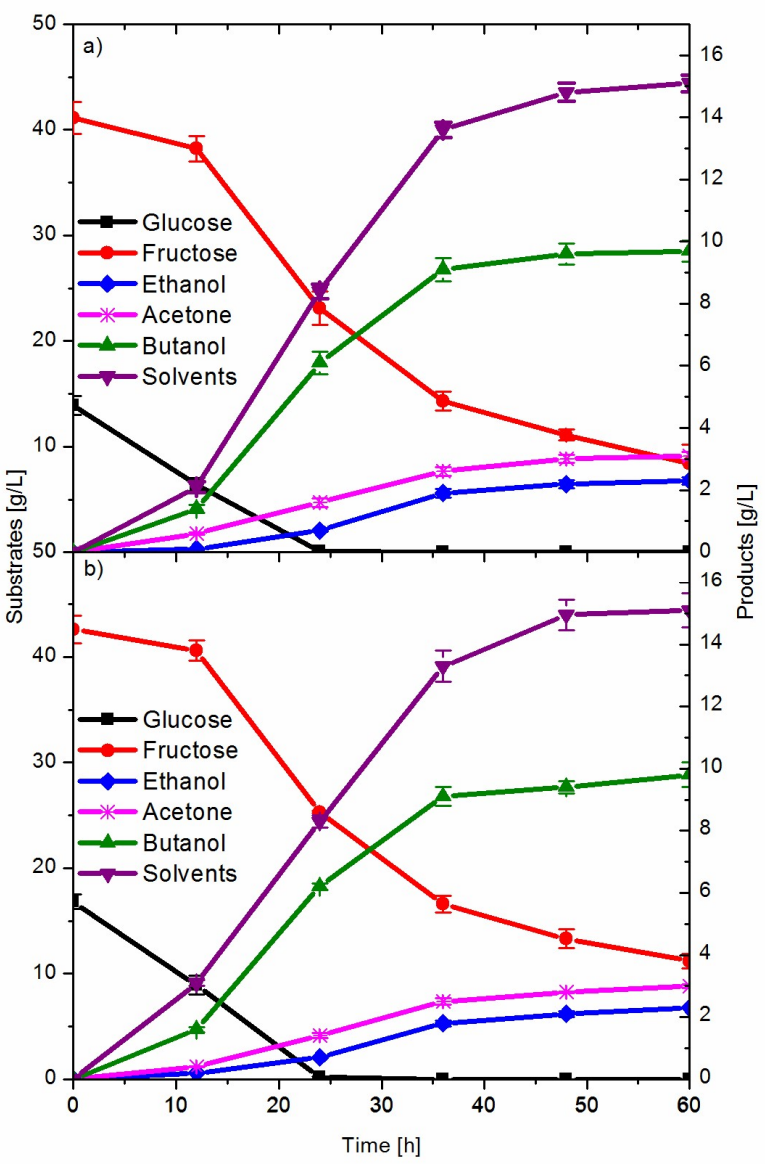

Figure 4-2 Profiles of solvents production and sugar utilization in a) mixed sugar b) hydrolysate of Jerusalem artichoke by *Clostridium saccharobutylicum* DSM 13864; A biomass increased was observed during the fermentation through an increase of turbidity from 0.185 to 1.654 OD units.

4.4 Conclusions

The optimal conditions for inulin hydrolysis to glucose and fructose are temperature of 97°C, pH of 2.0, for 35 minutes using H_2SO_4, which was the most suitable acid tested.

Phosphoric acid resulted in the highest amount of HMF followed by hydrochloric acid, whereas sulfuric acid results in the lowest HMF concentration. *Clostridium saccharobutylicum* DSM 13864 is able to ferment acid hydrolysate of Jerusalem artichoke similarly to fermenting synthetic medium with an equivalent carbohydrate composition. The culture converts 48.2 g L^{-1} sugar to 15.1 g L^{-1} ABE, resulting in a yield of 0.31 $g_{Solvent}$ g^{-1}_{sugar}. Therefore, the inulin of Jerusalem artichoke can be seen as a good raw material for butanol production after simple acid hydrolysis.

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Chapter 5

5 Optimization of fermentation condition favoring butanol production by *Clostridium pasteurianum* DSM 525

Tahereh Sarchami, Erin Johnson, Lars Rehmann.

Preface

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is substantially as it appears in Bioresource Technology, February 2016, Vol 208, pages 73-80.

Evaluating different feedstocks helps to understand the choices of substrates for butanol production in different regions, and promotes value-added by products for the processing industry. In the previous two chapters, Jerusalem artichoke tuber was evaluated as a low-cost fermentation substrate for butanol production. This chapter focused on biodiesel-derived glycerol as another potential substrate for butanol fermentation.

Glycerol is the principal by-product of the biodiesel production process, with production of ten liters of biodiesel resulting in one liters of glycerol by-products (Yazdani and Gonzalez 2007). Since the biodiesel industry has been expanding rapidly in recent years, a large amount of glycerol has been produced, leading to a significant fall in its market price. Also, the highly reduced nature of glycerol results in the production of twice the amount of reducing equivalents compared to the catabolism of sugars (Yazdani and Gonzalez 2007). This gives glycerol an advantage over sugars as a better substrate for butanol production. *Clostridium pasteurianum* can utilize glycerol and produces a unique product profile containing: butanol, ethanol, 1,3-propanediol (1,3-PDO), and trace amounts of organic acids (acetic and butyric). However, the effect of medium composition, process design, and operating conditions on yields and particularly product distribution (butanol vs 1,3-PDO) are not well understood; neither is the strains ability to ferment crude glycerol in comparison to pure glycerol.

In this study, the product profile of glycerol fermentation by *Clostridium pasteurianum* DSM 525 based on media composition using the two most common media for butanol production (MP2 and modified Biebl) were evaluated. Taking into consideration the efficiency of fermentation, expressed as butanol yield, it was concluded that modified Biebl medium seems to have better properties compared to MP2. Therefore, modified Biebl medium was used for all fermentation studies in this thesis using glycerol as substrate. Next, effects of different fermentation condition (inoculum age, initial cell density, initial pH of medium and temperature) on the butanol yield were studied. Statistical data obtained from RSM led to the development of an empirical model of butanol yield as function of all four investigated factors. This model was numerically optimized to obtain the fermentation conditions that maximize butanol yield. Finally, batch fermentations were performed at optimized conditions in a lab scale bioreactor using crude glycerol as substrates.

The results of this chapter indicate that biodiesel derived glycerol can be suitable substrate for butanol productions. The optimized fermentation condition will be used in the subsequent chapter and Jerusalem artichoke-derived sugar streams will be integrated as co-substartes.

Abstract

Butanol is a promising biofuel and valuable platform chemical that can be produced through fermentative conversion of glycerol. The initial fermentation conditions for butanol production from pure glycerol by *Clostridium pasteurianum* DSM 525 were optimized via a central composite design. The effect of inoculum age, initial cell density, initial pH of medium and temperature were quantified and a quadratic model was able to predict butanol yield as a function of all four investigated factors. The model was confirmed through additional experiments and via analysis of variance (ANOVA). Subsequently, numerical optimization was used to maximize the butanol yield within the experimental range. Based on these results, batch fermentations in a 7 L bioreactor were performed using pure and crude (residue from biodiesel production) glycerol as substrates at optimized conditions. A butanol yield of 0.34 $mole_{butanol}$ $mole^{-1}_{glycerol}$ was obtained indicating the suitability of this feedstock for fermentative butanol production.

5.1 Introduction

The worldwide demand for biofuels is rapidly growing with increasing focus on fuels beyond ethanol (Malaviya et al. 2012). Among various solvents produced through biological routes, the four-carbon alcohol butanol (n-butanol) is a biofuel of considerable interest due to its higher energy content compared to ethanol (Sarchami and Rehmann 2014a). Butanol also has a better miscibility with gasoline and a lower vapor pressure (Lee et al., 2008a; Sarchami and Rehmann, 2014a).

One of the major commercial challenges of butanol fermentation is the need for a cheap feedstock, readily and reliably available at industrial scale (Jones and Woods 1986; Sarchami and Rehmann 2014b). The butanol production cost and profitability of a plant largely depends upon the price of feedstock and is extremely sensitive to any price fluctuation (Green 2011). Therefore, transition towards low-cost (non-edible) feedstocks is crucially important from a process economics perspective and can offer the biggest opportunity for cost reduction and improved sustainability (Sabra et al. 2014).

While a number of low-cost fermentation substrates have previously been evaluated (Raganati et al. 2013; Gao et al. 2014; Gao and Rehmann 2014), glycerol as an alternative carbon source has recently been attracting much attention as a good substrate for bio-based butanol production as it is produced as a major by-product of the biodiesel industry (Dabrock et al. 1992; Yazdani and Gonzalez 2007; da Silva et al. 2009; Jensen et al. 2012a). So-called crude glycerol contains various impurities substantially reducing its value. Due to the worldwide increase in biodiesel production, surplus quantities of crude glycerol are being produced (da Silva et al. 2009). Disposal of crude glycerol has become a financial liability for the biodiesel industry, which also results in a significant decrease of the market price of glycerol (Khanna et al. 2013a; Yuan et al. 2015). Its abundance and cost competitiveness make glycerol an excellent alternative to other carbon substrates for butanol production (Khanna et al. 2013a; Sabra et al. 2014). Also, 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 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. *Clostridium pasteurianum*, a gram positive anaerobic bacteria, can utilize glycerol as a sole carbon source and convert it into butanol, 1,3-propanediol (1,3-PDO), and ethanol (Khanna et al. 2012; Jensen et al. 2012b).

Development of glycerol-based butanol production processes can add significant value to the biodiesel industry and presents excellent potential to establish industrial production of butanol near existing distribution infrastructure. *C. pasteurianum* is the most commonly used organism (Taconi et al. 2009; Ahn et al. 2011; Khanna et al. 2012; Jensen et al. 2012a,b; Khanna et al. 2013; Gallardo et al. 2014; Venkataramanan et al. 2014), however, the effect of medium composition, process design, and operating conditions on yields and particularly product distribution (butanol vs 1,3-PDO) are not well understood; neither is the strains ability to ferment crude glycerol in comparison to pure glycerol.

The objective of this study is therefore threefold: 1) to evaluate the product distribution for the two most common media compositions, 2) to optimize butanol fermentation conditions to maximize its corresponding production yield (both using pure glycerol), 3) to investigate butanol production from crude glycerol in a lab scale bioreactor at optimized conditions.

5.2 Materials and Methods

5.2.1 Crude glycerol preparation

Crude glycerol samples were obtained from Newalta Corporation, Ontario, Canada. Crude glycerol is a viscous inhomogeneous material with a gel-like appearance and a dark brownish color. First crude glycerol was homogenized using mechanical shaking. Then 250 g crude glycerol was mixed with 500 ml of deionized water for an aqueous solution. The obtained solution was filtered through a 0.2 μm filter three times to remove solids and was used as sole carbon source for butanol production. The final solution was diluted 250 fold and filtered using 0.2 μm grade filters. The resulting clear liquid was used for glycerol analysis (HPLC, see section 5.2.8 for conditions). It was found that the concentration of methanol was too low to have any significant negative effect on glycerol utilization.

5.2.2 Chemicals

Yeast extract, peptone and beef extract were obtained from BD- Becton, Dickinson and Company (New Jersey, USA). Soluble starch, sodium acetate, resazurin and thiamine were purchased from Alfa Aesar (Massachusetts, USA). Dextrose and $CaCO_3$ were from Amresco (Ohio, USA) and $CaCl_2$ was from EMD Millipore (Massachusetts, USA). $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, KH_2PO_4, and K_2HPO_4 were purchased from Caledon (Ontario, Canada). Pure glycerol, $FeSO_4$ $.7H_2O$, NaCl and L-cysteine were obtained from BDH (Georgia, USA). Biotin, 2-(N-morpholino) ethanesulfonic acid (MES), and P-aminobenzoic acid were from Sigma-Aldrich (Missouri, USA).

5.2.3 General microbiological conditions

Except for the bioreactor studies, all microbiological work was performed in an anaerobic chamber using aseptic techniques (Model 855-ACB, Plas Labs, Lansing, MI).

5.2.4 Strain and maintenance

C. pasteurianum DSM 525 was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cultures of this strain were grown in reinforced *Clostridium* medium (RCM) containing (per liter) 10 g peptone, 10 g beef extract, 3 g yeast extract, 5 g dextrose, 5 g NaCl, 1 g soluble starch, 0.5 g L-cysteine, 4 ml Resazurin, at pH 6.8. Cells were maintained as a glycerol (20%, w/v) stock, which was prepared after growing cells to an OD_{600} of 0.8, and were stored at -80 °C. Stock cultures were heat shocked for 2 minutes at 90°C and transferred to fresh RCM. The culture (inoculum) was allowed to grow for approximately 18 hours (mid exponential phase) at 37ºC when it was ready to be inoculated at 10% $v.v^{-1}$ into the butanol production medium.

5.2.5 Evaluation of two different fermentation media

Fermentations were carried out with two most common media: MP2 medium (Ahn et al., 2011; Baer et al., 1987) and modified Biebl medium (Biebl 2001; Taconi et al. 2009;

Venkataramanan et al. 2014). All fermentation studies were conducted in 150 ml flasks containing 45 ml of fermentation medium using pure glycerol. MP2 medium contained (per liter of distilled water): 50 g glycerol, 1 g yeast extract, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.02 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4.H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01 g NaCl, 0.01 mg biotin, 1 mg thiamin, and 1 mg p-aminobenzoic acid (Baer et al. 1987). It also contained 100 mM of 2-(N-morpholino) ethanesulfonic acid (MES) to prevent over-acidification (Lee et al. 2008a).

Modified Biebl fermentation medium contained (per liter of distilled water): 50 g glycerol, 1 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO4, 5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.1 g $FeSO_4.7H_2O$, 2 g $CaCO_3$, 0.01 mg Biotin, 1 mg Thiamine, 1 mg p-aminobenzoic acid, 4 ml of trace element solution (SL_7), as described before (Biebl 2001).

Fermentations were performed using 5 ml of actively growing cells inoculated into 45 ml of fermentation medium. Then the flasks were kept in an anaerobic chamber at 37ºC and placed on shaker at 200 rpm for 35 hours. The initial pH was adjusted to 6.3. Samples were taken intermittently and filtered using 0.2 µm grade filters. Clear liquid was stored at -20°C for solvent and glycerol analysis (HPLC, see section 5.2.8 for conditions). Bacterial growth was monitored by measuring the optical density at 600 nm (Microplate reader, see section 5.2.8 for conditions).

5.2.6 Optimization of butanol fermentation conditions

5.2.6.1 Butanol fermentation

All optimization studies were conducted in 150 ml flasks containing 50 ml of modified Biebl fermentation medium using pure glycerol. The required initial pH of each medium was adjusted using 1M HCl or NaOH and the required amount of actively growing cells was inoculated into each 45 ml aliquots of fermentation medium. The flasks were kept in an anaerobic chamber and placed on shaker at 200 rpm at the required temperature for 35 hours. Samples were taken intermittently and filtered using 0.2 µm grade filters. Clear

liquid was stored at -20°C for solvent and glycerol analysis (HPLC, see section 5.2.8 for conditions).

5.2.6.2 Identification of significant factors by ANOVA

An initial two-level full factorial design was employed to screen the influence of inoculum age, initial cell density, initial pH of medium, initial glycerol concentration and temperature on butanol yield (data not shown). The un-coded values for each parameter were as follows [low level, center point, high level]: Inoculum age in hours [13.5, 17.0, 20.5] (the cells were actively growing at the selected times, representing 25%, 55%, and 85% of the final growth), initial Cell density in g L^{1}_{CDW} [0.2, 0.6, 1.0], initial pH of medium [5.8, 6.4, 7.0], initial glycerol concentration in g L^{-1} [30, 50, 70], and Temperature in ºC [30, 33.5, 37]. The experimental design was developed using Design Expert 8.0.7.1 and resulted in 35 conditions including 3 center points. Analysis of variance (ANOVA) of the experimental results had shown significant curvature and confirmed the significance of inoculum age, initial cell density, initial pH of medium, and temperature (Table 5-1). As shown in Table 5-1 initial glycerol concentration had no significant effect on butanol yield. The significance of each parameter was determined based on an α of 0.05 using the F test. Therefore, the initial glycerol concentration of all further fermentation studies was limited to 50 g L^{-1}. Dabrock et al. (1992) showed that there was no substrate inhibition of glycerol using *C. pasteurianum* up to 17% (w vol^{-1}) or 170 g L^{-1} ; however, it was reported that at higher glycerol concentration (> 50 g L^{-1}), conversion was slower (Biebl 2001). Cell density (biomass concentration) refers to cell quantity which was measured based on grams of cell per unit volume where inoculum age refers to quality of the cells and was measure based on time of culture duration (hour).

Table 5-1 Analysis of variance of experimental results of full factorial design

Source	Remark	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value Prob>*F*
Inoculum Age (hr)	Significant	0.0023	1	0.0023	16.53	0.0455

Cell Density (g L^{-1})	Significant	0.0052	1	0.0052	38.28	0.0251
Initial pH of Medium	Significant	0.0055	1	0.0055	38.35	0.0263
Initial Glycerol Concentration (g L^{-1})	Not-Significant	0.000045	1	0.000045	0.031	0.8760
Temperature (°C)	Significant	0.027	1	0.027	185.28	0.0054

5.2.6.3 Central composite design and statistical analysis

After identification of significant factors, a central composite design (CCD) was selected to evaluate the response pattern and to determine the optimal combination of inoculum age, initial cell density, initial pH of medium, and temperature for maximizing butanol yield. The un-coded values for each parameter were as follows [low star point, low central point, center point, high central point, high star point]: Inoculum age in hours [12.05, 13.5, 17.0, 20.5, 21.95] (the cells were actively growing at the selected times, representing 13%, 25%, 55%, 85% and 99% of the final growth), initial Cell density in g $L^{-1}{}_{CDW}$ [0.03, 0.2, 0.6, 1.0, 1.17], initial pH of medium [5.55, 5.8, 6.4, 7.0, 7.25], and Temperature in °C [28.5, 30, 33.5, 37, 38.5]. The experimental design was developed using Design Expert 8.0.7.1 and resulted in 26 conditions. All conditions were tested in triplicated, including 3 center points. The resulting 87 conditions (16 * 3 factorial + 10 * 3 augmented + 3 * 3 center points) were fully randomized.

Linear regression analysis was used to fit the experimental data with a second-order model as given in equation (5-1):

$$Y= \beta_0+\sum_{i=1}^{4} \beta_i x_i+\sum_{i=1}^{4} \beta_{ii} x_i^2+\sum_{1\leq i\leq j}^{4} \beta_{ij} x_i x_j+\varepsilon \quad (5\text{-}1)$$

The experimental data was analyzed using Design Expert 8.0.7.1 as discussed in Chapter 3. Section 2.1.6

5.2.7 Butanol production in a lab scale bioreactor at optimized condition

A lab-scale stirred-tank bioreactor with a nominal volume of 7 L was used (Labfors, Infors, Quebec, Canada). The fermentations were conducted in 5 L working volume containing 4.5 L of modified Biebl medium and 0.5 L of culture. Temperature was controlled at 30ºC and 0.2 ml L^{-1} of antifoam was added to the fermentation medium to control foaming. Agitation was controlled at 150 rpm using one Rushton impeller and the fermenter was equipped with sensor probes monitoring pH (Hamilton EasyFerm, Switzerland), redox potential (Mettler Toledo, Switzerland), and cell density (TruCell2™, Finesse Solutions, LLC, USA). Nitrogen gas was used at a flow rate of 0.6 l min^{-1} throughout the experiment to purge the bioreactor and keep it at anaerobic condition. A carbon dioxide analyzer (Infors, Quebec, Canada) was connected to the outlet gas stream line. This system allowed online measurement of CO_2 content within the outlet gas stream. Temperature, gas flow rates (Red-y series flow controller, model GSC-C3SA-BB12, Vogtlin Instruments AG) and stirrer speed were regulated through control units (local loops). Iris software (Labfors, Infors, Quebec, Canada) was used to monitor and manage the process with good flexibility and total traceability. In addition to online determined parameters, samples were taken intermittently and filtered using 0.2µm grade filters for solvents and glycerol analysis (HPLC, see section 5.2.8 for conditions).

5.2.8 Analytical methods

Bacterial growth was monitored by measuring the optical density at 600 nm using a 200 pro infinite series microplate reader (Tecan, Switzerland) using 96 well microplates at 200 µl per well. Concentrations of glycerol as well as solvents produced in the fermentation were determined by high performance liquid chromatography on an Agilent 1260 infinity (Agilent USA, Santa Clara) using an Agilent Hi-plex H (7.7 × 300 mm) column (Agilent USA, Santa Clara) at 35°C. A refractive index detector was used for compound detection. 0.005 M H_2SO_4 was used as the isocratic mobile phase at a constant flow rate of 0.4 ml $min^{-1.}$ Before injection, samples were diluted to appropriate concentration with mobile phase and filtered through a 0.2 µm membrane filter. Total solvents and glycerol were quantified using pure glycerol (BDH, Georgia, USA), butanol (Sigma-Aldrich, St. Louis,

MO), 1,3-propanediol (Sigma-Aldrich, Missouri, USA), and ethanol (Sigma-Aldrich, Missouri, USA) as standards.

Product yield was calculated as the total amount of butanol produced, divided by the amount of fermentable glycerol utilized and is expressed as $mole_{butanol}$ $mole^{-1}_{glycerol}$.

5.3 Results and Discussion

5.3.1 Evaluation of two different fermentation media

The initial glycerol concentration was limited to 50 g L^{-1}. Dabrock et al. (1992) showed that there was no substrate inhibition of glycerol using *C. pasteurianum* up to 17% (w vol^{-1}) or 170 g L^{-1} ; however, it was reported that at higher glycerol concentration (>50 g L^{-1}), conversion was slower (Biebl 2001). As shown in Fig. 5-1a and b, in both media, the culture started to use glycerol directly after the inoculation. A biomass increase was observed during the fermentation from 0.42 to 3.52 g L^{-1}, and 0.39 to 3.81 g L^{-1} using MP2 and modified Biebl, respectively. A butanol concentration of 6.45±0.2 g L^{-1} and 8.56±0.3 g L^{-1} were achieved after 35 hours fermentation using MP2 and modified Biebl, respectively. Butanol yield in MP2 and modified Biebl were 0.20±0.02 $mole_{butanol}$ $mole^{-1}_{glycerol}$ and 0.26±0.03 $mole_{butanol}$ $mole^{-1}_{glycerol}$, respectively. Taking into consideration the efficiency of fermentation, expressed as butanol yield, it can be concluded that modified Biebl medium seems to have better properties compared to MP2. This can be potentially explained by the different compositions of the two media. It has been reported that the butanol concentration via glycerol fermentation using *C. pasteurianum* DSM525 is strongly dependent on the medium composition and higher concentration of $(NH_4)_2SO_4$ and $FeSO_4.7H_2O$, as was the case for Modified Biebl medium, will increase fermentation efficiency and yield (Moon et al. 2011). The yields obtained in this work are of similar values as elsewhere reported for butanol fermentation from glycerol by *Clostridium pasteurianum* DSM 525 (Biebl 2001; Malaviya et al. 2012). All experiments were carried out in duplicate and averages ± standard deviation are presented in the figure 5-1.

