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OPTIMIZATION OF WASTEWATER MICROALGAE PRETREATMENT FOR ACETONE,
BUTANOL, AND ETHANOL FERMENTATION

by

Yessica A. Castro

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biological Engineering

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Logan, Utah

2014

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ABSTRACT

Optimization of Wastewater Microalgae Pretreatment for Acetone, Butanol,
and Ethanol Fermentation

by

Yessica Castro, Master of Science

Utah State University, 2014

Major Professor: Dr. Ronald C. Sims
Department: Biological Engineering

The biological production of acetone, butanol, and ethanol (ABE), using wastewater microalgae from the Logan City Wastewater Lagoon System (LCWLS) as the carbon source, is an environmentally sustainable process that addresses the demand for liquid fuel. The fermentation of wastewater requires algal-fermentable sugars to be bioavailable and fermentable media to be enriched for proficient sugar digestion and cellular growth. The evaluation of 54 combinations of the acid concentration, retention time, and temperature in the acid hydrolysis defined the best conditions of the process parameters to increase ABE production while considering operational costs. Sulfuric acid concentrations ranging from 0-1.5 M, retention times of 40-120 min, and temperatures from 23°C- 90°C were combined to form a full factorial experiment. Additionally, the use of cheese whey as co-substrate and nutrient supplement of the medium reduces the costs of the pretreatment process by eliminating the need for some nutrients (i.e. potassium phosphate, magnesium sulfate, and

ferrous sulfate) and increases the concentration of solvents. The results of the project show a production of 11.4 g/L of ABE and 8.5 g/L of butanol with a cost reduction of USD\$0.33/gal of butanol produced. The optimization of wastewater microalgae pretreatment impacts positively the development and scale-up of ABE fermentation by enhancing yield and reducing costs of the process.

(80 pages)

PUBLIC ABSTRACT

Optimization of Wastewater Microalgae Pretreatment for Acetone, Butanol,
and Ethanol Fermentation

Yessica Castro

Acetone-butanol-ethanol (ABE) fermentation from wastewater microalgae by *Clostridium saccharoperbutylacetonicum* N1-4 is a novel bioprocess that utilizes waste substrate to generate valuable solvents. Butanol, the most abundant product resulting from ABE fermentation, is an environmentally safe and high performing fuel that can be utilized as a drop-in-fuel; however, high operational costs and low ABE yield present challenge in scale-up of the process. The utilization of algae as a substrate requires pretreatment prior to fermentation to increase the bioavailability of the algal fermentable sugars and to improve the conditions of the pre-fermentation medium. The purpose of this thesis was to optimize wastewater microalgae pretreatment through (1) the optimization of microalgae saccharification, and (2) the use of cheese whey as co-substrate and supplement.

Optimal conditions for sugar liberation from wastewater algae through acid hydrolysis were determined for subsequent fermentation to acetone, butanol, and ethanol (ABE). Acid concentration, retention time, and temperature were evaluated to define optimal hydrolysis conditions by assessing sugar and ABE concentrations, and the associated costs. Additionally, the effect of cheese whey as a supplement and substrate was determined for acetone, butanol, and ethanol (ABE) fermentation from wastewater microalgae. Three media

constituents, potassium phosphate, magnesium sulfate, and ferrous sulfate, were evaluated to assess their need as supplements in the medium to be inoculated, when 50 g/L of cheese whey was present. The optimization of wastewater microalgae pretreatment results in increasing ABE production and decreasing process costs.

To God almighty, Father, Son, and Holy Spirit:

That your perfect will on my life be accomplished to glorify your Name through my work.

To my parents, Julio Castro and Altagracia Estevez:

Thanks for your love, trust, and encouragement.

To my grandma, Edelmira Cedano

To my siblings, Julissa, Jennifer, Julio Francisco, and Stephany:

Thanks for being my unconditional friends.

To my beloved country, Dominican Republic:

That my life and work might contribute to your development.

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Yessica A. Castro

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CHAPTER 1

INTRODUCTION

There is no certainty regarding fossil fuel life expectancy, however global consumption of energy keeps increasing. Renewable engineering technologies based on solar, wind, hydro, and thermal sources have been successfully developed to address some of the electricity demand; conversely, approaches to cover the liquid fuel consumption still present problems. One limitation concerning the industrial production of alternative fuels such as biodiesel and bioethanol is the economic and environmental impact caused by the feedstock. The use of grains and oilseeds as feedstock biomass to produce biodiesel and bioethanol causes a decrease in world food supply and has a significant impact on food commodities prices (Timilsina et al., 2012; Zilberman et al., 2012). Additionally, pesticides connected with biofuel production are reported to contaminate water resources, give rise to health problems, and contribute to the shortage of a limiting nutrient (i.e. phosphorus) (Eide, 2008; UPI, 2013). The production of corn and soybean to generate biodiesel and bioethanol is expensive due to the use of supplies such as fertilizers, pesticides, and seeds (Tiffany, 2009). Therefore, studies to develop alternative and sustainable biofuels are important.