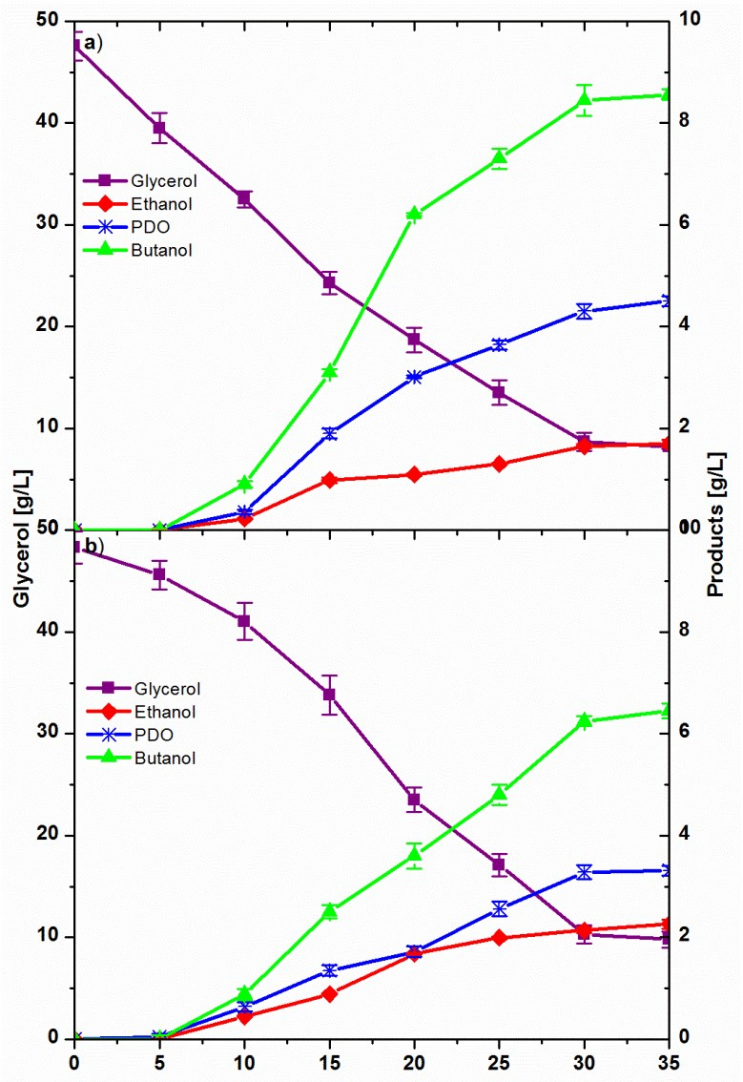

Figure 5-1 Profiles of solvent production and glycerol utilization in a) modified Biebl medium and b) MP2 medium by *C. pasterianum* DSM 525. Residual glycerol content (filled square), ethanol concentration (filled diamond), 1,3-PDO concentration (star), butanol concentration (filled triangle). The discrete data points are average of triplicate measurements ± standard deviation, the connecting lines are for visualization purposes only.

5.3.2 Optimization of butanol fermentation conditions

No optimization of the fermentation conditions for *C. pasterianum* can be found in the literature using the four aforementioned factors and it was anticipated that substantial

improvements of the butanol yields would be achievable. Experimental conditions were chosen based on a central composite design and the actual values of the independent variables and the measured responses are shown in Table 5-2. All experiments were carried out in triplicate and averages ± standard deviation are presented in the table.

Table 5-2 Butanol production yield (average of triplicates ± standard deviation) under conditions determined for CCD.

Initial pH of medium	Initial Cell density (g L^{-1}_{CDW})	Inoculum age (hr)	Temperature (°C)	Butanol yield (% $mole_{butanol}$ $mole^{-1}_{glycerol}$)
5.55	0.6	17.0	33.5	19.1±0.5
5.8	0.2	13.5	30	31.7±0.4
5.8	0.2	13.5	37	24.8±0.6
5.8	0.2	20.5	30	29.2±0.7
5.8	0.2	20.5	37	20.5±0.9
5.8	1.0	13.5	30	22.4±0.5
5.8	1.0	13.5	37	21.9±0.4
5.8	1.0	20.5	30	19.1±0.3
5.8	1.0	20.5	37	18.6±0.9
6.4	0.03	17.0	33.5	15.7±1.1
6.4	0.6	12.05	33.5	22.6±0.2
6.4	0.6	17.0	28.55	31.0±0.4
6.4	0.6	17.0	33.5	27.8±0.1
6.4	0.6	17.0	33.5	27.9±0.1
6.4	0.6	17.0	33.5	28.1±0.1
6.4	0.6	17.0	33.5	27.8±0.1
6.4	0.6	17.0	33.5	28.1±0.1
6.4	0.6	17.0	38.45	24.2±0.4
6.4	0.6	21.95	33.5	20.3±1.1
6.4	1.17	17.0	33.5	21.3±0.6
7.0	0.2	13.5	30	33.9±0.2
7.0	0.2	13.5	37	25.2±0.6
7.0	0.2	20.5	30	31.5±1.1

7.0	0.2	20.5	37	23.9±0.2
7.0	1.0	13.5	30	27.7±0.5
7.0	1.0	13.5	37	23.4±0.3
7.0	1.0	20.5	30	24.4±0.4
7.0	1.0	20.5	37	20.1±0.3
7.25	0.6	17.0	33.5	23.7±0.9

5.3.2.1 Response surface model validation

As observed from the experimental results in Table 5-2, the fermentation process was successful in producing butanol from glycerol within the ranges of the input variables. The complete dataset could be fitted with a quadratic model as describe in equation (5-1). The resulting model parameters are shown in Table 5.3. The *F* value of the model is 4.6 which is very high compared to the critical value, indicating that the model is highly significant. The significance of each parameter coefficient was determined by *P* values, the smaller the *P* values the more significance of the coefficient. In this case, all factors have great effect on butanol production yield. The quadratic effects of temperature, and cell density, as well as interaction effect of temperature-cell density have also significant effects on butanol yield. The goodness of fit of the model was confirmed by the coefficient of determination R^2=0.83 and adjusted determination coefficient Adj. R^2=0.66. A ratio of 8.4 of the adequate precision indicates an adequate signal to noise ratio for navigating the design space.

Based on the selected significant variables, the quadratic model for the butanol yield in terms of actual factors is shown as follows:

Butanol Yield = -10.40 + 65.99 * pH - 23.12 * Cell Density + 3.47 * Inoculum Age - 11.11 * Temperature + 1.09 * Cell Density * Temperature – 18.89 * Cell Density^2 + 0.17 * Temperature^2 (5-2)

The residuals can be judged as normally distributed based on a normal probability (data not shown).

Table 5-3 Analysis of variance of fitted model for butanol yield.

Source	Remark	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value Prob>*F*
Model	Significant	509.05	14	36.36	4.61	0.0045
Initial pH (A)	Significant	42.65	1	42.65	5.41	0.0368
Cell Density (B)	Significant	69.86	1	69.86	8.86	0.0107
Inoculum Age (C)	Significant	39.63	1	39.63	5.03	0.0430
Temperature (D)	Significant	151.40	1	151.40	19.21	0.0007
BD	Significant	37.52	1	37.52	4.76	0.04481
B^2	Significant	79.40	1	79.40	10.07	0.0073
D^2	Significant	37.47	1	37.47	4.75	0.0483
R-Squared						0.83
Adj-Squared						0.65
Adeq Precision						8.4

5.3.2.2 Combined effect of pH, Cell density, Inoculum age and Temperature

Response surface methodology was used to study the interaction effects of the four factors. The surface plot of the combined effect of initial pH of medium and cell density on butanol production yield at a constant inoculum age of 17 hours and temperature of 33.5°C is shown in Fig. 5-2A. The butanol yield is a function of both the initial pH of medium and

cell density. Fig. 5-2B shows the surface plots of the combined effect of initial pH and inoculum age on butanol yield at a constant cell density of 0.6 g L^{-1}_{CDW} and temperature of 33.5°C. The butanol yield is also a function of both the initial pH and inoculum age. The plots clearly indicate that an optimum exists within the observed design space with respect to cell density and inoculum age, increasing the initial pH (at the temperature of 33.5°C) appears to increase butanol yield over the observed design space.

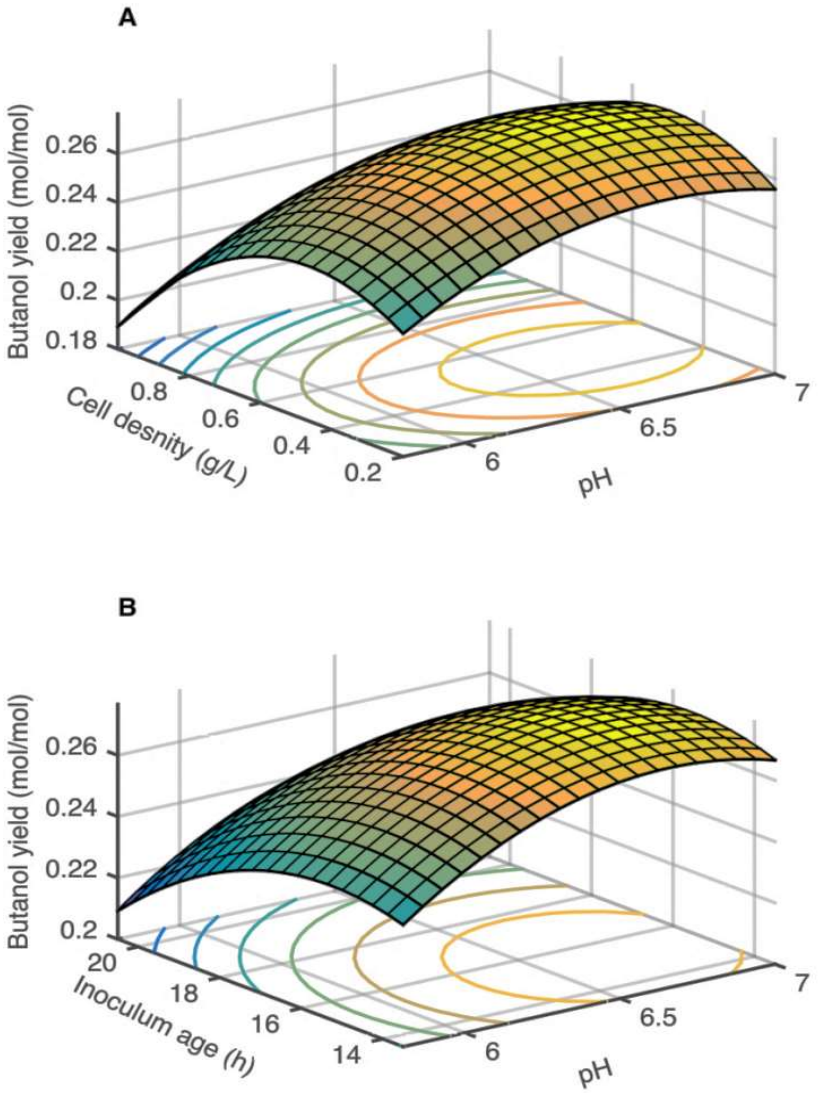

Figure 5-2 Surface plot of combined effect of process variables on butanol yield. A) pH and cell density, B) pH and inoculum age

5.3.2.3 Response optimization and model validation

Based on the model, numerical optimization was used to determine the optimal combination of process parameters for maximum butanol yield. The optimal conditions for butanol production yield were inoculum age of 16 hours (52% of the final growth), initial cell density of 0.4 g L^{-1}_{CDW}, initial pH of 6.8, and temperature of 30°C. To the best knowledge of the authors, this is the first attempt to optimize the fermentation conditions of butanol production from glycerol with the four mentioned factors using *C. pasteurianum* DSM 525. Khanna et al. (2013b) performed an optimization study with immobilized *C. pasteurianum* using the parameters: initial pH; temperature; agitation; and initial glycerol concentration. It was reported that for pure glycerol batch flask fermentations, the initial pH was the most critical factor and similar to the current study, the set of optimized parameters included a pH of 7.0 and a temperature of 30°C. Khanna et al. (2013b) reported 0.27 $mole_{butanol}$ $mole^{-1}_{glycerol\ added}$ butanol yield using immobilized *C. pasteurianum* at pH of 7.0, agitation rate of 200 rpm, temperature of 30ºC, and initial glycerol concentration of 25 g L^{-1} after 8 days of fermentation, which appears to be lower than the results obtained in this study. This deviation can be potentially explained by glycerol bioconversion with immobilized cells, unpublished media composition as well as unknown inoculum age and cell density in case of Khanna et al.'s (2013b) work. It was also reported that 0.3 $mole_{butanol}$ $mole^{-1}_{glycerol}$ butanol yield was achieved at temperature of 37ºC, initial glycerol concentration of 40 g L^{-1}, and pH of 7.0 under iron limitation condition (Dabrock et al. 1992). Higher fermentation temperature and iron limitation condition of Dabrock et al. (1992) work may justify its lower butanol yield compared to the results obtained in this study. In another study, butanol fermentation was carried out at a temperature of 37ºC under iron limitation conditions and optimal conditions were reported at an inoculum age of 18 hours (OD_{600} of 3.5), initial cell density of 0.42, and initial pH in the range of 5.5-6.0 (Malaviya et al. 2012). Under such conditions 0.31 $mole_{butanol}$ $mole^{-1}_{glycerol}$ butanol yield was reported after 50 hours of fermentation. Higher fermentation temperature, lower initial pH of medium and iron limitation condition of Malaviya et al. (2012) work may explain its lower butanol yield compared to the results obtained in this study. Taconi et al. (2009) reported maximum butanol concentration of 7 g L^{-1} and a butanol yield of 0.385 $mole_{butanol}$

mole$^{-1}_{glycerol}$ at temperature of 35ºC with an initial glycerol concentration of 25 g L^{-1} and pH of 7.0 after 10 days of fermentation. The slightly lower yield is this study is a direct result of the higher final butanol concentrations (12.3 g L^{-1}) and the organism dealing with additional stress through product inhibition at the end of the fermentation. However, higher maximum butanol concentration (12.3 g L^{-1} vs. 7 g L^{-1}), higher initial glycerol concentration (50 g L^{-1} vs. 25 g L^{-1}) and much shorter fermentation time (30 hours vs. 10 days) of this study make it more industrially advantageous. Biebl (2001) reported a butanol yield of 0.32 mole$_{butanol}$ 100 mole$^{-1}_{glycerol}$ at an initial pH of 6.0, initial glycerol concentration of 50 g L^{-1}, and a temperature of 37ºC. The optimized conditions obtained in this study resulted in one of the highest yield reported for the wild-type strain of *C. pasteurianum*. In comparison, lower temperature and higher initial pH (close to neutral) of this study coupled with high butanol yield might economically benefits the industrial production of butanol.

To validate the applicability of this RSM model, some confirming experiments were carried out around the estimated optimal conditions. The measured and predicted butanol yields of three conditions around the optimum are listed in Table 5-4. The predicted results were compared with the actual values obtained experimentally. *T* test at 95% confidence showed no significant difference between the predicted and actual values. In summary, the proposed RSM model could be a useful model for the prediction of maximum butanol production yield.

Table 5-4 Optimal conditions and model validation.

Initial pH of medium	**Cell density (g L$^{-1}_{CDW}$)**	**Inoculum age (OD_{600nm})**	**Temperature (°C)**	**Butanol yield % mole$_{butanol}$ mole^{-1} $_{glycerol}$**	
				Predicted	**Experimental**
6.71	0.39	16.0	30	35.0±0.3	34.6±0.6
6.76	0.38	15.5	30	34.9±0.2	34.9±0.5
6.85	0.39	15.5	30	35.1±0.5	34.9±0.4

5.3.3 Butanol production from crude glycerol in a lab scale bioreactor at optimized condition

The optimization study was carried out in shaking flasks due to the large number of experimental conditions (87 conditions). The validity of this method was verified by using the optimized fermentation conditions in a 7 L bench-top bioreactor, using crude glycerol as feedstock. Butanol production was performed by using 16 hr-old seed culture and starting the fermentation with an initial cell density of 0.4 g L^{-1}_{CDW}. The initial pH of the medium was adjusted to 6.8 using 1M NaOH and the fermentation temperature was controlled at 30°C. The butanol fermentation was also performed using 50 g L^{-1} pure glycerol as a control experiment and the results are presented in Fig. 5-3. In addition to offline determined concentrations, pH, CO_2 formation, cell density, and redox potential were measured online. As shown in Fig. 5-3a-c, a longer lag phase was observed in glycerol consumption, butyric acid, acetic acid, butanol, 1,3-PDO, and ethanol production using crude glycerol as a substrate compared to pure glycerol. A butanol yield of 0.34±0.01 $mole_{butanol}$ $mole^{-1}_{glycerol}$ was achieved after 35 hours fermentation using crude glycerol as substrate. Fermentation with crude glycerol showed identical yields (within error) with pure glycerol (0.35±0.01 $mole_{butanol}$ $mole^{-1}_{glycerol}$) as a control fermentation. As shown in Fig. 5-3d, the CO_2 signal is a result of biological CO_2 formation but is also influenced by the CO_2 release of the $CaCO_3$ buffer with decreasing pH. Therefore, the absolute value does not represent the overall biological activity. The CO_2 formation profile indicates the end of the fermentation using pure and crude glycerol as substrate at 30 and 35 hours, respectively (Fig. 5-3d). The pH profile of fermentation process was plotted in Fig. 5-3e. As mentioned previously, the pH of the fermentation medium was initially adjusted to 6.8 (optimal pH) but not controlled thereafter and the buffering capacity of the medium is clearly not sufficient to maintain a constant pH. As shown, pH continuously decreased until the pH value reached 5.23 and 5.19 using pure and crude glycerol as substrate, respectively. The decrease in pH was owing to the fact that in the acidogenesis phase of fermentation, organic acids such as acetic acid and butyric acid were produced, which resulted in a decrease in pH of the fermentation medium. The slow increase in pH later in fermentation was concurrent with solvent production (solventogenesis phase) which resulted in an

increase in pH of the medium to 5.4 for both substrates. As shown in Fig. 5-3f, the cell growth curve (continuously measure via online turbidity probe) using both substrates follows the typical profile of lag phase followed by exponential phase with a longer lag phase for crude glycerol compared to the pure glycerol. The cell density probe is suitable for transmission measurements within a fermenter. Due to the correlation of optical density and cell dry weight the output signal (AU) was analyzed as cell dry weight (g L^{-1}) with the aid of curve-fitting.

Also, at optimal conditions the butanol production was equal (within error) using pure glycerol in anaerobic shake flasks and 7 L bioreactor, justifying the necessary scale-down for the optimization experiments. These results indicate that crude glycerol is a reliable feedstock for butanol production.

The experimental work in the study largely focuses on the optimization of fermentation condition using glycerol as a potential feedstock for butanol production. The setup used in this study is not intended to represent a potential industrial process. More advanced fermentation process design, possibly including continuous fermentation and/or in-situ product removal, would likely have to be used in an industrial process, as evaluated for different feedstocks elsewhere (Lee et al. 2008b; Napoli et al. 2011).

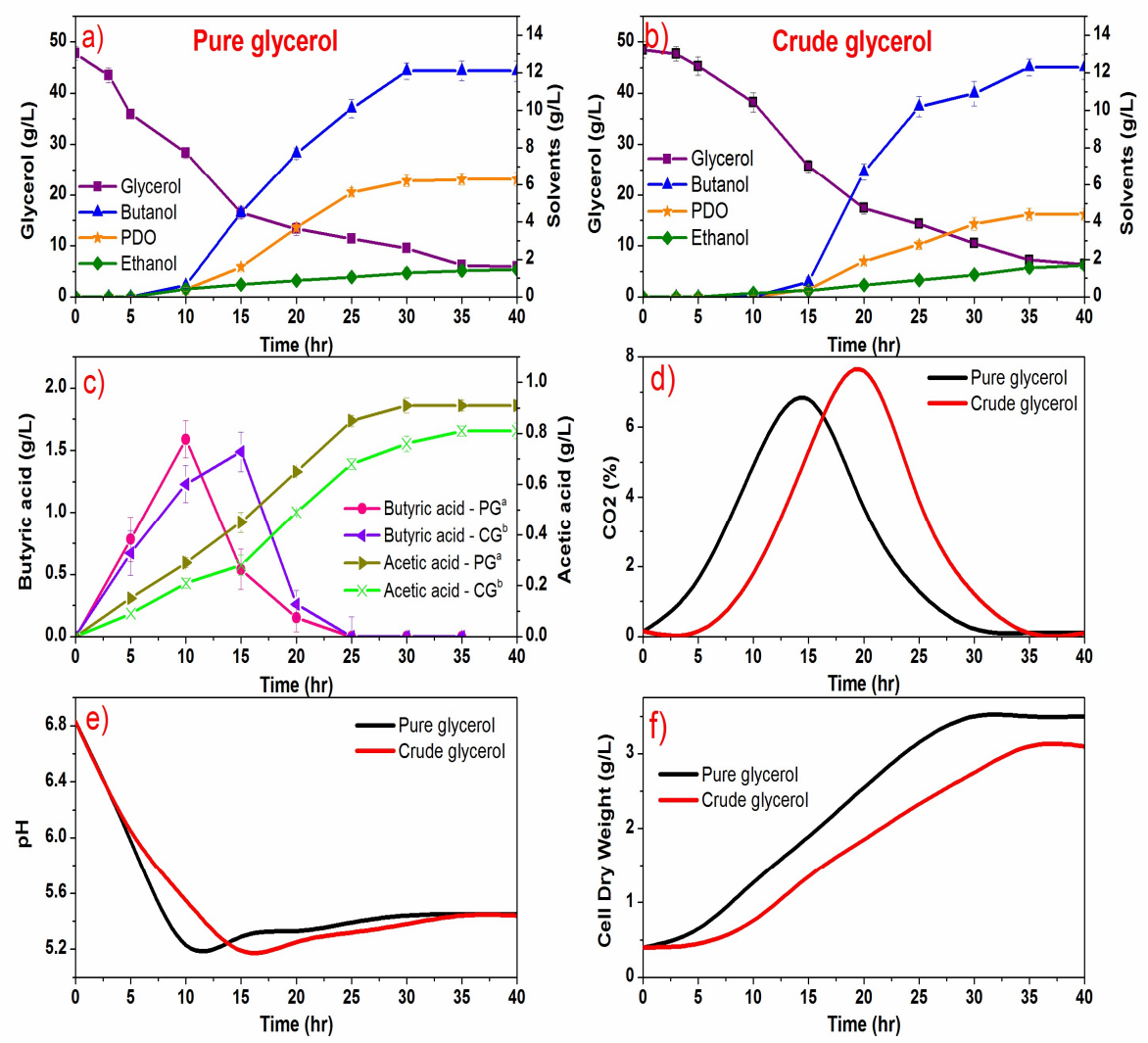

Figure 5-3 Profile of solvent production and substrate utilization using pure glycerol in a), and crude glycerol in b), organic acids production in c), CO_2 formation in d), pH profile in e), and Cell dry weight in f), for butanol production under optimal fermentation by *Clostridium pasterianum* DSM 525. The discrete data points (a-d) are average of triplicate measurements ± standard deviation, the connecting lines are for visualization purposes only. The smooth line sin d-f are the results of online measurements recorded every 10 s.

[a] Pure glycerol

[b] Crude glycerol

5.4 Conclusions

Out of the two most common media compositions for the conversion of glycerol to butanol, modified Biebl media was found to be the more appropriate medium and the optimal conditions for maximum butanol yield using an initial glycerol concentration of 50 g L^{-1} were inoculum age of 16 hours, initial cell density of 0.4 g L^{-1}_{CDW}, initial pH of 6.8, and temperature of 30°C.

Clostridium pasterianum DSM 525 was able to ferment crude glycerol under optimal condition resulting in a yield of 34 % $mole_{butanol}$ $mole^{-1}_{glycerol}$. Therefore, crude glycerol was found to be a good feedstock for butanol production.

5.5 References

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Chapter 6

6 Co-substrate fermentation of Jerusalem artichoke tubers and crude glycerol to butanol by *Clostridium pasteurianum* DSM 525

Tahereh Sarchami, Lars Rehmann.

Preface

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is ready for submission.

The work presented in the previous chapter demonstrates the suitability of biodiesel-derived glycerol as low-cost and available substrates for fermentative butanol production. However, to enhance butanol production from glycerol further, Regestein et al. (2015) showed that the addition of butyric acid to fermentative glycerol conversion shift its product distribution towards butanol (Regestein et al. 2015). In addition, it was shown that *C. pasteurianum* produces mainly organic acids such as acetic and butyric acids when using saccharides as its carbon source (Heyndrickx et al. 1991; Dabrock et al. 1992). Therefore, an alternative to adding butyrate to the glycerol fermentation medium is to co-utilize a sugar-based substrate, which can take advantage of substrate with lower costs than butyrate. The study conducted in Chapter 4 presents the suitability of Jerusalem artichoke acid hydrolysate as a low-cost substrate for butanol production. Therefore, Jerusalem artichoke acid hydrolysate as a sugar source and glycerol as the main carbon source could potentially be used as co-substrate for enhanced butanol production.

Therefore, this study was undertaken to assess the feasibility of employing the aforementioned co-substrate strategy for improved butanol production. In order to establish such a system, the effect of adding acetate and butyrate on butanol production was first investigated and confirmed. The product formation by the same strain using different mono-saccharide substrates was studied, followed by an optimization study of the co-substrate ratio. Based on the estimated optimal conditions, Jerusalem artichoke hydrolysate

and crude glycerol were used as low-cost carbon sources for the co-substrate based butanol production in a laboratory bench bioreactor.

The results of this chapter confirm that direct addition of acetate and butyrate into the fermentation broth enhances butanol yield and productivity. Also, Jerusalem artichoke acid hydrolysate was found to be a suitable sugar source to be fermented by *C. pasteuriaunm* DSM 525 to produce acetic and butyric acid. Biodiesel derived- glycerol and Jerusalem artichoke acid hydrolysate were found to be reliable feedstocks as low-cost carbon sources for co-substrate butanol production.

Abstract

Adding organic acids, especially butyric acid, enhancs butanol production from glycerol with *Clostridium pasteurianum* DSM 525. The strain predominantly produces acetic and butyric acid when grown on saccharides. Hence, the butyrate produced from assimilating sugars can be used to stimulate butanol production from glycerol under co-substrate cultivation with sugars and glycerol. In order to prevent cell growth inhibition due to high butyric acid concentration arising from a high sugar concentration, to prevent glycerol limitation, and to enhance butanol production yield without decreasing butanol production rate, the initial glycerol and sugar concentration were optimized. Under optimal condition (glycerol concentration of 50 g L^{-1} and sugar concentration of 15 g L^{-1}) a butanol yield and productivity of 0.27±0.01 $g_{butanol}$ $g^{-1}_{(glycerol+sugar)}$ and 0.74±0.02 g L^{-1} hr^{-1} was obtained, respectively. Based on these results, batch fermentation in a 5 L bioreactor was performed using Jerusalem artichoke hydrolysate and crude glycerol (residue from biodiesel production) as co-substrate at optimal condition. A butanol yield and productivity of 0.28±0.007 $g_{butanol}$ $g^{-1}_{(glycerol+sugar)}$ and 0.55±0.008 g L^{-1} hr^{-1} was achieved after 27 hours fermentation indicating the suitability of those cheap carbon sources as well as *C. pasteurianum* DSMZ 525 for co-substrate butanol production.

6.1 Introduction

Due to increased substrate costs and availability of less expensive petrochemically derived butanol in the 1950s, most of the acetone/butanol/ethanol (ABE) fermentation plants were closed (Jones and Woods 1986; Zverlov et al. 2006). However, in recent years, there has been a renewed interest in butanol fermentation, which has led to a large number of studies on strain development, fermentation improvement and in-situ product removal technologies (Ezeji et al. 2007; García et al. 2011). This has resulted in a dramatic reduction of butanol toxicity to the fermenting microorganisms, improved substrate utilization and overall bioreactor performance. Nevertheless, the high cost and availability concerns of conventional substrates (corn, molasses) still remains as one of the major hurdles for fermentative butanol to compete with the petroleum-based one (Jones and Woods 1986; García et al. 2011). In order to realize industrial-scale butanol fermentation, it is crucially important to identify inexpensive and available biomass feedstock that can be fermented by *Clostridium* species (Luque et al., 2014; Qureshi et al., 2008). Glycerol as a byproduct from biodiesel production has recently attracted much attention as a potential substrate for bio-based production of chemicals and fuels (Dabrock et al. 1992; Yazdani and Gonzalez 2007; da Silva et al. 2009; Jensen et al. 2012). As a result of worldwide increase in biodiesel production, surplus quantities of biodiesel-derived glycerol (crude glycerol) are being produced (da Silva et al. 2009). Crude glycerol is contaminated with various impurities which makes it unsuitable for conventional outlets (cosmetics, soaps). Also purification costs of this glycerol are excessively high which resulted in a significant decrease of its market price (Khanna et al. 2013a; Yuan et al. 2015). Hence effective utilization of crude glycerol is crucial to enhance the economy of biodiesel industry (Khanna et al. 2013a).

The mostly studied microorganism for biological production of butanol from glycerol is *Clostridium pasteurianum*. It can utilize glycerol as a sole carbon source and converts it into butanol, 1,3-propanediol (1,3-PDO), and ethanol (Khanna et al. 2012). However, when using saccharides as its carbon source, this microorganism produces mainly organic acids such as acetic and butyric acids (Heyndrickx et al. 1991; Dabrock et al. 1992). Butyrate is

an intermediate in the respective fermentation pathway leading to butanol, and the external addition of butyrate can significantly and efficiently enhance butanol production (Martin et al. 1983; Fond et al. 1985; Tashiro et al. 2004; Kao et al. 2013). The addition of acetate has also been reported to enhance butanol production for some clostridium species (Martin et al. 1983; Chen and Blaschek 1999). However, *Clostridium pasteurianum* appears to not fully convert these acids when growing on saccharides, a limitation that is not present with glycerol as the main carbon source. The addition of butyric acid to fermentative glycerol conversion by *Clostridium pasteurianum* has been shown to shift its product distribution towards butanol (Regestein et al. 2015). An alternative to adding butyrate to the fermentation medium is to utilize a co-substrate system, which can take advantage of substrate with lower costs than butyrate.

Jerusalem artichokes (Helianthus tuberosus L.) have been shown as an alternative source of saccharides for the fermentative production of butanol; they can grow well in non-fertile land and are resistant to plant diseases, not competing with grain crops for arable land (Szambelan et al. 2005; Dürre 2007; Raganati et al. 2013; Sarchami and Rehmann 2014a). Jerusalem artichoke tuber (as all member of the Asteraceae family) is a rich source of inulin, a biopolymer made up of linear chains of β (2→1)-linked D-fructose units terminated by a D-glucose linked to fructose by α (1→2) bond (Szambelan et al. 2005). Though the principal storage carbohydrate of Jerusalem artichoke tuber is inulin (15 to 20 %), monomeric sucrose, glucose, and fructose are also present (Matías et al. 2011). Most microorganisms cannot directly ferment inulin; therefore, inulin first needs to be hydrolyzed into fructose and glucose monomers. Hydrolysis can be achieved via an acid catalyst or enzymes. By comparison, hydrolysis via acid catalyst can hydrolyze up to 98.5 % of inulin within 35 minutes with non-inhibiting byproduct concentrations, while the same Jerusalem artichoke extract requires 24 hours to achieve similar numbers enzymatically (Sarchami and Rehmann 2014a). The shorter reaction times and lower catalyst costs would imply acid hydrolysis to be favorable over enzymatic conversion. Availability and cost competitiveness of crude glycerol and Jerusalem artichoke tubers make both excellent candidates for butanol production.

Using Jerusalem artichoke hydrolysate as a sugar source and glycerol as the main carbon source with a single culture of *C. pasteurianum* might lead to the formation of acids from the sugar source, stimulating the simultaneous butanol production from glycerol. Therefore, this study was undertaken to assess the feasibility of employing the aforementioned co-substrate strategy for the enhanced butanol production with the *C. pasteurianum* DSM 525. In order to establish such a system, the effect of adding acetate and butyrate on butanol production by C. *pasteurianum* DSM 525 was first investigated and confirmed. The product formation by the same strain using different mono-substrate was studied, followed by an optimization study of the co-substrate ratio. Based on the estimated optimal conditions, Jerusalem artichoke hydrolysate (JAH) and crude glycerol (from biodiesel manufacturing waste) were used as low-cost carbon sources for the co-substrate based butanol production in a 5L laboratory bench bioreactor.

6.2 Materials and Methods

6.2.1 Chemicals

See Chapter 5. Section 2.2

6.2.2 Crude glycerol preparation

Crude glycerol was prepared for fermentation following the protocol described in Chapter 5. Section 2.1

6.2.3 Jerusalem artichoke preparation

6.2.3.1 Preparation of Jerusalem artichoke flour

Jerusalem artichoke flour was prepared following the protocol described in Chapter 3. Section 2.1.1

6.2.3.2 Inulin extraction

Inulin extraction was performed following the protocol described in Chapter 3. Section 2.1.2

6.2.3.3 Acid hydrolysis of extracted inulin

Batch acid hydrolysis was performed in 500 ml storage/media bottles at optimal condition of pH 2.0, temperature of 97°C, and time period of 35 minutes (Sarchami and Rehmann 2014b). Each bottle contained 250 ml of water-extracted inulin from Jerusalem artichoke tubers obtained in 6.2.3.2 section and the pH was adjusted to 2.0 using sulphuric acid. The bottles were hermetically covered with Parafilm and aluminum foil to avoid evaporative loss, and the mixture was heated at 97°C while shaking at 300 rpm. Samples were taken at the end of hydrolysis at 35 minutes and filtered using 0.2 μm grade filters. Clear liquid was stored at -20°C for sugar analysis (HPLC, see section 6.2.3.9 for conditions). For the hydrolysate fermentation, the pH was adjusted to 6.8 using 1 M NaOH solution.

6.2.4 General microbiological conditions

Except for the bioreactor studies, all microbiological work was performed in an anaerobic chamber using aseptic techniques (Model 855-ACB, Plas Labs, Lansing, MI).

6.2.5 Strain and maintenance

Microorganism and cell culture condition previously described in Chapter 5. Section 2.4

6.2.6 Butanol fermentation

Fermentation studies were conducted in 150 mL flasks containing 50 ml of modified Biebl medium (Biebl 2001; Sarchami and Rehmann 2014a) at optimal fermentation conditions (Sarchami et al. 2016). Modified Biebl medium contained (per liter of distilled water): glycerol, 1 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO4, 5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.1 g $FeSO_4.7H_2O$, 2 g $CaCO_3$, 0.01 mg Biotin, 1 mg Thiamine, 1 mg p-aminobenzoic acid, 4 ml of trace element solution (SL_7), as described before (Biebl 2001). 0.4 g L^{-1}_{CDW} of actively growing cells were inoculated into 50 ml of fermentation medium. Then the flasks were kept in an anaerobic chamber at 30ºC and placed on shaker at 200 rpm for 40 hours. The initial pH of fermentation medium was adjusted to 6.8 using NaOH but was not controlled thereafter. Samples were taken

intermittently and filtered using 0.2 μm grade filters. Clear liquid was stored at -20°C for solvent and glycerol analysis (HPLC, see section 6.2.3.9 for conditions).

6.2.7 Central composite design and statistical analysis

A central composite design (CCD) was selected to evaluate the response pattern and to determine the optimal combination of glycerol and sugar concentration for maximizing butanol yield and productivity. The un-coded values for each parameter were as follows [low star point, low central point, center point, high central point, high star point]: Glycerol concentration in g L^{-1} [23.6, 30, 50.0, 70, 76.4] and Sugar concentration in g L^{-1} [6.8, 10, 20, 30, 33.2]. The experimental design was developed using Design Expert 8.0.7.1 and resulted in 8 conditions. All conditions were tested in triplicated, including 3 center points. The resulting 33 conditions (4 * 3 factorial + 4 * 3 augmented + 3 * 3 center points) were fully randomized.

Linear regression analysis was used to fit the experimental data with a second-order model as given in equation (6-1):

$$Y= \beta_0+\sum_{i=1}^{2} \beta_i x_i+\sum_{i=1}^{2} \beta_{ii} x_i^2 +\sum_{1\leq i\leq j}^{2} \beta_{ij} x_i x_j+\varepsilon \quad (6\text{-}1)$$

The experimental data was analyzed using Design Expert 8.0.7.1 as discussed in Chapter 3. Section 2.1.6

6.2.8 Butanol production in a lab scale bioreactor

A lab-scale stirred-tank bioreactor with a nominal volume of 5 L was used (Labfors, Infors, Quebec, Canada). The fermentations were conducted in 3 L working volume containing 2.7 L of modified Biebl medium and 0.3 L of culture. The temperature was controlled at 30ºC and 0.2 ml L^{-1} of antifoam was added to the fermentation medium to control foaming. Agitation was controlled at 150 rpm using one Rushton impeller and the fermenter was equipped with sensor probes monitoring pH (Hamilton EasyFerm, Switzerland), redox potential (Mettler Toledo, Switzerland), and cell density (TruCell2^{TM}, Finesse Solutions, LLC, USA). Nitrogen gas was used at a flow rate of 0.3 L min^{-1} throughout the experiment to purge the bioreactor and keep it at anaerobic condition. A carbon dioxide analyzer

(Infors, Quebec, Canada) was connected to the outlet gas stream line. This system allowed online measurement of CO_2 content within the outlet gas stream. Temperature, gas flow rates (Red-y series flow controller, model GSC-C3SA-BB12, Vogtlin Instruments AG) and stirrer speed were regulated through control units (local loops). Iris software (Labfors, Infors, Quebec, Canada) was used to monitor and manage the process with good flexibility and total traceability. In addition to online determined parameters, samples were taken intermittently and filtered using 0.2 µm grade filters for solvents, glycerol and sugar analysis (HPLC, see section 6.2.3.9 for conditions).

6.2.9 Analytical methods

Bacterial growth was monitored by measuring the optical density at 600 nm using a 200 pro infinite series microplate reader (Tecan, Switzerland) using 96 well microplates at 200 µl per well. Concentrations of glycerol, sugars, and solvents produced in the fermentation were determined by high performance liquid chromatography on an Agilent 1260 infinity (Agilent USA, Santa Clara) using an Agilent Hi-plex H (7.7 × 300 mm) column (Agilent USA, Santa Clara) at 35°C. A refractive index detector was used for compound detection. 0.005 M H_2SO_4 was used as the isocratic mobile phase at a constant flow rate of 0.4 ml $min^{-1.}$ Before injection, samples were diluted to appropriate concentration with mobile phase and filtered through a 0.2µm membrane filter. The analytes were quantified using pure glycerol (BDH, Georgia, USA), inulin (Sigma Aldrich Co.), fructose, glucose, and sucrose (VWR Co.), butanol (Sigma-Aldrich, St. Louis, MO), 1, 3-propanediol (Sigma-Aldrich, Missouri, USA), and ethanol (Sigma-Aldrich, Missouri, USA) as standards.

Product yield was calculated as the total amount of butanol produced, divided by the amount of fermentable glycerol and sugar utilized and is expressed as $g_{butanol}\ g^{-1}$ $_{(glycerol+sugar).}$ In case of adding organic acids directly to the fermentation medium the butanol yield was calculated as the total amount of butanol produced, divided by the amount of fermentable glycerol utilized and organic acid added and is expressed as $g_{butanol}\ g^{-1}$ $_{(glycerol+added\ acid).}$ Productivity was calculated as the maximum butanol concentration achieved divided by the fermentation time and is expressed as $g\ L^{-1}\ hr^{-1}$.

6.3 Results and Discussion

The secondary carbon source for the proposed process are Jerusalem artichoke-derived carbohydrates. Monomeric sugars were produced through acid hydrolysis of Jerusalem artichoke tubers.

6.3.1 Acid hydrolysis of Jerusalem artichoke-derived inulin

The total solid content of Jerusalem artichoke tuber used in this study was about 30% of the fresh weight. Inulin, fructose, glucose, and sucrose composition of the material before and after acid hydrolysis are shown in Table 6-1. Samples for analysis were randomly taken from the available material and the small standard deviation indicates the compositional homogeneity of the tubers.

Table 6-1 Jerusalem artichoke carbohydrate composition (average of triplicates ± standard deviation) of raw material and water extract after acid hydrolysis (hydrolysate)

Compound	$g_{sugar}\ g^{-1}_{Jerusalem\ artichoke}$	
	Raw material	**Hydrolysate**
Inulin	0.52±0.05	0.008±0.003
Fructose	0.16±0.02	0.60±0.09
Glucose	0.10±0.01	0.15±0.06
Sucrose	0.05±0.008	0.072±0.003

6.3.2 Effect of acetic and butyric acid addition on butanol yield and productivity

The goal of this study was to increase butanol formation from glycerol through the co-metabolism of organic acids produced by the same organism from sugars. Therefore, initially the effect of acetic and butyric acid addition was investigated in batch cultures with a medium containing 50 g L^{-1} pure glycerol. Due to powerful odor, volatility and corrosive nature of these acids, butyrate and acetate were added in the form of sodium salts at concentrations of 1, 2, 3, 4, and 5 g L^{-1}. The initial pH of fermentation medium was

adjusted to 6.8 using NaOH but was not controlled thereafter. As a control, *C. pasteurianum* was first cultivated on pure glycerol as the sole carbon source. As shown in Fig. 6-1a, the addition of acetate improved the butanol production yield from 0.28±0.008 to 0.31±0.015 $g_{butanol}\ g^{-1}_{(glycerol+acetate)}$, as the acetate concentration added was increased from 0 to 5 g L^{-1}. However, the butanol production rate started to decrease from 0.36±0.006 to 0.32±0.008 g L^{-1} hr^{-1} as the acetate added was increased from 3 to 5 g L^{-1} (Fig. 6.1a). The results show that the addition of 3.0 g L^{-1} acetate improved the butanol production yield by 10.7% without decreasing butanol production rate. In another study, Chen and Blascheck (1999) investigated the effect of acetate addition on solvents production on a fermentation medium containing 60 g L^{-1} of glucose supplemented with 0, 0.3, 1.2, 2.4, 3.5, 4.7, and 5.9 g L^{-1} of acetate by *C. beijerinckii NCIMB 8052* and *C. beijerinckii BA 101* (Chen and Blaschek 1999). The results indicate that the addition of acetate could improve the butanol production, but for acetate addition greater than 4.7 g L^{-1} the butanol production started to decrease. It was also reported that the effect of acetate on increasing butanol production was correlated to the increase in the coenzyme-A transferase, an enzyme that plays a key role in the butanol pathway (Chen and Blaschek 1999).

As shown in Fig. 6-1b, the addition of butyrate improved the butanol production yield from 0.28±0.008 to 0.37±0.005 $g_{butanol}\ g^{-1}_{(glycerol+butyrate)}$, as the butyrate concentration added was increased from 0 to 5 g L^{-1}. More significantly, the addition of butyric acid had an influence on the 1,3-PDO formation and consequently on the butanol/1,3-PDO ratio (data not shown), as also reported elsewhere (Regestein et al. 2015; Johnson and Rehmann 2016). The concentration of 1,3-PDO decreased from 6.4±0.17 to 4.6±0.12 g L^{-1} as the butyrate concentration added was increased from 0 to 5 g $L^{-1.}$ The butanol production rate also started to decrease from 0.74 to 0.52 g L^{-1} hr^{-1} as the butyrate added was increased from 4 to 5 g L^{-1}. The results show that the addition of 4.0 g L^{-1} butyrate improved the butanol production yield to 0.36±0.004 $g_{butanol}\ g^{-1}_{(glycerol+butyrate)}$ (22%), without decreasing butanol production rate. In a closely related study, Kao et al. (2013) investigated the effect of butyrate addition using a fermentation medium containing 100 g L^{-1} of glycerol supplemented with 0, 2, 4, 6, 8, and 10 g L^{-1} of butyrate by *C. pasteurianum* CH4. The results show that the addition of butyrate could improve the butanol production yield but

any further addition of butyrate greater than 6 g L^{-1} decrease the butanol production rate. In another study, Regestein et al. (2015) studied the impact of butyric acid on butanol formation by *C. pasteurianum* DSM 525 under pH-controlled condition using 45 g L^{-1} glycerol supplemented with butyric acid. It was concluded that the addition of butyric acid could improve the butanol production yield in moderate amounts (3 g L^{-1}) without decreasing the production rate but elevated concentrations (> 4 g L^{-1}) decrease the metabolic rate of the organisms and result in a prolonged lag-phase while the butanol yield is increased. The result obtained in this work is in agreement with the results of both studies. Therefore, this study confirms that acetate and butyrate addition especially butyrate is beneficial to butanol production with *C. pasteurianum* DSM 525 using pure glycerol as carbon source.