The production of butanol using wastewater algae as the carbon source may be an environmentally and economically competitive process. The removal of algae from wastewater municipal facilities helps to mitigate the environmental pollution of the water to be disposed into the natural reservoirs and stimulates domestic economic growth. The harvesting of algae from wastewater municipal systems would result in a reduction of total

dissolved phosphorus (TDP) and total dissolved nitrogen (TDN) in discharges from these systems (Christenson and Sims, 2012). According to Jones and Woods (1986), 60% of the overall ABE production costs were due to the costs of substrates in the 20th century. The microalgae harvested from wastewater systems is a low cost substrate for biobutanol fermentation. Furthermore, butanol is environmentally competitive with biodiesel, and safer to transport and combust than gasoline and ethanol. Low vapor pressure point, high flash point, and low corrosive properties make butanol feasible to transport in pipelines (Jin et al., 2011; Ramey, 2007). Studies have shown greenhouse emissions of n-butanol blends to be lower than emissions of pure diesel (Durre, 2007; Rakopoulos et al., 2010). Butanol can be used effectively alone or blended in diesel fuel in commercial vehicle engines without engine modification (Rakopoulos et al., 2010; Ramey, 2007). Furthermore, butanol has a competitive performance as liquid fuel. Some advantages of butanol over ethanol are the higher energy content, lower water adsorption, and better blending ability (Dürre, 2007). Therefore, butanol from wastewater algae may be a suitable sustainable fuel in transportation.

Solvents obtained from ABE fermentation can be commercially used to manufacture several products. Butanol not only is useful as liquid fuel for transportation, but also as auxiliary material in the production of goods. In 1919 butanol began to be used as solvent for nitrocellulose lacquer of low viscosity (Jones and Woods, 1986). Butanol can be used to produce butyl acetate, which is a substitute of amyl acetate for the automotive industry (Dürre, 2007). Similarly, acetone has been used for military, cosmetic, and food industries. In the last century, the large scale production of ABE was triggered by the demand of cordite, which was a material used in World War I as a substitute for gunpowder. The production of cordite required the use of acetone (Dürre, 2007; Jones and Woods, 1986). Acetone is also

widely used as an ingredient in cosmetics such as nail polishes and polish remover, hair tonics, and tanning lotions and oils. Also, acetone is classified as GRAS grade “generally recognized as safe,” and is present in beverages, baked food, and desserts in a concentration range of 5-8 mg/L (Hernandez, 1999). Thus, ABE fermentation from wastewater algae represents an alternative for sustainable and valuable solvents such as acetone and butanol.

The fermentation of butanol was first achieved by Louis Pasteur in 1861. Since then, the anaerobic process had been modified to obtain not only butanol, but also acetone and ethanol (Jones and Woods, 1986). As a result, the production of these solvents by biological means is well known as acetone-butanol-ethanol (ABE) fermentation. The ABE fermentation is accomplished by a bacterial strain, generally of the *Clostridium* species, which commonly use hexoses as the carbon source. However, studies show that ABE can be produced using pentose as either the sole carbon source or co-substrate (Raganati et al., 2012; Yoshida et al., 2012; Zheng et al., 2013). When the substrates are raw materials (i.e. non-monosaccharides), pretreatment is required. The general ABE production process is performed in three main stages: (1) pretreatment of biomass, (2) fermentation, and (3) recovery (see Figure 1-1). The pretreatment main objectives are to lyse the cells to make the sugars bioavailable and to hydrolyze polysaccharides into monosaccharides (Hemming, 2011). As a result, the pretreatment stage is of most interest for researchers due to the pretreatment effect on the system outcome.

Parameters used in algae pretreatment affect the economic success and performance of the ABE production. Activities related to biomass pretreatment including hydrolysis, and nourishment have an impact on the total production cost of ABE. Kumar et al. (2012) stated that ABE capital cost increases by about 37% of the due to pretreatment steps when raw

materials instead of sugars are used as the substrate. In addition, the fermentation medium must meet certain conditions prior to inoculation, such as supplementation and sterilization. The supplementation of the pre-fermentation medium for ABE depends on the substrate used. When ABE was industrially produced at the beginning of the 20th century, the use of molasses as substrate required the addition of nutrients at industrial scale. However, the use of maize mash as a carbon source required minimal supplementation (see Table A-1, Appendix A). The determination of optimal parameters for hydrolysis, nourishment, and sterilization phases is necessary for improvements in the yield and total production cost of ABE. Thus, studies to achieve both optimization of the saccharification of the algae and supplementation of the medium in the pretreatment process must be conducted prior to the ABE scale-up.