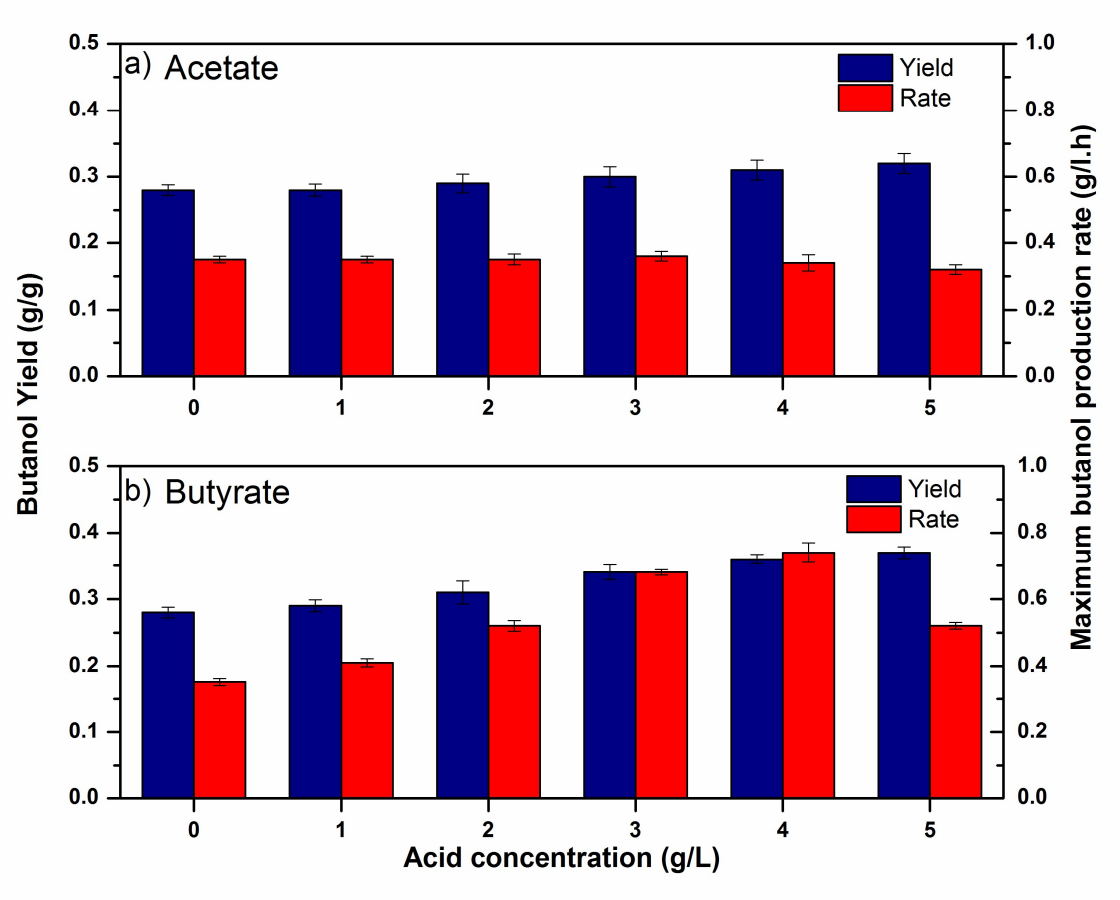

Figure 6-1 The effect of acetate and butyrate addition on butanol yield and productivity. The values are average of triplicate measurements ± standard deviation.

6.3.3 Product formation by *C. pasteurianum* DSM 525 using different substrates

The product formation by *C. pasteurianum* was studied using pure glycerol, crude glycerol, fructose, glucose, fructose & glucose (same ratio as in JAH), and JAH as the sole carbon sources. The fermentations were performed in a batch culture with a medium containing 50 g L^{-1} of each substrate under optimal fermentation conditions (Sarchami et al. 2016). The initial pH of fermentation medium was adjusted to 6.8 using NaOH but was not controlled thereafter. As shown in Fig. 6-2, the major products of *C. pasteurianum* utilizing both pure and crude glycerol as substrate are butanol and 1,3-PDO. However, when this

strain is cultivated on sugar (fructose, glucose, fructose & glucose, JAH) it produces mostly acetic and butyric acid. The highest butyric acid concentration was achieved using glucose as substrate, whereas using JAH resulted in the highest acetic acid titer (Fig. 6-2a). The highest butanol and 1,3-PDO concentrations were obtained using pure glycerol as substrate, however, 1,3-PDO concentration decreased significantly using crude glycerol (from 6.4 g L^{-1} to 4.4 g L^{-1}) and no 1,3-PDO was produced when this bacteria was cultivated on sugar (Fig. 6-2b). These results indicate that JAH (sugar source) can be utilized by *C. pasteurianum* to appropriately produce acids from sugar source to serve as the precursor to stimulate the subsequent butanol production from glycerol.

The measured values obtained from pure glycerol, crude glycerol, and glucose fermentation are in agreement with values typically found for these feedstocks fermented with *C. pasteurianum* DSM 525 (Sabra et al. 2014; Sarchami et al. 2016). To the best knowledge of the authors, this is the first attempt to use fructose, mixture of fructose and glucose, and Jerusalem artichoke hydrolysate as a substrate for fermentative production by *C. pasteurianum* DSM 525.

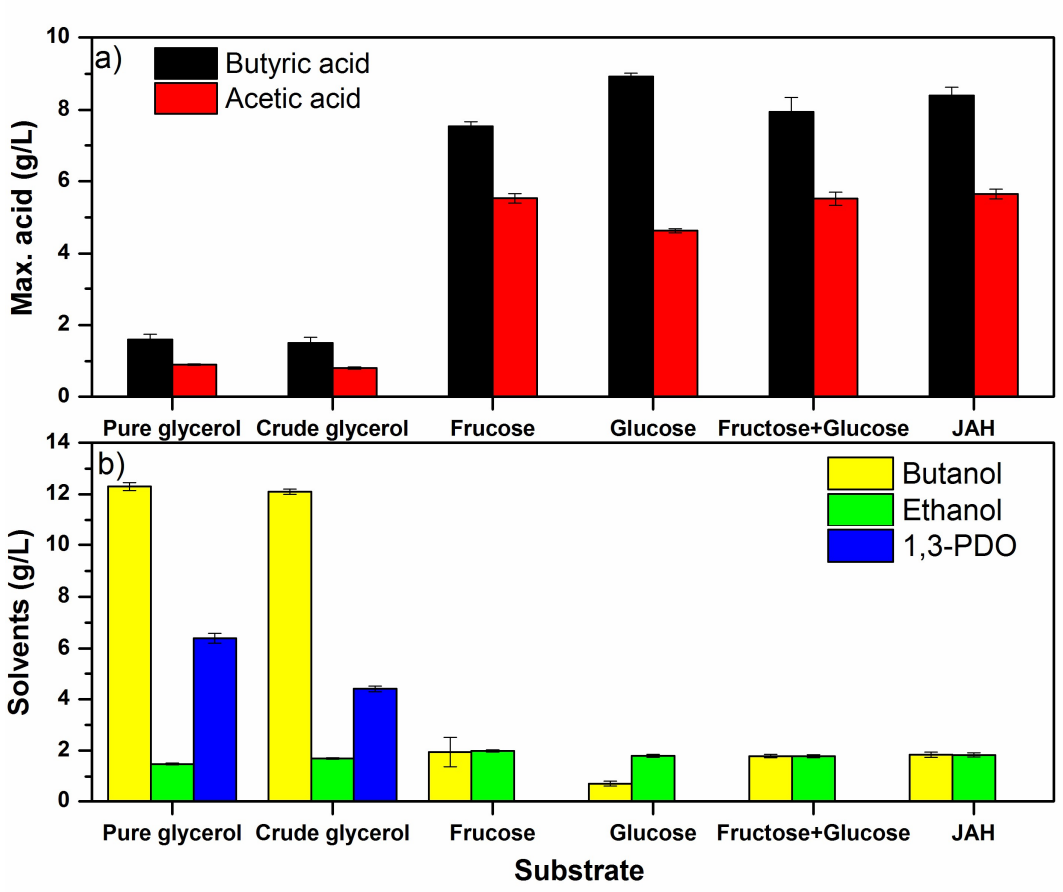

Figure 6-2 Product formation by *C. pasteurianum* DSM 525 using different substrates. The values are average of triplicate measurements ± standard deviation.

6.3.4 Optimization of glycerol and sugar concentration in co-substrate strategy

The effect of acetic and butyric acid addition on butanol fermentation was investigated above, showing that direct addition of acetate and butyrate into the fermentation broth enhances butanol yield and productivity (Figure 6-1), but suffers the drawback of the high cost of acetate and butyrate. The results shown in Figure 6-2 indicate the suitability of JAH as a sugar source to be fermented by *C. pasteuriaunm* DSM 525 to produce acetic and butyric acid. Therefore, as already mentioned, JAH (sugar source) and crude glycerol can be fermented at the same time (co-substrate strategy) to conduct acetate and butyrate formation and butanol fermentation simultaneously. To prevent cell growth inhibition due to high acid concentration arising from a high sugar concentration and to enhance butanol

production yield without decreasing butanol production rate, the initial glycerol and sugar concentration should be optimized to maximize butanol yield and productivity.

All optimization studies were performed using pure glycerol and a synthetic media simulating JAH at optimal butanol fermentation condition (Sarchami et al. 2016). Experimental conditions were chosen based on a central composite design and the actual values of the independent variables and the measured responses are shown in Table 6-2. All experiments were carried out in triplicate and averages ± standard deviation are presented in the table.

Table 6-2 Butanol yield and productivity (average of triplicates ± standard deviation) under conditions determined for CCD.

Glycerol Concentration ($g\ L^{-1}$)	**Sugar Concentration ($g\ L^{-1}$)**	**Butanol Yield ($\%g_{butanol}\ g^{-1}_{(glycerol+sugar)}$)**	**Butanol Productivity ($g\ L^{-1}\ hr^{-1}$)**
23.60	15	18.6±0.2	0.4±0.03
30	5	23.3±0.8	0.58±0.009
30	25	19.4±0.4	0.49±0.02
50	1.80	25.3±0.3	0.68±0.03
50	15	27.3±0.2	0.74±0.03
50	15	26.8±0.2	0.74±0.03
50	15	26.9±0.2	0.74±0.03
50	15	27.1±0.2	0.74±0.03
50	15	26.8±0.2	0.74±0.03
50	28.20	24.1±0.3	0.54±0.02
70	5	24.3±0.2	0.56±0.02
70	25	23.8±0.2	0.55±0.006
76.4	15	23.3±0.4	0.54±0.01

6.3.5 Response surface model validation

As observed from the experimental results in Table 6-2, the fermentation process was successfully producing butanol from pure glycerol and a synthetic media simulating JAH as co-substrate, within the ranges of the selected input variables. The complete dataset could be fitted with a quadratic model for both butanol yield and productivity as describe in equation (6-1). The resulting model parameters are shown in Table 6-3. The *F* values of the models are 57.8 and 41.4 for butanol yield and productivity, respectively, which is very high compared to the critical value, indicating that both models are highly significant. The significance of each parameter coefficient was determined by *P* values, the smaller the *P* values the more significance of the coefficient. In this case, both factors have great effect on butanol yield and productivity. The goodness of fit of the models was confirmed by the coefficient of determination R^2 and adjusted determination coefficient Adj. R^2 as shown on Table 6.3.

Based on the selected significant variables, the quadratic model for the butanol yield and productivity in terms of actual factors are shown as follows:

Butanol Yield = 0.016420 + 0.001236 * Sugar concentration + 0.0092511 * Glycerol concentration + 0.0000375 * Sugar concentration * Glycerol concentration – 0.00013 * Sugar concentration^2 – 0.00009 * Glycerol concentration^2 (6-2) (1)

Butanol Productivity = -0.2567 + 0.010630 * Sugar concentration + 0.03629 * Glycerol concentration – 0.00064 * Sugar concentration^2 – 0.00036 * Glycerol concentration^2 (6-3)

The residuals can be judged as normally distributed based on a normal probability (data not shown).

Table 6-3 Analysis of variance of fitted model for butanol yield and productivity

Response	Source	Remark	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value Prob>*F*
Yield	Model	Significant	0.011	5	0.0023	57.84	<0.0001
Productivity		Significant	0.17	5	0.034	41.4	<0.0001
Yield	Glycerol	Significant	0.00053	1	0.0005	13.4	0.0080
Productivity	concentration	Significant	0.011	1	0.011	13.07	0.0086
	(A)						
Yield	Sugar	Significant	0.0018	1	0.0018	42.23	0.0003
Productivity	concentration	Significant	0.0067	1	0.0067	8.14	0.0246
	(B)						
Yield	AB	Significant	0.0002	1	0.0002	5.66	0.0489
Productivity		Not-Significant	0.0016	1	0.0016	1.93	0.2073
Yield	A^2	Significant	0.0010	1	0.0010	26.46	0.0013
Productivity		Significant	0.025	1	0.025	30.62	0.0009
Yield	B^2	Significant	0.0080	1	0.0080	198.89	<0.0001
Productivity		Significant	0.013	1	0.13	153.76	<0.0001
Yield	R-Squared	Significant					0.98
Productivity		Significant					0.97
Yield	Adj-Squared	Significant					0.96
Productivity		Significant					0.94
Yield	Adeq	Significant					18.85
Productivity	Precision	Significant					15.27

6.3.6 Combined effect of glycerol concentration and sugar concentration

Response surface methodology was used to study the interaction effects of the two factors. The surface plots of the combined effect of glycerol concentration and sugar concentration

on butanol yield and productivity are shown in Fig. 6-3a and 3b, respectively. The butanol yield and productivity are both a function of glycerol concentration and sugar concentration. The plots clearly indicate that an optimum exists within the observed design space for both responses.

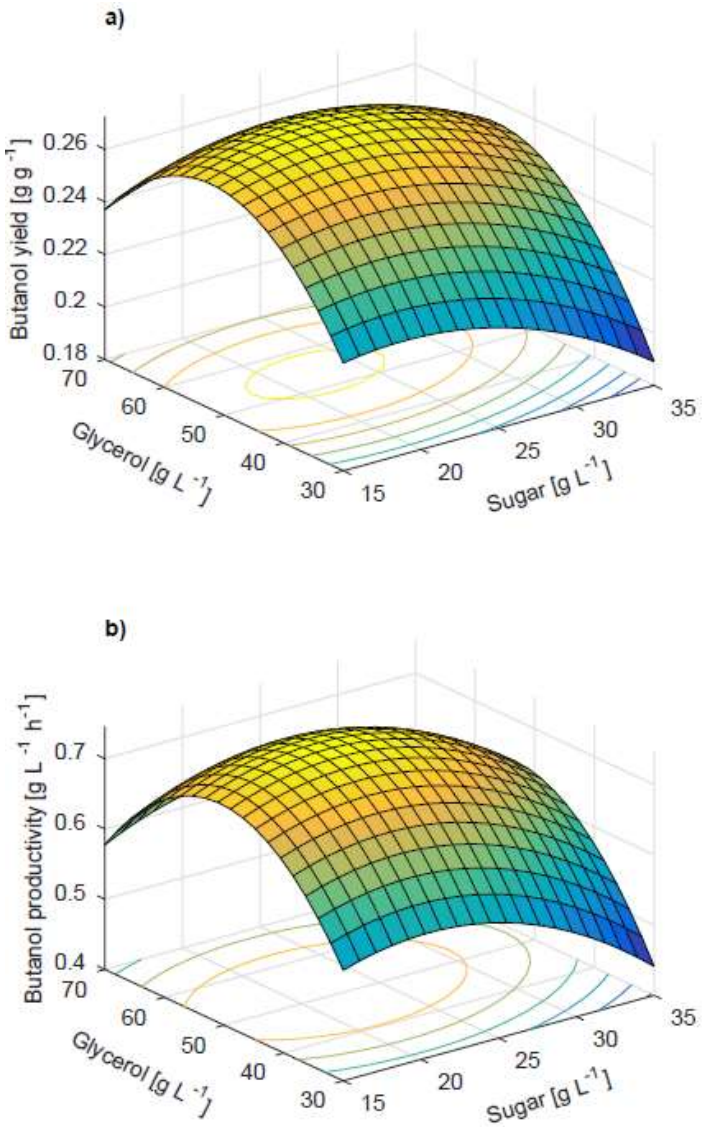

Figure 6-3 Surface plot of combined effect of glycerol concentration and sugar concentration on a) butanol yield, b) butanol productivity.

6.3.7 Response optimization and model validation

Based on the both models, numerical optimization was used to determine the optimal combination of process parameters for maximum butanol yield and productivity. The optimal conditions for butanol production yield and productivity were glycerol concentration of 50 g L^{-1} and sugar concentration of 15 g L^{-1}. To validate the applicability of this RSM model, some confirming experiments were carried out around the estimated optimal conditions. The measured and predicted butanol yields and productivity of three conditions around the optimum are listed in Table 6-4. The predicted results were compared with the actual values obtained experimentally. *T* test at 95% confidence showed no significant difference between the predicted and actual values. In summary, the proposed RSM models could be useful for the prediction of maximum butanol production yield and productivity.

Table 6-4 Optimal conditions and model validation

Glycerol Concentration (g L^{-1})	Sugar Concentration (g L^{-1})	Butanol Yield ($g^{-1}_{butanol}$ $g^{-1}_{(glycerol+sugar)}$)		Butanol Productivity (g L^{-1} hr^{-1})	
		Predicted	Experimental	Predicted	Experimental
53	13	0.273±0.005	0.268±0.004	0.72±0.03	0.71±0.04
54	15	0.274±0.006	0.271±0.004	0.74±0.008	0.74±0.01
50	11	0.262±0.009	0.269±0.005	0.71±0.014	0.70±0.012

6.3.8 Co-substrate fermentation using Jerusalem artichoke hydrolysate and crude glycerol as feedstock

The conditions estimated through the optimization experiments were used to conduct fermentation with JA acid hydrolysate and crude glycerol, obtained from the waste of the biodiesel manufacturing process. The fermentation was initially performed at the same

scale that was used during the optimization experiments and compared to a co-substrate fermentation using pure substrates as shown in Fig. 6-4. For the pure substrates the culture started to use sugars and glycerol directly after the inoculation and all the sugar were utilized by the culture within 15 hours. However, a slight lag phase was observed in glycerol and sugar consumption, as well as organic acids and solvents production using JAH and crude glycerol as substrate compared to control experiment (Fig. 6-4c-d). On the pure substrates the *C. pasteurianum* DSM 525 culture produced 14.2±0.4 g $L^{-1}$of butanol, 3.9±0.15 g L^{-1} of 1, 3-PDO, and 2.95±0.18 g $L^{-1}$of ethanol at the end of fermentation, as shown in Fig. 6-4c. The butanol yield and overall productivity were 0.27±0.01 $g_{butanol}$ $g^{-1}_{(glycerol+butyrate)}$ and 0.74±0.02 g L^{-1} hr^{-1}, respectively. At the end of fermentation, the acid concentration was 0.52±0.04 g L^{-1} for butyrate, 2.47±0.02 g L^{-1} for acetate (Fig. 6-4d). Acetate did not appear to be used by *C. pasteuriaunm* DSM 525 in co-substrate fermentation. Fermentation with Jerusalem artichoke hydrolysate and crude glycerol showed identical butanol production yield (within error) compared to using pure substrates. However, butanol productivity decreased from 0.74 to 0.56 g L^{-1} hr^{-1} using crude glycerol and JAH as co-substrate. This deviation can be potentially explained by longer lag phase microorganism needs to take up crude glycerol and JAH as substrate. These results clearly demonstrate the feasibility of using Jerusalem artichoke hydrolysate and crude glycerol as low cost carbon sources to enhance butanol production. To the best knowledge of authors, this the first attempt to use Jerusalem artichoke hydrolysate and crude glycerol as carbon sources for fermentative butanol production in a co-substrate system.

In another study, Kao et al. (2013) investigated the optimal glucose to pure glycerol ratio (20:60 g L^{-1}) for the strain *C. pateurianum* CH4 (an isolate from anaerobic sludge. The simultaneous co-substrate strategy obtained a butanol titer, an overall productivity and a yield of 13.2 g L^{-1}, 0.19 g L^{-1} hr^{-1}, and 0.21 $g_{butanol}$ $g^{-1}_{(glycerol+glucose)}$, respectively, whereas using pure glycerol as the only carbon source resulted in a butanol titer, an overall productivity and a yield of 11.5 g L^{-1}, 0.13 g L^{-1} hr^{-1}, and 0.16 $g_{butanol}$ $g^{-1}_{(glycerol+glucose)}$ respectively. Moreover, bagasse and crude glycerol as co-substrates were also converted into butanol with a butanol concentration, an overall productivity and a yield of 11.8 g L^{-1}, 0.14 g L^{-1} hr^{-1}, and 0.19 $g_{butanol}$ $g^{-1}_{(glycerol+glucose)}$, respectively, with a fermentation time

of 4 to 5 days (96 to 120 hours), substantially longer than the 35 hours used in this study. Higher fermentation temperature and iron limitation condition of Kao et al.'s (2013) work may explain its lower butanol yield and productivity. It has been reported that the optimal fermentation temperature for butanol production by *C. pateurianum* is 30ºC (Khanna et al. 2013b; Sarchami et al. 2016) and iron limitation condition enhance 1,3-PDO production over butanol (Dabrock et al. 1992). Additional deviation can be potentially explained by strain characteristics of the *Clostridia* (CH4 vs. DSM 525). In a closely related study, Sabra et al. (2014) also reported that using pure glycerol and glucose as carbon source (wt. ratio 1:1) fermented by *C. pateurianum* DSM 525, butanol concentration, overall productivity and yield achieved were 21.1 g L^{-1}, 0.69 g L^{-1} hr^{-1}, and 0.23 $g_{butanol}$ $g^{-1}_{(glycerol+glucose)}$, respectively. This is one of the highest reported butanol titers in conventional batch fermentation since the product butanol itself is toxic to the *Clostridia spp.* and thus with the wild-type strain of *C. pasteurianum*, a maximum concentration of butanol exceeding 17 g L^{-1} is rarely achieved. Furthermore, biomass hydrolysate and pure glycerol as co-substrates were also fermented into butanol and a butanol concentration, an overall productivity and a yield of 17.4 g L^{-1}, 0.62 g L^{-1} hr^{-1}, and 0.2 $g_{butanol}$ $g^{-1}_{(glycerol+glucose)}$ were achieved, respectively, with a fermentation time of 50 hours. Higher fermentation temperature and lower glycerol to glucose ratio (1:1, 50 g L^{-1} glycerol+50 g L^{-1} glucose) of Sabra et al.'s (2014) work may explain its lower butanol yield and productivity compared to the results obtained in this study. Higher butanol yield (0.27 g L^{-1} vs. 0.2 g L^{-1}), higher butanol productivity (0.74 g L^{-1} hr^{-1}vs. 0.69 g L^{-1} hr^{-1}), shorter fermentation time (35 hours vs. 50 hours) of this study make it more industrially advantageous. Utilizing crude glycerol instead of pure glycerol in a co-substrate strategy is also a more relevant carbon source.

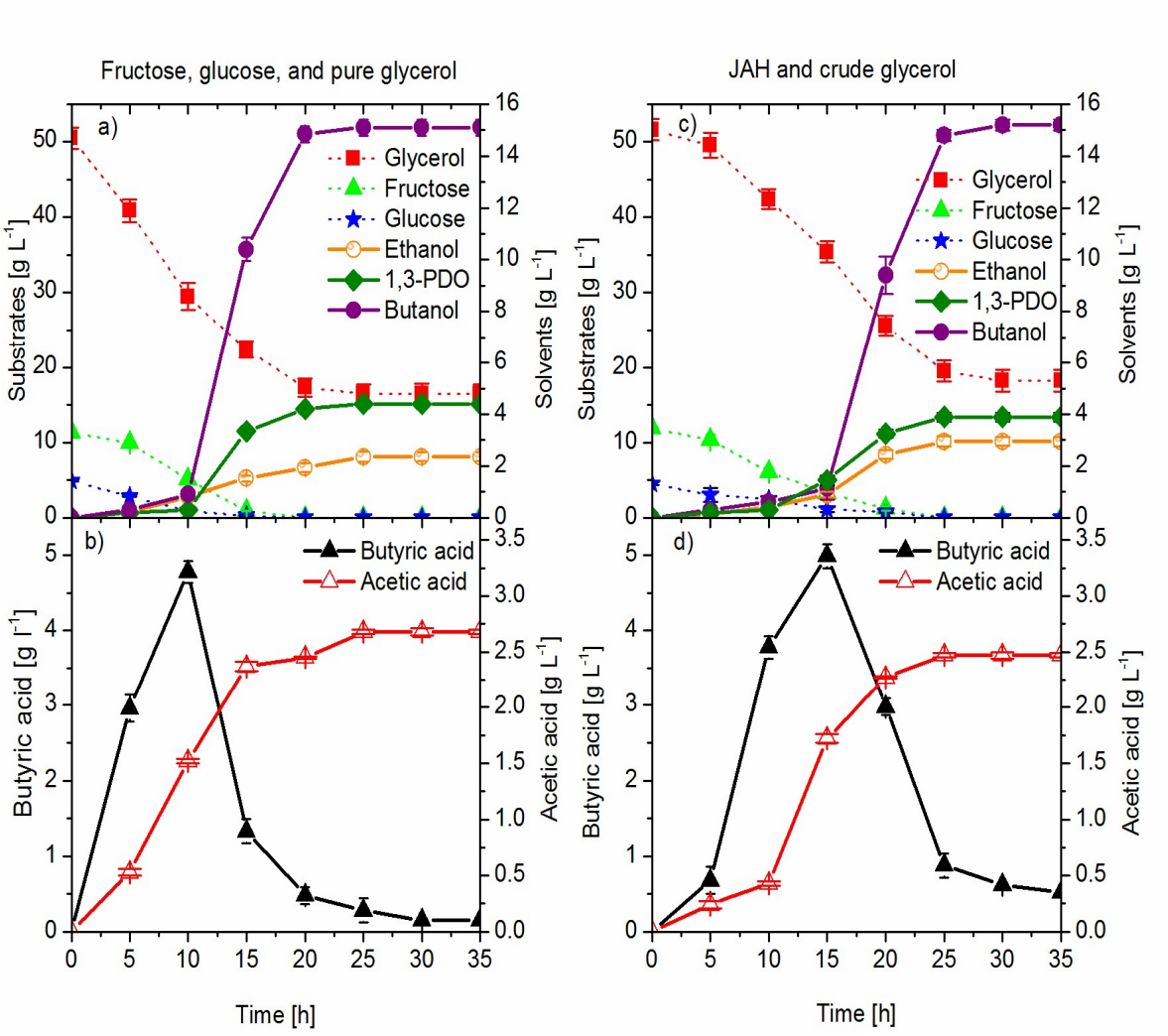

Figure 6-4 Profile of substrate utilization, solvent production, and organic acids production using fructose, glucose, and pure glycerol as feedstock in a) and b), using Jerusalem artichoke hydrolysate and crude glycerol in c) and d), under optimal fermentation and co-substrate condition by *Clostridium pasterianum* DSM 525

6.3.9 Butanol production from Jerusalem artichoke hydrolysate and crude glycerol in a lab scale bioreactor

To verify the validity of co-substrate fermentation method by *Clostridium pasterianum* DSM 525 in larger scale, fermentations were carried out in a 5 L bench-top bioreactor using

Jerusalem artichoke hydrolysate and crude glycerol as feedstock and the results are presented in Fig. 6-5.

Butanol production was performed by using 16 hr-old seed culture and starting the fermentation with an initial cell density of 0.4 g L^{-1}_{CDW}. The fermentation temperature was controlled at 30°C. In addition to offline determined substrate and product concentrations, pH, CO_2 formation, and cell density were measured online. A butanol yield and productivity of 0.28±0.007 $g_{butanol}$ $g^{-1}_{(glycerol+sugar)}$ and 0.55±0.008 g L^{-1} hr^{-1} were achieved after 27 hours fermentation using co-substrate strategy. The fermentation was judged complete after 27 hours based on the online signals of CO_2 formation and CDW. The CO_2 signal is a result of biological CO_2 formation but is also influenced by the CO_2 release of the $CaCO_3$ buffer with decreasing pH (Fig. 6-5b). Therefore, the absolute value does not represent the overall biological activity. The pH of the fermentation medium was initially adjusted to 6.8 (optimal pH) but not controlled thereafter and the buffering capacity of the medium is clearly not sufficient to maintain a constant pH, as shown in Fig. 6-5b, the pH continuously decreased until it reached 5.01. The cell growth curve (continuously measure via online turbidity probe) follows the typical profile of a short lag phase followed by exponential phase with a higher cell dry weight (Fig. 6-5b).

At optimized conditions the butanol production was equal (within error) in anaerobic shake flasks and in the 5 L bioreactor, justifying the necessary scale-down for the fermentation and optimization studies.

The experimental work in the study largely focuses on the co-substrate fermentation using Jerusalem artichoke tubers and crude glycerol as potential feedstocks for butanol production. The setup used in this study is not intended to represent a potential industrial process. More advanced fermentation process design, possibly including continuous fermentation and/or in-situ product removal, would likely have to be used in an industrial process, as evaluated for different feedstocks elsewhere (Lee et al. 2008; Napoli et al. 2011).

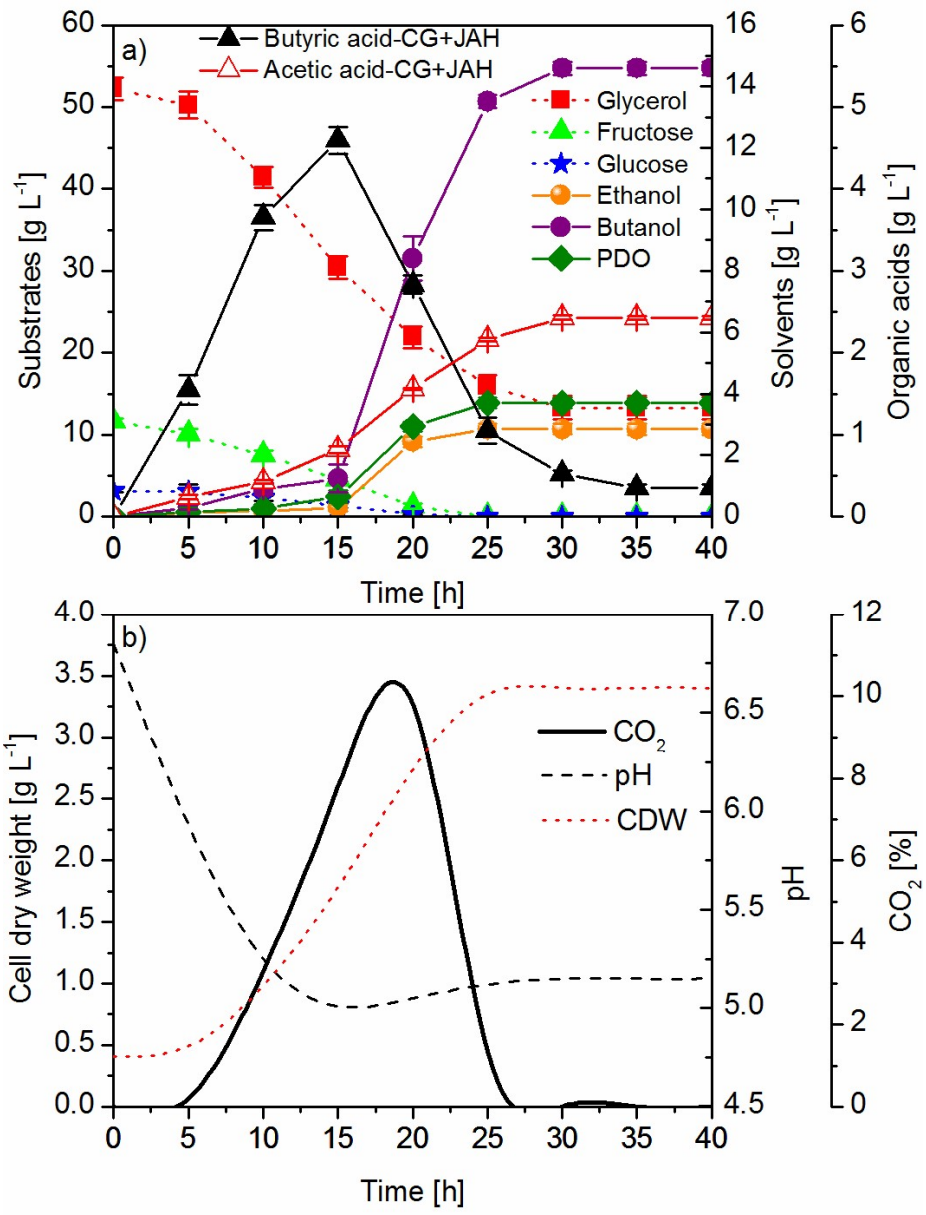

Figure 6-5 Profile of substrate utilization, solvent, and organic acids production using Jerusalem artichoke hydrolysate and crude glycerol (a), CO_2 formation pH and cell dry weight formation (b), under optimized fermentation conditions and glycerol-to-sugar concentration by *Clostridium pasterianum* DSM 525. The discrete data points (a) are average of triplicate measurements ± standard deviation, the connecting lines are for visualization purposes only. The smooth lines in (b) are the results of online measurements.

6.4 Conclusions

Butanol production by *C. pasteurianum* DSM 525 from glycerol was significantly enhanced by adding organic acids especially butyric acid directly to the fermentation medium. These organic acids can be directly produced by *C. pasteurianum* DSM 525 through the conversion of sugars. A co-substrate system was characterized and optimized for compounds and could directly be transferred to the relevant carbon sources of crude glycerol and JA hydrolysate. Under optimal condition (glycerol concentration of 50 g L^{-1} and sugar concentration of 15 g L^{-1}) a butanol yield and overall productivity of 0.27 $g_{butanol}$ $g^{-1}_{(glycerol+sugar)}$ and 0.74 g L^{-1} hr^{-1} was obtain, respectively, and the results could successfully be scaled to a 5L bench top bioreactor. The system is a potential way to utilize an industrial waste stream and a dedicated energy crop for the efficient production of an advanced biofuel.

6.5 References

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Chapter 7

7 Enhanced butanol production in the integrated fed-batch fermentation process with pervaporation

Tahereh Sarchami, Erin Johnson, Sascha Kießlich, and Lars Rehmann.

Preface

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is ready for submission.

The microbial butanol production from the co-substrate system developed in previous chapter is limited by butanol toxicity to the microbial culture. In order to address toxicity problem, pervaporation has been widely used as an efficient technique for in-situ butanol recovery (Qureshi and Blaschek 1999; Wu and Liu 2012; Ikegami et al. 2014; Qureshi et al. 2014; Rozicka et al. 2014; Shin et al. 2015). However, to the authors' knowledge there are no reports on integrated PBE fermentation with pervaporation. Therefore, more research is needed to investigation the effect of the by-products in PBE fermentation broth, more specifically the effect of glycerol and 1,3-PDO on the performance and efficiency of pervaporation.

In this study, the effectiveness of Pervap 4060 membrane for selective separation and concentration of butanol from binary butanol/water solution was first investigated and confirmed. The intrinsic membrane properties in terms of permeance and selectivity coefficient of binary solution revealed that the investigated membrane is selective toward butanol. The effects of different fermentation by-products and hydrocarbon sources of PBE fermentation on pervaporation performance were the investigated using a novenary mixture. Glycerol, glucose, fructose, acetic acid, and 1,3-PDO did not cross the membrane. Based on the fluxes of organic compounds, butanol was the most efficiently transported compound due to its high vapour pressure.

Finally, pervaporation was directly integrated with fed-batch fermentations of *Clostridium pasteurianum* to remove butanol from fermentation broths and to increase the productivity and efficiency. Enhanced overall butanol productivity was achieved in the integrated process. Pervaporation was able to efficiently recover butanol from fermentation broth and relieve the inhibition caused by butanol. Concentrated substrate feeding was possible in the fed-batch PBE fermentation when coupled with online butanol removal, increasing the volumetric productivity. More efficient substrate conversion was achieved in the integrated process than the non-integrated process due to relieved inhibition and stress on the bacteria.

Abstract

Butanol has the potential to become an important renewable transportation fuel and feedstock chemical in the future. However, product inhibition and low productivity are the main obstacles in feasible, industrial-scale, fermentative butanol production. Both problems can be overcome by using pervaporation as an in-situ product removal technique. In this work, the performance and suitability of the PDMS-based membrane Pervap 4060, was investigated for butanol separation in contact with binary butanol/water solutions followed by experiments with aqueous novenary mixtures. Results obtained indicated that the tested membrane has potential to be used in the butanol fermentation process. Therefore, pervaporation was directly integrated with fed-batch 1,3-propanediol butanol ethanol (PBE) fermentation. Membrane performance and its effect on the fed-batch PBE fermentation process were assessed by measuring flux, separation factor, concentrations of different components in the feed and permeate, and cell density as a function of time. Volumetric butanol productivity increased from 0.55 g L^{-1} hr^{-1} in simple batch fermentation to 0.60 g L^{-1} hr^{-1} in the case of pervaporative fermentation. Overall, total butanol production improved by a factor of 2.6, viable fermentation time increased by a factor of 2.2, and cell density increased by a factor of 1.3 upon applying pervaporation.

7.1 Introduction

Butanol is an important solvent with many applications in the chemical industry. Butanol contains more energy than ethanol, and is less hygroscopic. Hence it can be easily mixed with gasoline in any proportion (Liu et al. 2011; Ikegami et al. 2014), and is therefore regarded as a suitable biofuel (Wu and Liu 2012; Shin et al. 2015). Butanol can be produced through fermentation from renewable resources by *Clostridia* spp., which has received increased attention in recent years (Niemisto et al. 2013; Ikegami et al. 2014; Rozicka et al. 2014).

In order to achieve sustainable butanol production, the availability of renewable and cost-effective raw material is essential (Qureshi et al. 2008; Gao et al. 2014; Qureshi et al. 2014; Luque et al. 2014). An enhanced butanol production has been reported recently using Jerusalem artichoke hydrolysate and crude glycerol simultaneously as low-cost and available feedstocks (Sarchami and Rehmann 2016b). This co-substrate system is a potential way to utilize an industrial waste stream and a dedicated energy crop for the efficient production of an advanced biofuel. *Clostridium pasteurianum* utilized those two carbon sources to produce butanol, 1,3-propanediol (1,3-PDO), and ethanol while no acetone is formed.

However, microbial butanol production from renewable sources such as the aforementioned co-substrate system is limited by its toxicity to the microbial cultures. Due to product toxicity, butanol concentration in the fermentation broth in excess of 10-20 g L^{-1} is rarely achieved which results in low butanol productivity and high product removal costs (Johnson et al. 2016; Sarchami et al. 2016a). In order to address toxicity problem, a number of novel product recovery techniques have been developed including gas stripping, vacuum stripping, pervaporation, liquid-liquid extraction, perstraction, and adsorption (Qureshi et al. 2005; Ha et al. 2010; Mariano et al. 2011; Mariano et al. 2012; Abdehagh et al. 2014; Errico et al. 2016).

Among these techniques, pervaporation (PV) has been widely reported as an efficient butanol recovery method (Yen et al. 2012a; Yen et al. 2012b; Shin et al. 2015; Wu et al.

2015; Kong et al. 2016). Low energy consumption and no solvents requirements make pervaporation a green process which has no harmful effect on the involved microorganisms (Sarchami et al. 2016a). Many studies have been reported in the literature investigating the ABE fermentation process being integrated with pervaporation (Qureshi and Blaschek 1999; Wu and Liu 2012; Ikegami et al. 2014; Qureshi et al. 2014; Rozicka et al. 2014; Shin et al. 2015). However, to the authors' knowledge there are no reports on integrated PBE fermentation with pervaporation. Therefore, more research is needed to investigation the effect of the by-products in PBE fermentation broth, more specifically the effect of glycerol and 1,3-PDO on the performance and efficiency of pervaporation.

The purpose of this study is therefore threefold, 1) to investigate and confirm the effectiveness of pervaporation of PDMS-based membrane, namely Pervap 4060, for selective separation and concentration of butanol from binary butanol/water solution, 2) to elucidate the effects of different fermentation by-products and hydrocarbon sources of PBE fermentation on pervaporation performance using a novenary mixture, and 3) to directly integrate pervaporation with fed-batch fermentations of *Clostridium pasteurianum* to remove butanol from fermentation broths and to increase the efficiency of butanol production.

7.2 Materials and Methods

7.2.1 Chemicals

See Chapter 5. Section 2.2

7.2.2 Membrane

Commercial dense flat-sheet polymeric thin film composite membranes were purchased from Sulzer Chemtech (Switzerland) with the trade name Pervap 4060. The Pervap 4060 consists of a thin separation layer (PDMS, 6 μm) on top of a porous support layer (70 –100 μm), coated on a mechanical support layer (100 –150 μm).

7.2.3 Preparation of binary and novenary mixture

The binary solution used in the first set of experiments consisted of water and butanol with initial butanol concentrations of about 20 g L^{-1}. The novenary mixture contained (initial concentration of components per liter of water): 50 g glycerol, 10 g fructose, 5 g glucose, 5 g acetic acid, 5 g butyric acid, 10 g ethanol, 15 g 1,3-PDO, 15 g butanol to mimic the concentration range relevant for the fed-batch PBE fermentation process. In preparation of novenary mixture organic compounds were weighed and mixed together before adding them into distilled water. Solutions were blended with a magnetic stirrer over night to ensure proper mixing and heated to 35ºC before being used in pervaporation studies.

7.2.4 Crude glycerol preparation

Crude glycerol was prepared for fermentation following the protocol described in Chapter 5. Section 2.1

7.2.5 Jerusalem artichoke preparation

Jerusalem artichoke flour preparation, inulin extraction, and acid hydrolysis of extracted inulin were performed following the protocol described in Chapter 3. Section 2.1.1 to 2.1.3

7.2.6 General microbiological conditions

Except for the bioreactor studies, all microbiological work was performed in an anaerobic chamber using aseptic techniques (Model 855-ACB, Plas Labs, Lansing, MI).

7.2.7 Strain and maintenance

Microorganism and cell culture condition previously described in Chapter 5. Section 2.4

7.2.8 Pervaporation experiments

Pervaporation experiments were conducted using a cross-flow membrane unit with a stainless steel test cell. The Pervap 4060 membrane with an effective area of 170 cm^2 was placed on a porous sintered support of the test cell and sealed with an O-ring. The volume of the feed tank was 1 L and the feed solution volume of 1 L was used with all the experiments (binary solution and model solution). Feed solutions were kept at constant

temperature of 35ºC using a stirring hotplate. The feed solution was circulated continuously through the membrane module by a peristaltic metering pump (Flex-Pro Norprene, model A4F24-MNHH, Blue-White Ind., Huntington Beach, USA) with a flow rate of 4.0 L min^{-1}. The pressure difference acting as the driving force for the system was enabled by keeping the pressure in the permeate side below 100 Pa (1mbar) with a vacuum pump (RV5, Edwards, Crawley, UK) while the feed side was under atmospheric pressure during the experiments. The system obtained isothermal conditions after 15 minutes from the beginning of the experiment and the permeate stream was condensed in a cold trap using dry ice/ethanol at -70ºC. Two parallel cold traps were used in this study which allowed a continuous work of the system. The permeate was collected in one cold trap for 30 minutes before switching to another one. The permeate phase separates into a butanol-rich phase and a water-rich phase. After measuring the mass, the permeate was diluted with water to form a single phase solution. The compositions of feed and permeate streams were monitored by HPLC as a function of time (See section 7.2.11 for conditions). Fig. 7-1 shows a schematic of the pervaporation setup used in this study to separate butanol from feed solutions.

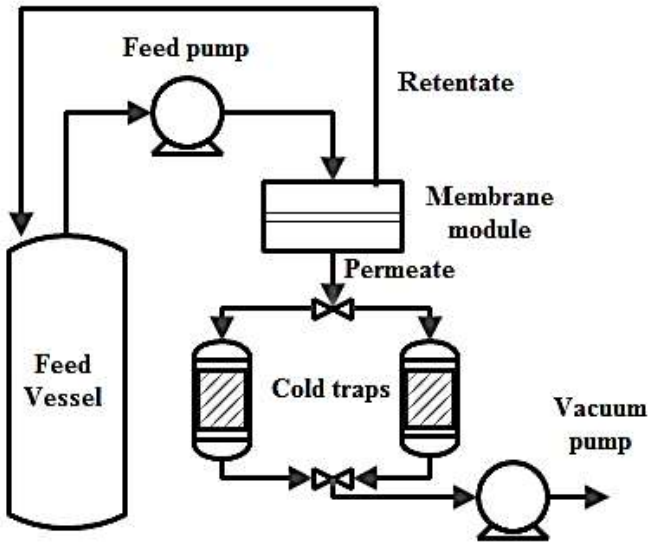

Figure 7-1 Schematic diagram of pervaporation set up

7.2.9 Calculations

The obtained data were used for characterization of the membrane performance. Equations used in this work for the determination of partial fluxe (J_i) and separation factor (β_i) are shown below:

$$J_i = \frac{m_i}{At} \quad (7\text{-}1)$$

$$\beta_i = \frac{y_i/(1-y_i)}{x_i/(1-x_i)} \quad (7\text{-}2)$$

Where m_i is the weight of the compound i in the permeate (g), A is the effective area of the membrane (m^2), t is the time of permeation (h), y_i and x_i are the mass fractions of the compound i in the permeate and feed, respectively. Based on the solution–diffusion model, the pervaporation transportation equation for a compound i through the membrane can be expressed as follows:

$$J_i = PM_i(p_{i,f} - p_{i,p}) = [\frac{p_i}{l}](p_{i,f} - p_{i,p}) \quad (7\text{-}3)$$

Where PM_i is the membrane permeance (g m^{-2} h^{-1} kPa^{-1}), obtained by the membrane permeability (p_i) divided by the membrane thickness l (m), $p_{i,f}$ and $p_{i,p}$ are the partial vapor pressures (kPa) of component i in the feed and permeate, respectively. Pressures for the compound i in the feed and permeate sides can be determined by the Raoult's law:

$$p_{i,f} = \gamma_i X_{i,f} p_i^{sat} \quad (7\text{-}4)$$

$$p_{i,p} = Y_i p^p \quad (7\text{-}5)$$

Where γ_i is the activity coefficient and X_i is the mole fraction of the compound i in the feed, p_i^{sat} is the saturated vapor pressure of the pure compound i at given temperature (35ºC). Y_i is the mole fraction of the compound i in the permeate and p^p is the permeate pressure. By rearrangement of the equations above, the membrane permeance can be determined as:

$$\left[\frac{p_i}{l}\right] = \frac{J_i}{X_i \gamma_i p_i^{sat} - Y_i p^p} \quad (7\text{-}6)$$

Permeate pressure (p^p) during pervaporation experiments is usually very low, therefore the term ($Y_i p^p$) in Eq. (6-7) can be neglected and the latter equation can be rewritten as follows:

$$\left[\frac{p_i}{l}\right] = \frac{J_i}{X_i \gamma_i p_i^{sat}} \quad (7\text{-}7)$$

The saturated vapor pressure p_i^{sat} of the pure component i at a given temperature can be determined with the Antoine equation:

$$\log p_i^{sat} = A - \frac{B}{T + C - 273.15} \quad (7\text{-}8)$$

Where A, B and C are the component specific Antoine constants and T(K) is the temperature.