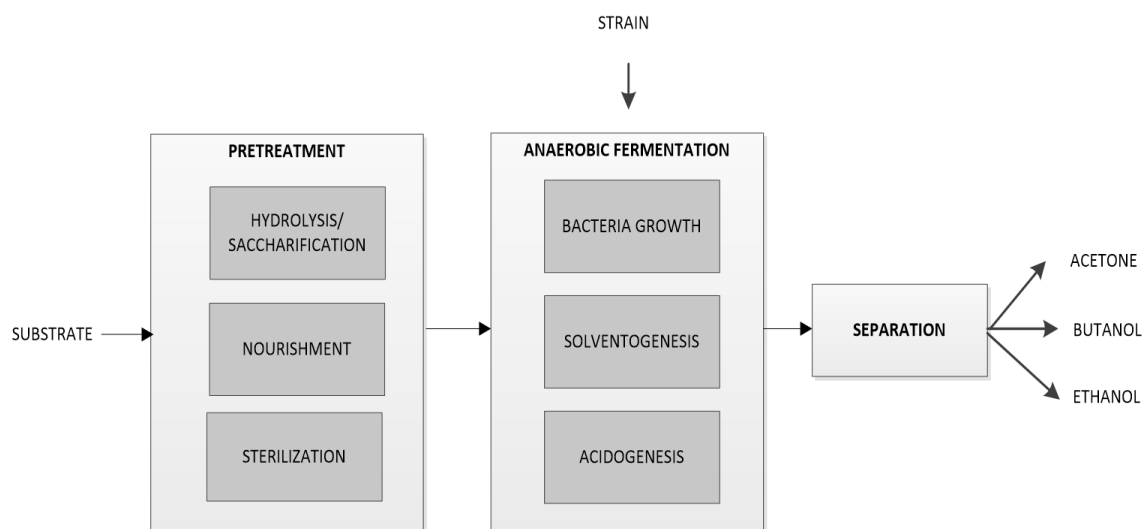


Figure 1-1. Flow of Acetone-Butanol-Ethanol (ABE) production. Figure modified from Hemming (2011).

CHAPTER 2
WASTEWATER MICROALGAE SACCHARIFICATION USING ACID
HYDROLYSIS FOR ACETONE, BUTANOL, AND ETHANOL
(ABE) FERMENTATION¹

2.1. Abstract

Exploring and developing sustainable and efficient technologies for biofuel production are crucial for averting global consequences associated with fuel shortages and climate change. Optimization of sugar liberation from wastewater algae through acid hydrolysis was determined for subsequent fermentation to acetone, butanol, and ethanol (ABE) by *Clostridium saccharoperbutylacetonicum* N1-4. Acid concentration, retention time, and temperature were evaluated to determine optimal hydrolysis conditions by assessing the sugar and ABE yield as well as the associated costs. Sulfuric acid concentrations ranging from 0-1.5 M, retention times of 40-120 min, and temperatures from 23°C- 90°C were combined to form a full factorial experiment. Acid hydrolysis pretreatment of 10% dried wastewater microalgae using 1.0 M sulfuric acid for 120 min at 80-90°C was found to be the optimal parameters, with a sugar yield of 166.1 g for kg of dry algae, concentrations of 5.23 g/L of total ABE, and 3.74 g/L of butanol at a rate of USD \$12.54 per kg of butanol.

¹Co-authors: Joshua Ellis, Charles Miller, and Ronald Sims

2.2. Introduction

While the global consumption of petroleum based products keeps increasing, the demand for alternative, renewable, and efficient energy technologies gains interest. Renewable energy technologies such as solar, wind, hydro, and thermal sources have been successfully developed to mitigate the energy demand; however, renewable approaches to cover liquid fuel consumption, such as biodiesel and bioethanol production, have economic and environmental impacts due to the feedstocks used. The use of corn and soybeans as feedstocks for biofuel production requires fertilizers, pesticides, and seeds, which increases the final cost of the bioproducts (Tiffany, 2009). Growing these crops for biofuel production not only affects food prices, but also triggers the contamination of water by the indiscriminate use of pesticides and fertilizers (Eide, 2008; Timilsina et al., 2012; Zilberman et al., 2012). As a result, studies to find alternative, low cost, and environmentally friendly feedstocks for the production of biofuels are being widely established.