The activity coefficient γ_i for binary systems can be calculated using the Non-Random Two-Liquid (NRTL) method and the following equations:

$$\ln \gamma_i = X_j^2 \left[\tau_{ji} \left(\frac{G_{ji}}{X_i + X_j G_{ji}}\right)^2 + \left(\frac{\tau_{ij} G_{ji}}{(X_j + X_i G_{ij})^2}\right)\right] \quad (7\text{-}9)$$

$$\tau_{ij} = \frac{g_{ij} - g_{jj}}{RT} = A_{ij} + \frac{B_{ij}}{T} \quad (7\text{-}10)$$

$$G_{ij} = \exp(-o_{ij} \tau_{ij}) \quad (7\text{-}11)$$

Where gij and gjj are interaction parameters between the components i and j or j and j, respectively, and oij is a non-randomness parameter. The values of specific Antoine constants and the interaction parameters as well as non-randomness parameter of activity coefficient were estimated by Aspen Tech Aspen One 8.4. A Non-Random Two Liquid (NRTL) model was selected as the property method of calculations due to it applicability for dilute solutions of organic compounds [52]. With the permeances calculated for a binary system, the selectivity coefficient (α_{ij}) for that system can be determined as follows:

$$\alpha_{ij} = \frac{p_i/l}{p_j/l} \quad (7\text{-}12)$$

The selectivity coefficient α can be seen as the driving force-normalized equivalent to the separation factor β.

7.2.10 Pervaporation integrated with fed-batch PBE fermentation

A lab-scale stirred-tank bioreactor with a nominal volume of 7 L (Labfors, Infors, Quebec, Canada) containing 3.6 L of modified Biebl medium (Biebl 2001; Sarchami and Rehmann 2014b) was used. Modified Biebl medium contained (per liter of distilled water): 50 g crude glycerol, 15 g sugars derived from Jerusalem artichoke acid hydrolysate, 1 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.1 g $FeSO_4.7H_2O$, 2 g $CaCO_3$, 0.01 mg Biotin, 1 mg Thiamine, 1 mg p-aminobenzoic acid, 4 ml of trace element solution (SL7), as described before (Biebl 2001). 0.4 L of actively growing cells was inoculated into the fermentation medium. The temperature and pH were controlled at 35ºC and 5.0, respectively, and 0.2 ml L^{-1} of antifoam was added to the fermentation medium to control foaming. Agitation was controlled at 200 rpm using one Rushton impeller and the fermenter was equipped with sensor probes monitoring pH (Hamilton EasyFerm, Switzerland). Nitrogen gas was used at a flow rate of 1 L min^{-1} throughout the experiment to purge the bioreactor and keep it at anaerobic condition. Temperature, gas flow rates (Red-y series flow controller, model GSC-C3SA-BB12, Vogtlin Instruments AG) and stirrer speed were regulated through control units (local loops). Iris software (Labfors, Infors, Quebec, Canada) was used to monitor and manage the process with good flexibility and total traceability. Samples were taken intermittently and filtered using 0.2 μm grade filters for solvents, glycerol and sugar analysis (HPLC, see section 7.2.11 for conditions).

The pervaporation unit described above was also used in the integrated process as well. The pervaporation membrane was sterilized by circulating 70% ethanol through PV unit for 1 hour. The excess ethanol was removed by flushing the PV unit by sterile deionized water for 2 hours at a fixed flow rate of 4 L min^{-1}. The permeate vapor was collected in a cold trap using dry ice/ ethanol mixture while the retentate was returned to fermenter. The

cold trap was exchanged every 5 hours with a consecutive permeate collection. Butanol was removed from the fermentation broth by pervaporation at 35ºC. Samples were taken from the fermenter and permeate for solvents, carbohydrate, and acid analysis. Fig. 7-2 shows a schematic of the pervaporative-fed batch fermentation setup used in this study.

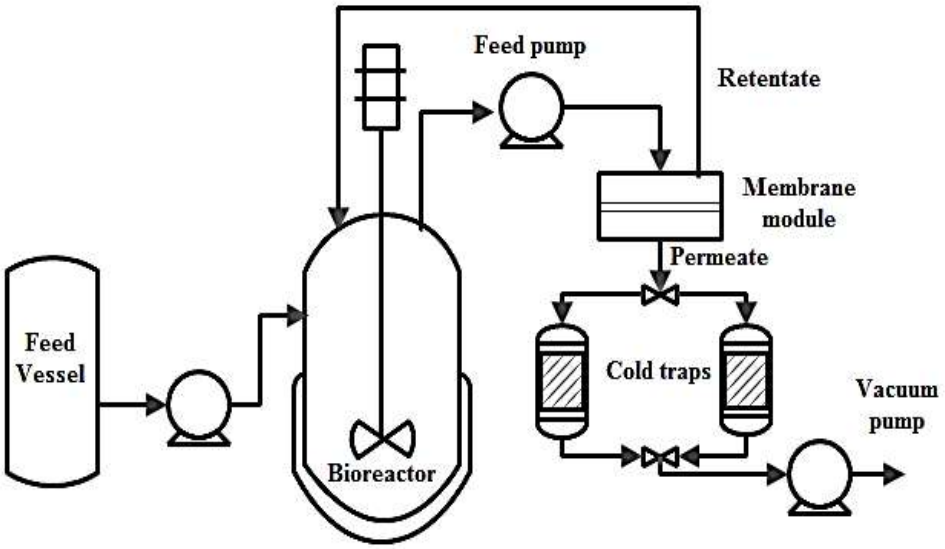

Figure 7-2 Schematic diagram of pervaporative-fed-batch fermentation set up

7.2.11 Analytical methods

Bacterial growth was monitored by measuring the optical density as previously discussed in Chapter 6. Section 2.3.9

Concentrations of glycerol, sugars, and solvents produced in the fermentation were determined by high performance liquid chromatography as also previously discussed in Chapter 6. Section 2.3.9

Product yield was calculated as the total amount of butanol produced, divided by the amount of fermentable glycerol and sugar utilized and is expressed as $g_{butanol}\ g^{-1}$ $_{(glycerol+sugar)}$. Productivity was calculated as the maximum butanol concentration achieved divided by the fermentation time and is expressed as $g\ L^{-1}\ hr^{-1}$.

7.3 Results and Discussion

A binary butanol/water solution and a novenary mixture were tested in order to investigate the performance and suitability of the membrane for separating the main products of a typical PBE fermentation. The data obtained was used for direct integration of pervaporation with fed-batch fermentations of *Clostridium pasteurianum*.

7.3.1 Binary mixture

Flux, permeate concentration, permeance, selectivity coefficient, and separation factor as a function of butanol concentration in the feed for the pervaporation of binary butanol/water mixture are presented in Fig. 7-3. The butanol permeation flux decreased linearly with a decrease in the concentration of butanol in the feed solution, as shown in Fig. 7-3A. This can be explained by the solution-diffusion model where the concentration gradient between the two sides of the membrane creates the thermodynamic driving force for pervaporation (Wijmans and Baker 1995). This result is in good agreement with previous reports, where linear relationships were found to exist between butanol flux and its concentration in the feed using PDMS membranes and binary butanol/water solutions (Niemisto et al. 2013; Rozicka et al. 2014). The water flux was constant during the experiment with values around 500–550 g m^{-2} hr^{-1}, indicating that water transport through the membrane is independent of the feed composition in the studied concentration range. Similar observations with water alcohol separations have been reported elsewhere (Favre and Nguyen 1996; Liu et al. 2005; Niemisto et al. 2013. As shown in Fig. 7-3B, with a decrease of butanol concentration in the feed, a nonlinear decrease of butanol content in permeate is observed (Fig. 7-3A). With water flux being constant and butanol flux decreasing over time, the water concentration in permeate increased continuously.

It has been suggested that the driving force normalized permeation properties (permeability or permeance) could be a better way of reporting the experimental results in order to decouple the effect of operating conditions, e.g. concentration and temperature of the feed, and permeate pressure (Wijmans 2003; Fen et al. 2004; Fen et al. 2004). Hence, membrane efficiency and pervaporation performance of various membranes and experimental

conditions can be compared using the values reported for permeance. The permeance data obtained by normalizing the partial fluxes with respect to the corresponding driving force is shown in Fig. 7-3C. The butanol permeance was constant throughout the pervaporation experiment. This is in contrast to the butanol flux, which increased with the butanol concentration in the feed. The water permeance was also constant throughout the experiment, corresponding to the constant water flux during the pervaporation experiments mentioned above. The average butanol permeance was 546 g m^{-2} hr^{-1} kPa^{-1}, whereas the average water permeance was 90 g m^{-2} hr^{-1} kPa^{-1}. These results indicate that butanol has a higher permeance compared to water.

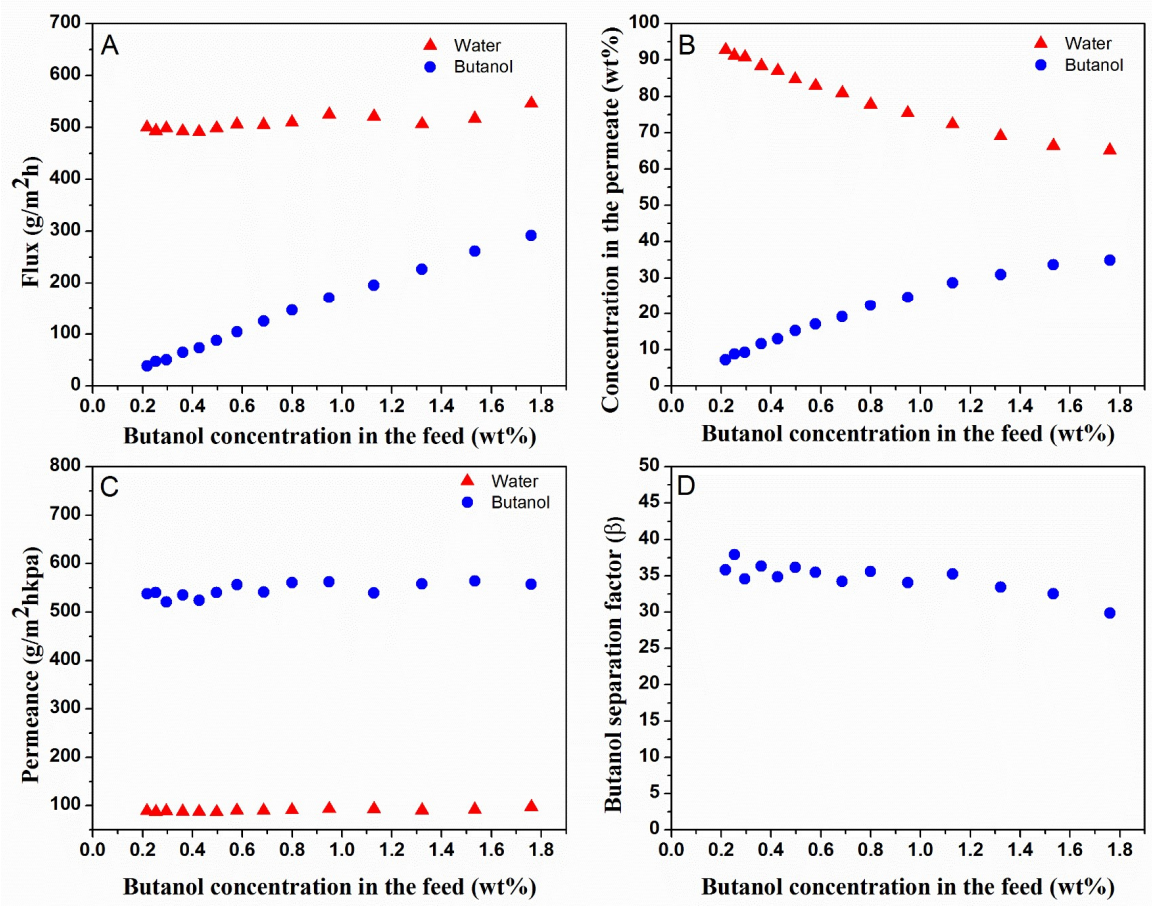

Figure 7-3 Flux (A), Concentration in the permeate (B), Permeance (C) of butanol and water, and Selectivity and butanol separation factor (D) in binary butanol/water solution solution with initial butanol concentration of 20 g L^{-1}.

The separation factor α has commonly been used as an indicator of membrane selectivity (Fouad and Feng 2008). A separation factor of greater than 1 indicates that enrichment occurred during the separation process. As shown in Fig. 7-3D, the separation factor slightly increased with a decreasing mass fraction of butanol in the feed and was in the range of 29-38 for butanol. Table 7-1 presents the values of separation factors calculated for various membranes in contact with aqueous binary mixtures according to Eq. (7-2). The pervaporation performance of the PDMS membranes seems to be superior for the recovery of butanol (Table 7-1). The studied Pervap 4060 membrane had better separation factors than PDMS-PAN and Polyetherblockamide (PEBA) 2533 membranes. PDMS and Pervap 4060 had about the same separation factor for butanol in binary mixtures. Also, the temperature difference should be taken into consideration in comparison of these results.

Table 7-1 Separation factors of pervaporative butanol separation from binary butanol/water solution.

Membrane	**Butanol content in feed solution (wt%)**	**Temperature (ºC)**	**Separation factor β**	**Ref.**
PEBA 2533	0.03-0.4	40	19-24	(Fouad and Feng 2008)
PDMS: Pervap 1060	1	40	27	(Jonquieres and Fane)
PDMS	4	50	34	(Hickey et al. 1992)
PMS	5	50	14	(Hickey et al. 1992)
PERV 2200	0.6-5	33	2-14	(El-Zanati et al. 2006)
PDMS-PAN	3.5	42	22	(Niemisto et al. 2013)
PDMS: Pervap 4060	5	25	35	(Rozicka et al. 2014)
Pervatech	5	25	9	(Rozicka et al. 2014)
PolyAn	5	25	8	(Rozicka et al. 2014)

PDMS: Pervap 4060	0.2	35	29-38	This work

The separation factor is a parameter which depends on experimental conditions, therefore with the change of temperature, the separation factor value is also changed. Another approach to present pervaporation results is to use selectivity coefficient (α_{ij}). Selectivity coefficients relate to the intrinsic properties of the membrane and allowed to compare properties of various membranes without taking into account experimental conditions or physicochemical properties of solvents (Wijmans and Baker 1995). As shown in Fig. 7-3D, the selectivity coefficient of butanol was rather constant with value of 6.2 during the PV experiment. Table 7-2 presents the values of selectivity coefficients calculated for various membranes in contact with aqueous binary mixtures according to Eq. (7-8). All membranes presented in Table 7-2 are selective toward butanol (selectivity coefficient is higher than 1) and evidently it can be seen that selectivity of butanol transport determined in contact with Pervap 4060 is the highest which indicates that this membrane is the most suitable among these membranes for butanol recovery.

Table 7-2 Selectivity coefficient of pervaporative butanol separation from binary butanol/water solution.

Membrane	**Butanol content in feed solution**	**Temperature (ºC)**	**Selectivity coefficient α**	**Ref.**
PolyAn	0.01 M	25	3.4	(Rozicka et al. 2014)
Pervatech	0.01 M	25	3.6	(Rozicka et al. 2014)
PDMS: Pervap 4060	0.2 wt%	35	6.05	This work

7.3.2 Model solution (novenary mixture)

A typical fermentation broth contains multiple additional organic compounds, hence experiments with novenary mixtures were conducted in order to evaluate how the feed composition affects pervaporation and if coupling phenomena exists. Coupling effects are

caused by mutual interactions between the permeating compounds in the membrane, as well as by interactions between different components and the membrane material. Coupling phenomena may be divided into a kinetic and a thermodynamic part (Raisi and Aroujalian 2011). The kinetic part takes place when the faster permeating component drags also the slower permeating component(s) through the membrane. Consequently, the slower component can show a higher permeability in comparison to the permeation of a single component. The thermodynamic part is defined as the concentration change of one component in the membrane caused by the presence of other components (Ren and Chengzhang 1998; Raisi and Aroujalian 2011).

The novenary mixture consisted of glycerol, fructose, and glucose as substrates, acetic acid, butyric acid, ethanol, and 1,3-PDO as by-products, and butanol as main product of PBE fermentation. The initial concentration of these components in the model solution can be found in section 7.2.3. The analysis of the feed and permeate composition indicates that some of these components did not cross the membrane. These components were glycerol, fructose, glucose, acetic acid, and 1,3-PDO. None of these components were detected in the permeate and their concentration in the feed was constant over time (data not shown). Fluxes and permeate concentration of butanol, ethanol, and butyric acid as well as water flux are shown in Fig. 7-4.

Permeation fluxes of butanol, ethanol, and butyric acid decreased almost linearly as the function of a decrease in the concentration of that compound in the feed, as shown in Fig. 7-4A, B, and C. The partial flux of these three compounds followed the order of butanol > ethanol > butyric acid. Butyric acid flux was very low compared to butanol and ethanol. Also, the permeate concentration of butanol was greater than ethanol and butyric acid, with butyric acid having the lowest permeate concentration. Butyric acid concentration reduced from 5.1% w.w-1 to 4.8% $w.w^{-1}$ after 6 hours of pervaporation, whereas the butanol mass fraction in the feed decreased from 1.38 to 0.25 % $w.w^{-1}$. Only a minor variance occurred in the butanol flux from the novenary mixture compared to the binary butanol/water solution, indicating that coupling effects are negligible in this case. The main cause of this is due to the dilute solutions, where the large amount of water molecules is present and

hinders the interactions between the different molecules presented in the solution. As shown in Fig. 7-4D, the water flux was rather constant during the experiment with values around 542–604 g m^{-2} hr^{-1}. The minor variance in transport of water from binary and novenary mixtures indicates that water flux through Pervap 4060 is constant regardless of the type and concentration of compound present in the feed.

The model solution represents a more complex system than a binary solution and it is difficult to analyse a system of such complexity in terms of permeance and selectivity coefficient. In order to calculate the permeance, activity coefficient parameters should be available. However, to the authors' knowledge not all the binary interaction parameters of this model solution are available which makes permenace calculation impossible. Hence, less detailed information can be gained from model solution.

The data demonstrated that not only butanol but also the ethanol and butyric acid can be selectively separated from a model solution by using the Pervap 4060 membrane system. The separation factor of butyric acid is very low compared to ethanol and butanol. The average separation factor of butyric acid is 1.6-1.3, whereas the average separation factor of butanol and ethanol are in the range of 15.2-26.3 and, 5.1- 9.0, respectively. Butyric acid is an intermediate in the respective PBE fermentation pathway leading to butanol. Therefore, it is important to retain butyric acid in the fermentation broth. The data obtained in this study show that butyric acid permeation across membrane is low and should not have a substantial effect on butanol yield or productivity. It was also found that glycerol, fructose, and glucose as substrates of PBE fermentation did not cross the membrane which indicates the suitability of Pervap 4060 as a PDMS-based membrane in an integrated process. In addition, 1,3-PDO as one of the by-products of PBE fermentation did not cross the membrane and remained in the feed solution. There is a lack of information in the literature on the toxicity of 1,3-PDO to *C. pasteurianum*. Therefore, more research is needed to investigation the effect of the accumulation of this by-product in the fermentation broth on *C. pasteurianum.*

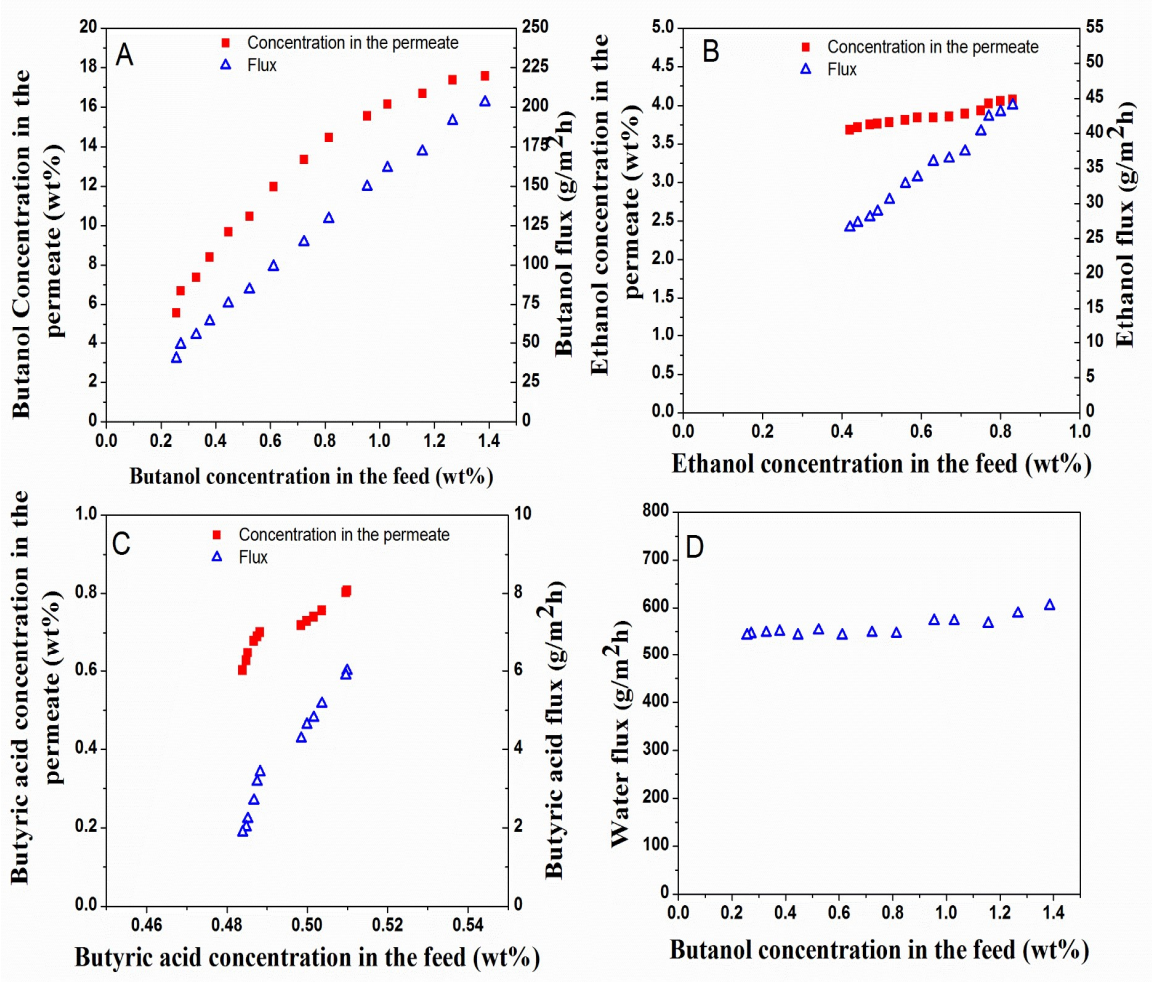

Figure 7-4 Butanol concentration in the permeate and flux (A), Ethanol concentration in the permeate and flux (B), Butyric acid concentration in the permeate and flux (C), and Water flux (D) of model solution.

7.3.3 Membrane performance in fermentation–pervaporation coupled process

To mitigate butanol toxicity, fed-batch PBE fermentation was integrated with pervaporation to further concentrate butanol while removing it from the fermentation broth and improve its productivity. The profiles of PBE production, substrate concentration and cell density in the coupled process are shown in Fig. 7-5A. Fermentation was allowed to proceed for 15 hr in batch mode, when butanol concentration in the fermentation broth was 3.6 g L^{-1}, when coupled with pervaporation. Based on the available substrate, a similar fermentation without pervaporation would have resulted in butanol concentrations of 14.6

g L^{-1} (Sarchami and Rehmann 2016b). When the glycerol concentration in fermenter decreased below 20 g L^{-1}, concentrated substrate and additional nutrients were fed to fermenter. A total of three successive feeding cycles where conducted while butanol was constantly removed via integrated pervaporation over a total time period of 65 hr. The butanol concentration in the fermentation broth remained around 3.7 g L^{-1} during the first 35 hr and gradually increased to 4.5 g L^{-1} afterwards, well below the inhibiting levels of approximately 13 g L^{-1}. Similar to novenary mixture, glycerol, fructose, glucose, acetic acid, and 1,3-PDO did not cross the membrane, while butanol, ethanol, and butyric acid passed through the membrane. Substrates were consumed during the fermentation but 1,3-PDO started accumulating in the fermenter.

The profiles of total solvents and biomass production and substrate consumption are shown in Fig. 7-5B. Total butanol and ethanol production was estimated based on the amounts of solvent present in the fermentation broth and permeate. 192.50 g butanol and 15.7 g ethanol were produced from 552.7 g crude glycerol and 274.65 g Jerusalem artichoke-derived sugars (fructose+glucose). A butanol productivity of 0.60 g L^{-1} hr^{-1} was obtained, while a comparable simple batch process resulted in 0.55 g L^{-1} hr^{-1} of butanol productivity. 171.5 g butanol was recovered by pervaporation which accounts for 89.1% of total butanol produced during this process. Despite accumulation of acetic acid and 1,3-PDO in the fermenter a high glycerol consumption rate of 1.9 g L^{-1} hr^{-1} was observed, suggesting that there was no 1,3-PDO inhibition on *C. pasteurianum* DSM 525 up to 1,3-PDO concentration of 12.5 g L^{-1}. Butanol, 1,3-PDO and ethanol yields of 0.23 $g_{butanol}$ $g^{-1}_{(glycerol+sugars)}$, 0.08 $g_{1,3\text{-}PDO}$ $g^{-1}_{(glycerol+sugars)}$, 0.019 $g_{ethanol}$ $g^{-1}_{(glycerol+sugars)}$, were obtained after 65 hours of PBE fermentation, respectively (Fig. 7-5C). Also, this integrated process resulted in biomass yield of 0.026 $g_{biomass}$ $g^{-1}_{(glycerol+sugars)}$ which is 33% higher than biomass yield of non-integrated batch process. Overall, total butanol production improved by a factor of 2.6 and viable fermentation time increased by a factor of 2.2 upon applying Pervap 4060 membrane pervaporation, relative to the batch process.

The performance of Pervap 4060 in fed batch PBE fermentation with PV is shown in Fig. 7-5D. Permeate samples were taken every 4 to 5 hours to measure permeant concentration,

while the fluxes and separation factors of permeants were calculated according to Eqs. 7-1 and 7-2. Also, permeate concentration of butanol was greater than ethanol and butyric acid, with butyric acid having the lowest permeate concentration. Butanol and ethanol concentration in the permeate were up to 97.8 and 15.6 g L^{-1}, which was 5.19 and 5.47 times higher than that in batch fermentation. The butyric acid concentration in the permate was up to 0.2 g L^{-1}. Butanol, ethanol, and butyric acid separation factors ranged from 30.4 to 36.8, 7.8 to 9.2, and 1.5- 2.1, respectively, while the total flux varied from 575 to 640 g m^{-2} hr^{-1}. More research is needed to investigate membrane fouling and cleaning behavior of Pervap 4060.

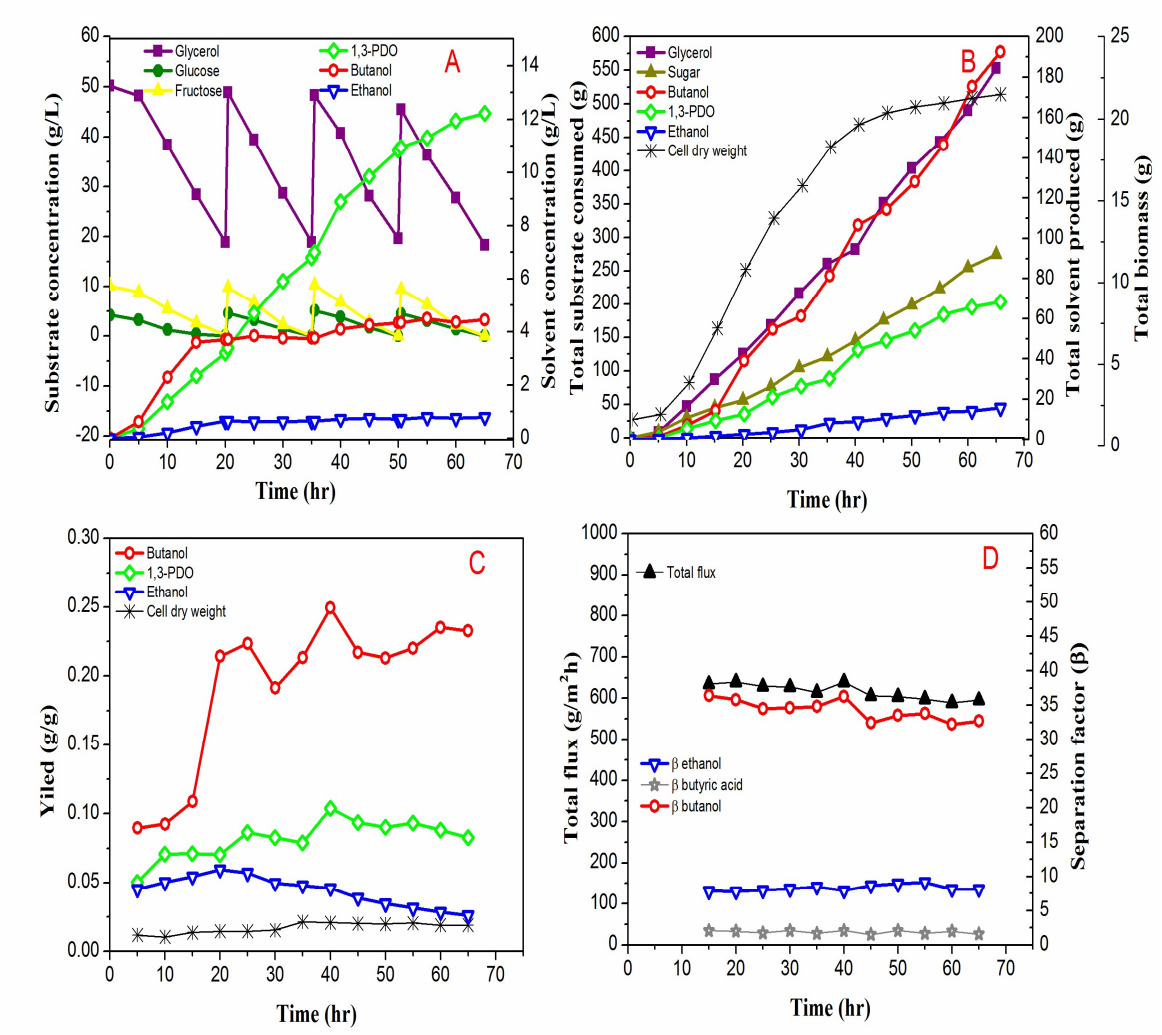

Figure 7-5 Concentration of solvents, substrates, and cell density of fermentation–PV coupled process in (A), Total solvent and biomass production and substrate consumption in (B), Solvents and biomass yield in (C), and Total flux and separation factors of butanol, ethanol, and butyric acid in (D).

To the authors' knowledge there are no studies in the literature on fermentation integrated with pervaporation using glycerol as substrate for *C. pasteurianum*. This is the first attempt to integrate PBE fermentation with pervaporation. Therefore, data reported on PEB fermentation is compared to available data for ABE fermentations.

In a recent study, Kong et al. (2016) investigated a PV coupled ABE fermentation using a butanol-tolerant mutant (*C. beijerinckii* BT14) and PDMS/ceramic composite membrane

for a duration of 95 hours. An overall butanol productivity of 0.36 g L^{-1} hr^{-1} with an average total flux of 524-707 g m^{-2} hr^{-1} and butanol separation factor of 11-19 was achieved. This process generated glucose consumption rate of 2.64 g L^{-1} hr^{-1}. In another study, Shin et al. (2015) studied cross-linked polydimethylsiloxane (PDMS) membrane for in-situ product removal of ABE biofuels in *C. acetobutylicum* fermentations operated in a semi-continuous mode. Butanol volumetric productivity increased from 0.27 g L^{-1} hr^{-1} in simple batch fermentation to 0.40 g L^{-1} hr^{-1} in the case of pervaporative-fermentation with the PDMS membrane with an average total flux of 941 g m^{-2} hr^{-1}. Overall, both total butanol production and viable fermentation time improved by a factor of two applying PDMS membrane pervaporation, relative to the batch process.

Wu et al. (2015) studied the effects of pH and cell immobilization on ABE production coupled with a PDMS/ceramic membrane using *C. acetobutylicum* XY16. A butanol productivity of 0.35 g L^{-1} hr^{-1} was obtained using a two-stage controlled-pH in the coupled process, which was 11% higher than a control process without pH control, however, membrane fouling was a limitation, which was alleviated when using sugarcane bagasse as a cell immobilization carrier. This resulted in the average permeation flux of 676 g m^{-2} hr^{-1} and butanol separation factor of 15.8. A maximum butanol productivity of 0.35 g L^{-1} hr^{-1} was also obtained. Yen et al. (2012a) and Yen et al. (2012b) investigated the application of PEBA and PEBA-CNTs membranes in separation of butanol from ABE fermentation broth. The process involved one stage fed-batch ABE fermentation using *C. acetobutylicum* BCRC 1063 (glucose as substrate). The results of these studies indicated that the addition of CNTs in PEBA membrane had better butanol removal flux and separation factor than the control trial without CNTs addition (Table 7-3). Table 7-3 compares the results of the present study with published results on fed-batch ABE pervaporative-fermentation.

The total flux obtained in Shin et al. (2015) work using PDMS membranes is higher than that of other studies including this work. This may be attributed to use of a thinner PDMS membrane in Shin et al. (2015) work. However, the butanol separation factor obtained in this work is higher than that of prior studies. The butanol yield of this work is comparable

to results reported by Kong et al. (2016) using PDMS/ceramic membrane, however, the butanol productivity obtained in this work in significantly higher than all other ABE pervaporative-fermentations. By comparison, Pervap 4060 have good potenital as membrane for butanol removal.

Table 7-3 Pervaporation performances of different PV membranes in fed-batch ABE fermentation coupled process.

Membrane	Membrane area (cm^2)	Membrane thickness (μm)	Total flux ($g\ m^{-2}\ hr^{-1}$)	Butanol separation factor $\beta_{butanol}$	Butanol productivity ($g\ L^{-1}\ hr^{-1}$)	Butanol yield ($g\ g^{-1}$)	Ref.
PDMS/ceramic	160	--	524-707	11-19	0.36	0.23	(Kong et al. 2016)
PDMS	37	0.5	941	19	0.40	0.16	(Shin et al. 2015)
PDMS/ceramic	160	--	676	15.8	0.35	0.17	(Wu et al. 2015)
PEBA	800	50	161	14	0.27	0.17	(Yen et al. 2012a)
PEBA-CNTs	800	50	147	18	0.34	0.20	(Yen et al. 2012b)
PDMS-Pervap 4060	170	6	575-640	30.4-36.8	0.60	0.23	This work

7.4 Conclusion

Pervaporation performance of PDMS-based membrane, Pervap 4060, was investigated in order to observe its suitability for the PBE production process. Water flux of Pervap 4060 membrane did not change with the change of concentration or type of organic compound present in tested aqueous mixtures. The intrinsic membrane properties discussed in terms of permeance and selectivity coefficient of binary solution reveal that the investigated membrane is selective toward butanol. Glycerol, glucose, fructose, acetic acid, and 1,3-PDO did not cross the membrane. Based on the fluxes of organic compounds, butanol was the most efficiently transported compound due to its high vapour pressure. The data obtained was used for direct integration of pervaporation with fed-batch PBE fermentation.

This is the first report on PV coupled PBE fermentation using crude glycerol and Jerusalem artichoke acid hydrolysate as substrate. During PV coupled fed-batch PBE fermentation, *Clostridium pasteurianum* DSM 525 grew to a high cell dry weight of 4.3 g L^{-1}. The fed-batch fermentation with in-situ recovery by pervaporation continued for 65 hours, 89.1% of butanol produced during the fermentation was extracted, and butanol productivity of 0.60 g L^{-1} hr^{-1} was achieved. The total flux ranged from 575 to 640 g m^{-2} hr^{-1} and the separation factor of butanol ranged from 30.4 to 36.8 in this process. The results of this work could be helpful to develop an efficient continuous process for butanol production via integrated PV-PBE fermentation.

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Chapter 8

8 Conclusions and Recommendations

This chapter outlines the conclusions of the study. In addition, some recommendations for future work are proposed.

8.1 Conclusions

The work presented in this thesis investigated fermentative butanol production from Jerusalem artichoke tubers and biodiesel-derived glycerol, and evaluated pervaporation as an alternative separation technique for online butanol recovery.

The results presented in this work indicated that both acid and enzymatic hydrolysates of Jerusalem artichoke tubers are suitable substrates for butanol fermentation using the optimal hydrolysis conditions developed in the present study. The shorter reaction times and lower catalyst costs would imply acid hydrolysis to be favorable over enzymatic conversion if conducted as separate process steps. In addition, robust butanol yield comparable to control fermentation with pure glycerol as substrates was obtained from biodiesel derived- glycerol in a lab scale bioreactor using the optimal fermentation condition developed in this study. This result indicated that crude glycerol is also a suitable feedstock for butanol production.

It was found that direct addition of acetic and butyric acid into the glycerol fermentation broth enhances butanol yield and productivity. Also, Jerusalem artichoke acid hydrolysate was found to be a suitable sugar source to be fermented by *C. pasteuriaunm* DSM 525 to produce acetic and butyric acid. Therefore, Jerusalem artichoke acid hydrolysate as a sugar source and glycerol as the main carbon source were used for the co-substrate based butanol production in a laboratory bench bioreactor using optimal co-substrate ratio developed in the present work. The co-substrate system was found to be a potential way to utilize an industrial waste stream and a dedicated energy crop for the efficient production of an advanced biofuel.

The co-substrate system was used for direct integration of pervaporation with fed-batch butanol fermentation. Enhanced overall butanol productivity was achieved in the integrated process. Pervaporation was able to efficiently recover butanol from fermentation broth and relieve the inhibition caused by butanol. Concentrated substrate feeding was possible in the fed-batch PBE fermentation when coupled with online butanol removal, increasing the volumetric productivity. More efficient substrate conversion was achieved in the integrated process than the non-integrated process due to relieved inhibition and stress on the bacteria.

Overall, during this research a process to produce butanol via fermentation of Jerusalem artichoke tubers and biodiesel-derived glycerol integrated with online pervaporation was developed. To the author' knowledge this is the first attempt to integrate PBE fermentation with pervaporation. Therefore, the work presented in this thesis is a very useful contribution for mitigation of butanol toxicity and improving its volumetric productivity in PBE fermentation.

8.2 Recommendations

Although an integrated process for butanol production has been developed and demonstrated in this project, many areas still require continuing research endeavors for improvement and perfection before this process can be industrialized on a commercial scale and compete with petrochemically-derived butanol. Some suggestions and recommendations for future research work are listed below:

- In addition to the feedstocks that have been investigated in this study, other biomass can be evaluated in the future for butanol production to broaden the substrate pool and gain more information on the performance of each type of feedstock. Evaluating different feedstocks helps to understand the choices of substrates for butanol production in different regions, and promotes value-added by products for the processing industry.
- More research attention should also be paid on medium formula for PBE fermentation. Soybean meal, cotton seed protein, corn steep liquor and molasses were investigated elsewhere as potential nitrogen sources to replace the expensive

yeast extract currently used in the medium formula. In the future, an optimized medium formula using these alternative nitrogen sources and supplementation of additional chemicals can be developed in search for a cost-effective medium formula for industrial process for economical butanol production.

- Besides batch and fed-batch fermentations investigated in this study, continuous fermentation using both crude glycerol and Jerusalem artichoke hydrolysate as co-substrate can be studied in the future. Cell-recycle continuous fermentation may offer many advantages, including high reactor productivity and reduced inhibition due to a constant flow of fresh medium.
- Performance of pervaporation is highly dependent on the choice of membranes. Therefore, more research is needed to study other advanced membranes that have high butanol selectivity and high butanol recovery efficiency in PBE fermentation.
- Besides pervaporation, there exist many alternative butanol recovery techniques, such as liquid-liquid extraction, adsorption, and gas-stripping. More research is needed to study in-situ butanol removal (one-stage and hybrid) for PBE fermentations using other separation techniques.

Appendix

A. *Clostridium pasterianum* DSM 525 dry cell weight calibration

Clostridium pasterianum DSM 525 was grown 17 hours at 37°C in 100ml of seed medium. 1.0 µm pore size glass fiber membrane filters were dried at 100 °C for 24 hours. 6.25ml, 12.5 ml, 25mL, 50mL, 100 mL, 150mL and 200ml of the grown bacteria where added to water to complete 200mL. Optical density was measure at 600 nm using a 200 pro infinite series microplate reader (Tecan, Switzerland). Solutions were later vacuumed filtered. Filter was removed and dried at 100 °C for 48 hours. Dry filter weight was measured previously to filtration and subtracted from the final weight to obtain cell dry weight.

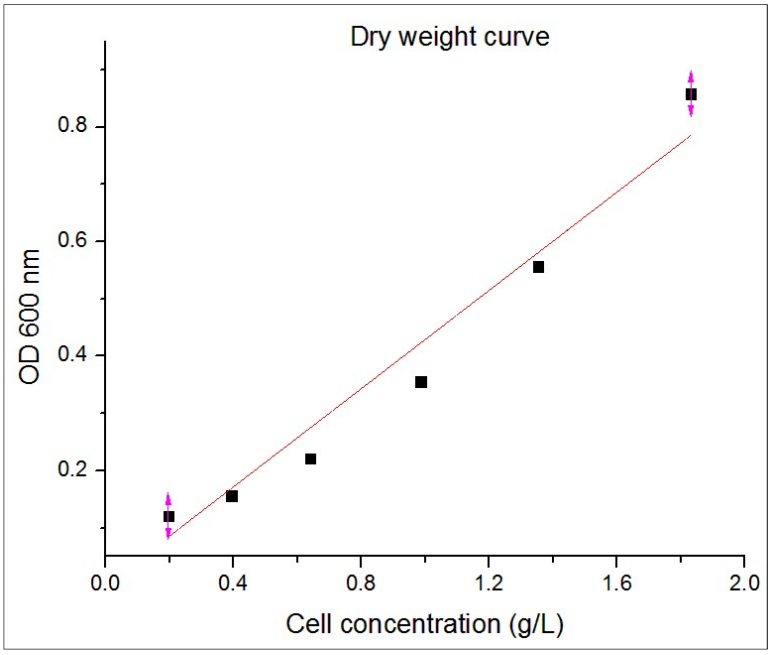

Figure A.1 Calibration curve of for.

Table A.1 Linear regression equation and statistics for the *Clostridium pasterianum* DSM 525 standard curve

Equation	y = a + b*x		
Adj. R-Square	0.98579		
		Value	**Standard Error**
OD	Intercept	0	---
OD	Slope	0.42917	0.02101

B. *Clostridium pasterianum* DSM 525 growth curve

Clostridium pasterianum DSM 525 was grown at 37°C in 200 µl of reinforced Clostridium medium (RCM). Cultures of this strain were grown in RCM containing (per liter) 10 g peptone, 10 g beef extract, 3 g yeast extract, 5 g dextrose, 5 g NaCl, 1 g soluble starch, 0.5 g L-cysteine, 4 ml Resazurin, at pH 6.8. Optical density was measure at 600 nm using a 200 pro infinite series microplate reader (Tecan, Switzerland).

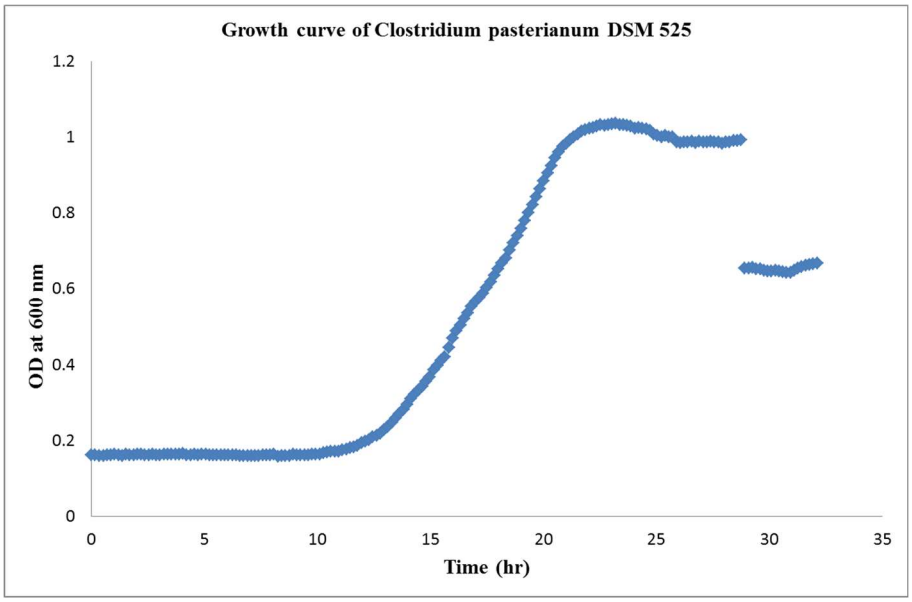

Figure B.1 Growth curve of *Clostridium pasterianum* DSM 525 on 15 g L^{-1} of glucose in reinforced Clostridium medium.

C. HPLC calibration curves

HPLC was used to monitor glucose, fructose, and glycerol consumption and butanol, ethanol, 1,3-propanediol, acetic acid and butyric acid production. At the same time 5-hydroxymethylfurfural was monitored with a diode array detector set to a wavelength of 280 nm. The following figures show the calibration curves for each of the compounds. All the calibration curves were linear in the range of concentrations studied.

Figure C.1 Refractive index calibration curves for glucose, fructose, glycerol, butanol, acetone, 1,3-propanediol, ethanol, butyric acid and acetic acid. mRIU stands for micro refractive index units, standard units rendered by Agilent software.

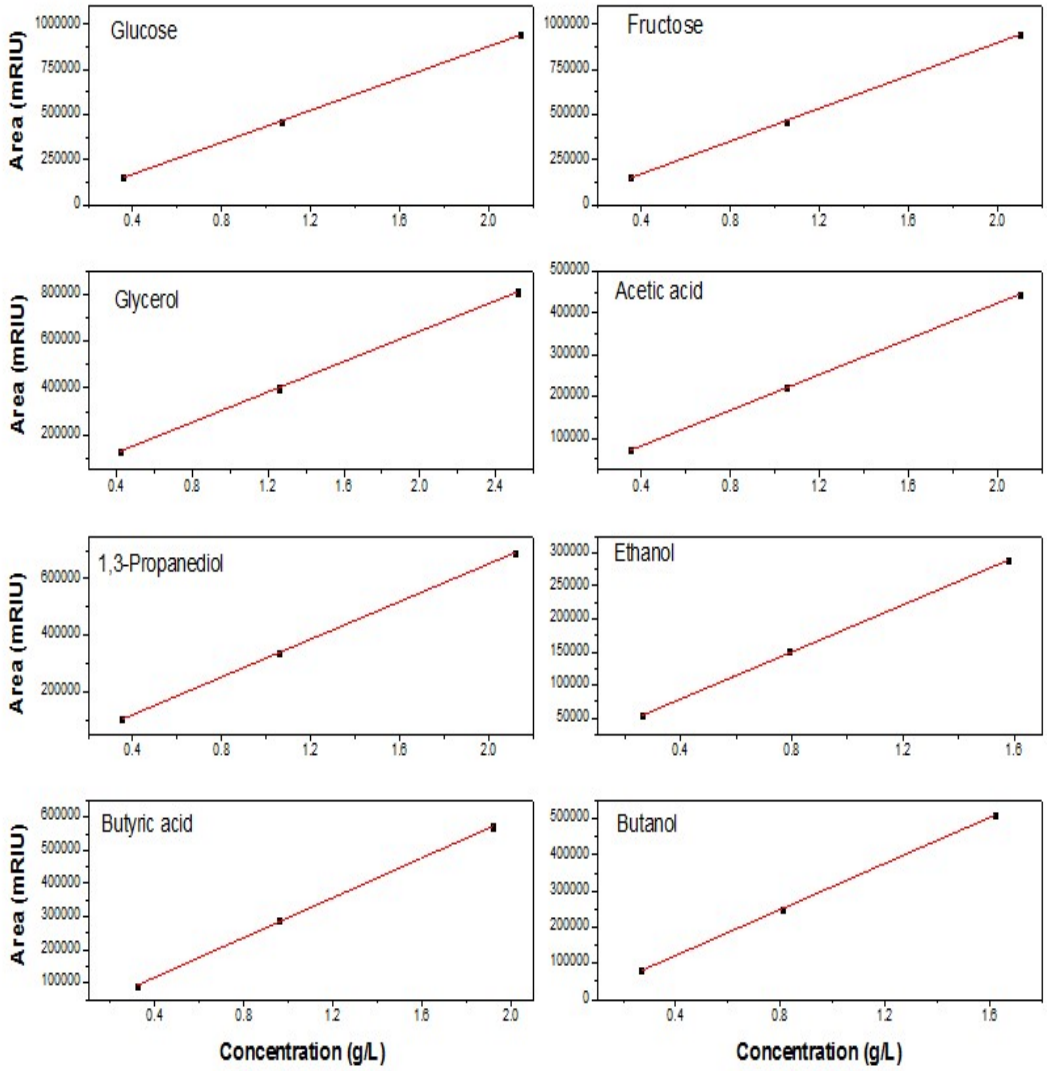

Table C.1 Retention time, slope, Y-intecept, and R^2 values for the calibration curves of eight different compounds analyzed by refractive index.

Compound	Retention time [min]	Slope	Y-intercept	R-square
Glucose	16.695	442861.70202	-6911.13817	0.9999
Fructose	18.031	453963.909	-8987.89474	0.9998
Glycerol	23.602	324673.7817	-6220.83102	0.9999
Acetic acid	27.517	213531.95489	-3298.94737	1.0
1,3-	30.391	332496.21661	-	0.9999
Ethanol	37.077	178054.43266	7512.94737	0.9995
Butvric acid	42.429	300482.54643	-3096.22201	0.9998
Butanol	69.87	318935.67251	-5842.10526	0.9998

Figure C.2 Diode array detector calibration curves for 5-HMF and furfural. mAU stands for array units, standard units rendered by Agilent software.

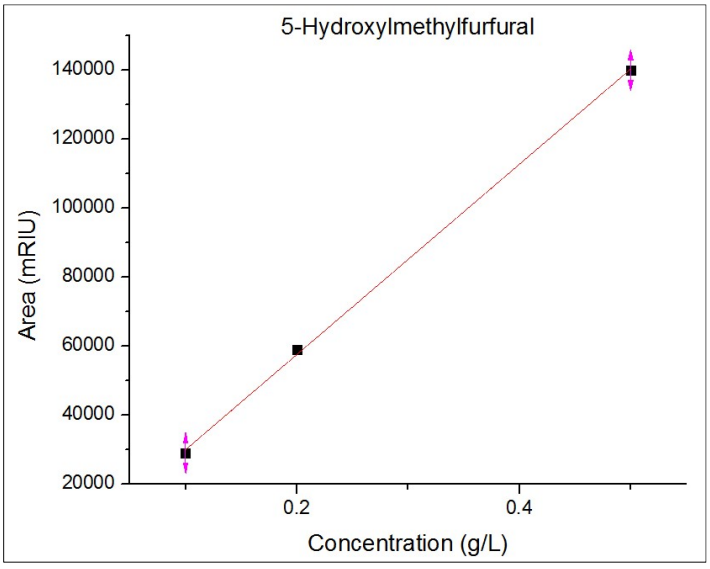

Table C.2 Retention time, slope, Y-intercept and R^2 values for the calibration curve of 5- hydroxymethylfufural analyzed by absorbance at 280 nm on the diode array detector.

Compound	Retention time [min]	Slope	Y-intercept	R-squared
5-hydroxymethylfufural	31.001	275769.23077	2461.53846	0.99906

D. Pervap 4060 membrane system

The Pervap 4060 membrane system consisted of a flat sheet membrane which was located inside a rectangular stainless steel test cell. If necessary, the test cell could be opened for cleaning procedures and to replace the membrane sheet.

The Pervap 4060 was a composite membrane consisting of a thin separation layer (PDMS, 6 µm (Rozicka et al. 2014)) on top of a porous support layer (70–100 µm), coated on a mechanical support layer (100–150 µm).

When assembling the membrane system, the membrane sheet was put on top of a sintered metal plate inside the permeate part of the module, separation layer facing the liquid side. Using vacuum, the membrane sheet now could be sucked flat and the O-ring gasket was positioned so that it was pulled into the corners to seal test cell and membrane (Figure D.1). The test cell was closed by screwing on the top part.

Figure D.1 Pervap 4060 membrane and test cell: (A) Assembled test cell showing permeate (vacuum) connection, (B) assembled test cell showing feed and retentate

connections, (C) test cell permeate part showing sinter plate, membrane and O-ring installed.

The Pervap test cell had inlet and outlet ports for the liquid (Figure D.1 B) as well as ports to connect vacuum to the permeate side (Figure D.1 A). Liquid entered the test cell as feed through the inlet port, passed the membrane tangentially in a cross-flow and left the cell on the other side as retentate. Vacuum was applied to the permeate side and emerging vapors were collected further downstream. The maximum pressure and temperature were never reached throughout the experiments and the liquid feed flow rate was only limited by the speed of the peristaltic metering pump.

E. Cleaning protocol of the membrane and pervaporation unit

Since the carbohydrates in the feed could potentially be a substrate for any microorganism that can grow inside the equipment, tubing or onto the membrane, any fouling had to be prevented. Therefore, a protocol for cleaning in place (CIP) was developed.

At first, the model solution was removed from the system by pumping the feed into an empty container. Then, the system was flushed thoroughly with deionized water for 30 minutes using the pump at a medium high flow rate at room temperature. Next, the system was washed with NaOH (1% w/w) by recirculating it for 30 minutes. After this caustic cycle, the system was rinsed with deionized water until the pH was neutral. Then, the system was washed with citric acid (1% w/w) by recirculating it for 30 minutes. When finished with the acid cycle, the system was rinsed again with deionized water until the pH was neutral. The cleaning water was removed by pumping it into an empty container. The membrane module was disconnected from the liquid pump and blow dried with filtered air for 10 minutes.

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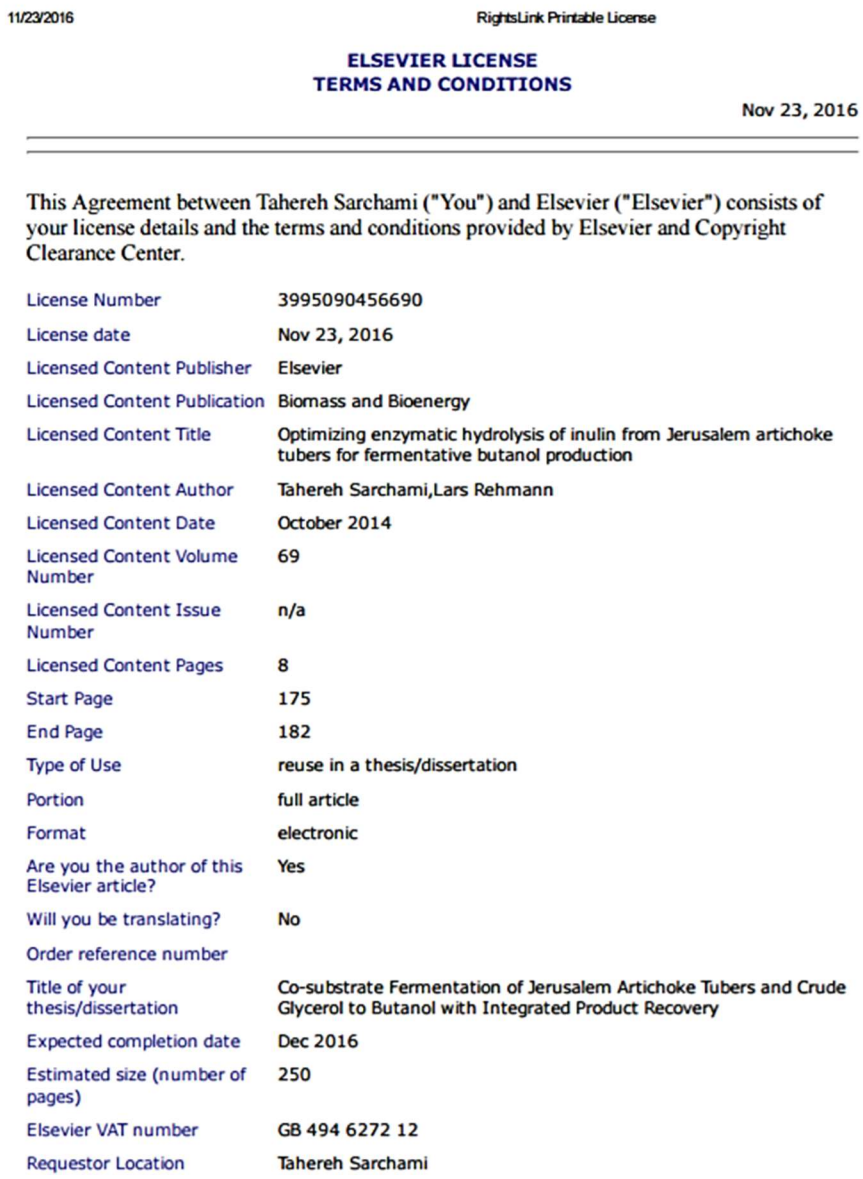

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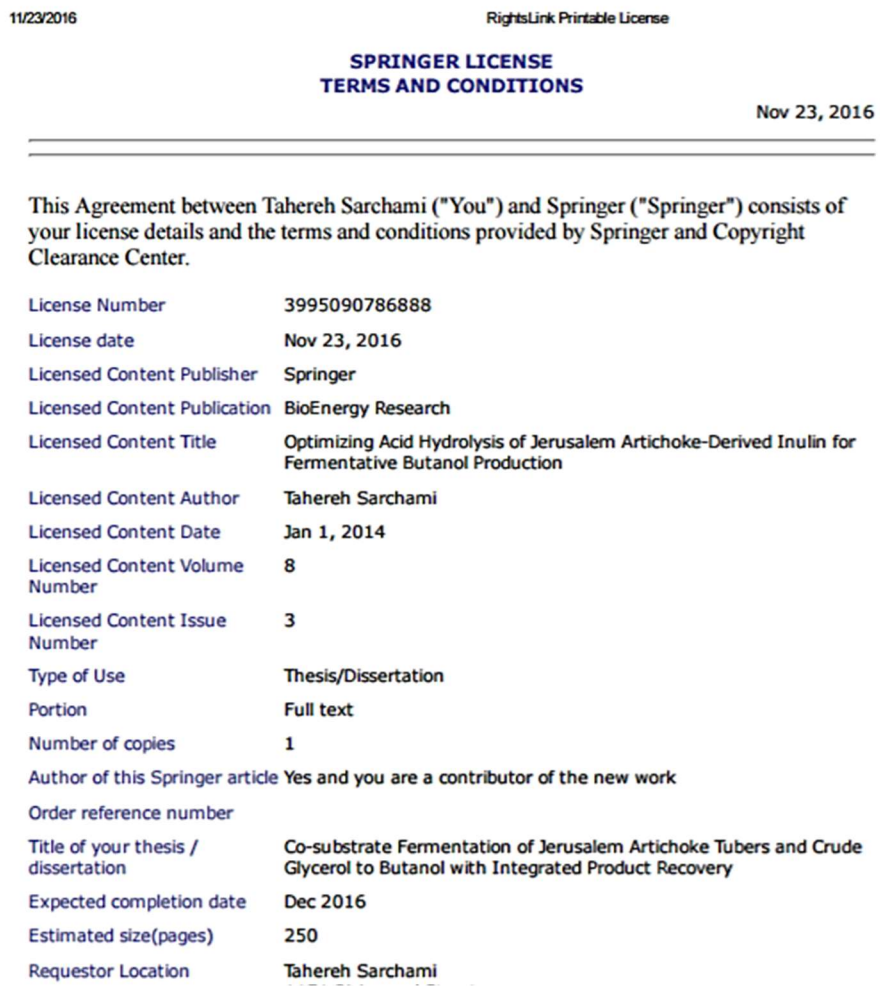

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Curriculum Vitae

EDUCATION

PhD, Chemical and biochemical engineering, University of Western Ontario, Canada 2011- 2016

- First student who got admitted directly into PhD program in the department of chemical and biochemical engineering at university of Western Ontario.

BS, Chemical engineering, Ferdowsi University of Mashhad, Mashhad, Iran 2007- 2010

TEACHING EXPERIENCE

SUMMER ACADEMY INSTRUCTOR (Mentoring high school students), University of Western Ontario, London, Ontario, Canada, 2012-2015.

GRADUATE TEACHING ASSISTANT, Department of Chemical and Biochemical Engineering, University of Western Ontario, London, Ontario, Canada:

- Computational methods for engineers (Second year undergraduate course), Winter 2012.
- Chemical process and plant design (Fourth year undergraduate course), 2012-2013.
- Staged unit operations (Third year undergraduate course), Fall 2013.
- Computer process control (Graduate course), Winter 2014.
- Staged operations (Third year undergraduate course), Fall 2014.
- Chemical kinetics and equilibrium (Graduate course), Fall 2015.

PROFESSIONAL EXPERINECE

ENGINEERING PhD STUDENT RESERACHER

- **Chemical and biochemical engineer, RWTH, Aachen, Germany. October 2015- January 2016.** Collaboration in a research on anaerobic fermentation.
- **Chemical and biochemical engineer, Newalta, Ontario.**
  January 2014- September 2015. Collaboration in a research on "Fermentative Butanol Production from Biodiesel Derived Glycerol".

- **Chemical and biochemical engineer, Agriculture and Agri-Food Canada, Ontario. September 2011- December 2012.** Collaboration in a research on "Fermentative Butanol and Ethanol Production from Jerusalem Artichoke".

INTRENSHIPS

- **Chemical Engineer Intern, Poly pipe manufacturer Co., Mashhad, Iran. June 2009- September 2009.**
- **Chemical Engineer Intern, Metro paper industries tissue group**, **Toronto, Canada. May 2008- September 2008**

VOLUNTEERING EXPERIENCE

- **Judge,** Chemical and biochemical engineering graduate seminar, University of Western Ontario, Winter 2014
- **Chair,** Chemical and biochemical engineering graduate seminar, University of Western Ontario, Fall 2014
- **Co-Chair,** Research Bridge, Sarnia Research park, Canada, Summer 2014
- **Chair,** Research project undergraduate seminar (Capstone), Sarnia Research park, Canada, April 2013

PUBLICATIONS

PUBLISHED MANUSCRIPTS

- **Tahereh Sarchami,** Lars Rehmann **(2014);** Optimizing enzymatic hydrolysis of inulin from Jerusalem artichoke tubers for fermentative butanol production, Biomass and Bioenergy.
- **Tahereh Sarchami,** Lars Rehmann **(2014);** Optimizing acid hydrolysis of Jerusalem artichoke-derived inulin for fermentative butanol production, BioEnergy Research.
- **Tahereh Sarchami,** Erin Johnson, Lars Rehmann **(2016);** Optimization of fermentation condition favoring butanol production from glycerol by *Clostridium pasteurianum,* Bioresource Technology.

- Erin Johnson, **Tahereh Sarchami,** Sascha Kiesslich, Garret Munch, Lars Rehmann **(2016);** Consolidating biofuel platforms through the fermentative bioconversion of crude glycerol to butanol: A Review, World Journal of Microbiology and Biotechnology (WIBI)
- **Tahereh Sarchami,** Garret Munch, Sascha Kiesslich, Erin Johnson, Lars Rehmann **(2016);** A Review of process-design challenges for industrial fermentation of butanol from crude glycerol by non-biphasic *Clostridium pasteurianum*, Fermentation Journal.

PREPARED FOR SUBMISSION

- **Tahereh Sarchami,** Erin Johnson, Lars Rehmann**;** Co-substrate fermentation of Jerusalem artichoke tubers and crude glycerol to butanol by *Clostridium pasteurianum* DSM 525.
- **Tahereh Sarchami,** Erin Johnson, Lars Rehmann; Enhanced butanol production in the integrated fed-batch fermentation process with pervaporation.
- Sascha Kiesslich, Erin Johnson, Garret Munch, Gao Kai, **Tahereh Sarchami**, Lars Rehmann; Pervaporative butanol removal from model solutions and fermentation broths from bioconversion of glycerol using *Clostridium pasteurianum.*

RESEARCH PRESENTATIONS

CONFERENCE PRESENTATIONS

- **(Co-substrate fermentation of crude glycerol and Jerusalem Artichoke tubers to butanol by *Clostridium pasteurianum* DSM 525),** 18th CSChE Quebec-Ontario Biotechnology meeting, Waterloo, Ontario, Canada 2016
- **(Improved butanol production by *Clostridium pasteurianum* using Jerusalem artichoke tubers and glycerol as co-substrate),** Chemical and biochemical engineering graduate seminar, London, Canada 2016
- **(Optimization of fermentation condition favoring butanol production from glycerol by *Clostridium pasteurianum*),** 65th Canadian Chemical Engineering conference, Calgary, Canada 2015

- **(Optimizing enzymatic hydrolysis of inulin from Jerusalem artichoke tubers for fermentative butanol production),** Chemical and biochemical engineering graduate seminar, London, Canada 2015
- **(Comparison of acid and enzymatic hydrolysis of Jerusalem artichoke tubers for fermentative butanol production),** 64th Canadian Chemical Engineering conference, Niagarafalls, Canada 2014
- (**Acid hydrolysis of Jerusalem artichoke for bio-fuel production**), 15th CSChE Quebec-Ontario Biotechnology meeting, Quebec City, Quebec, Canada 2013
- (**Volumetric measurements of pure gas adsorption**), Chemical engineering annual meeting, Mashhad, Iran 2010

POSTER

- (**Pervaporation of butanol from model solutions and fermentation broth using PDMS membrane system)**, BiofuelNet Canada, Vancouver, Canada 2016
- (**Bio-ethanol production from Jerusalem artichoke**), 61th Canadian Chemical Engineering conference, London, Canada 2011

AWARDS

- **Ontario Graduate Scholarship (OGS), 2015-2016.**
- **Western Graduate Research Scholarship (WGRS), 2011-2016.**
- **Queen Elizabeth II Graduate Scholarships in Science and Technology (QEIIGSST), 2012-2013.**
- **E.G.D. Murray Scholarship, 2012.**