The biological production of acetone, butanol, and ethanol (ABE) using wastewater algae as the carbon source is an environmentally sustainable process that could mitigate the demand for petroleum fuel. Butanol, the most abundant solvent produced in ABE fermentation, is an environmentally friendly and competitive drop-in-fuel that can be directly used in vehicles and has a comparable energy density to gasoline (Dürre, 2007; Jin et al., 2011; Rakopoulos et al., 2010). In addition, n-butanol is a superior transportation fuel over ethanol because of its higher energy content, immiscible properties, lower volatility, lower corrosibility, and lower hygroscopicity (Srirangan et al., 2012). The production of ABE from wastewater algae takes advantage of the substrate source to minimize derived costs of production. Some of the advantages of wastewater algae over other terrestrial biomass

include lower nutrient requirements for growth, higher growth rate, higher yield per cultivation area, less land area requirement, non-fresh water required for growth, and non-competition with food crop demand (Pate et al., 2011; Rawat et al., 2013; Sayadi et al., 2011; Vasudevan and Briggs, 2008). In addition, microalgae harvested from municipal wastewater lagoons are considered a bioremediation technique for removing phosphorus and nitrogen to prevent downstream eutrophication (Christenson and Sims, 2012; 2011). Similarly, concentrated CO₂ emissions from industrial sources can be redirected to municipal wastewater lagoons to be used as supplemental CO₂ for algae growth; thus, mitigating CO₂ emissions (Pate et al., 2011). ABE fermentation using microalgae from the Logan City Wastewater Lagoon System (LCWLS) by *C. saccharoperbutylacetonicum* was previously demonstrated (Ellis et al., 2012).

The production of ABE from a raw material such as wastewater algae requires pretreatment prior to fermentation to make the sugars bioavailable. The sugar content in algae is reported to be up to 50% dry weight (Chen et al., 2013). According to preliminary studies (Figure B-1 and B-2, Appendix B), wastewater microalgae yields xylan, maltose, glucose, and xylose after thermal and dilute acid hydrolysis. Glucose, maltose, and xylose have been reported to be fermentable sugars by using various strain of *C. saccharoperbutylacetonicum* (Al-Shorgani et al., 2011; Ferchichi et al., 2005; Jones and Woods, 1986; Kumar and Gayen, 2011; Yoshida et al., 2012). Methods used to achieve saccharification of recalcitrant feedstocks are enzyme digestion, thermolysis, dilute acid hydrolysis, and concentrated acid hydrolysis (Harrison et al., 2003; Kang et al., 2012). Ellis et al. (2012) conducted experiments to compare different pretreatment conditions used to produce ABE from wastewater algae. The ABE fermentation using dilute acid hydrolysis for

wastewater microalgae pretreatment produced the lowest ABE yield, 2.74 g/L. The highest ABE yield (9.74 g/L) was obtained with the combination of acid hydrolysis and enzymatic digestion. However, enzymatic digestion is an expensive method that increases the cost of ABE production (Kumar and Murthy, 2013). In general, the costs associated to pretreatment process of feedstock for biofuel production range from 40 to 70% of the selling prices of biofuel (Srirangan et al., 2012). Thus, the selection of methods for wastewater microalgae saccharification needs to take into account the cost involved.

Acid hydrolysis of wastewater algae as pretreatment for ABE fermentation by *Clostridium* spp. is a potentially effective and low cost method. The negligible content of lignin into microalgae reduces the costs, time, and difficulty of the conversion process (Harun et al., 2014). Similarly, because *C. saccharoperbutylacetonicum* is an amylolytic microorganism, the enzymatic hydrolysis step for the conversion of starch into fermentable sugars is not required (Thang et al., 2010). Studies focused on acid hydrolysis as pretreatment process to digest cellulose and hemicellulose in algae have been already conducted (Kang et al., 2012; Khambhaty et al., 2012; Setyaningsih et al., 2012; Wang et al., 2011; Yazdani et al., 2011). However, there are no studies to date regarding the optimization of wastewater microalgae for ABE fermentation using acid hydrolysis as a saccharification method. The optimization of algae saccharification through acid hydrolysis will result in increased fermentable sugar yields from microalgae while accounting for the cost of the process. Kinetic studies on the dilute acid hydrolysis of cellulosic materials indicate that the acid hydrolysis efficiency depends on substrate, acid concentration, temperature, and retention time (Wang et al., 2011). The optimization of microalgae acid hydrolysis through evaluation of these parameters will result in an increased yield of fermentable sugars in the

medium. Currently, there are no statistically detailed studies that describes the cost analysis associated with the evaluated parameters for acid saccharification of wastewater microalgae. The aim of this study was to optimize acid hydrolysis using wastewater microalgae for subsequent ABE fermentation by determining the conditions that yields the highest ABE concentration while controlling the costs of the process.

2.3. Materials and methods

Algae biomass

Mixed microalgae biomass from the LCWLS was grown in SE media containing 850 mg NaNO₃, 350 mg KH₂PO₄, 150 mg MgSO₄·7H₂O, 150 mg K₂HPO₄, 50 mg CaCl₂·2H₂O, 50 mg NaCl, and 15 mg C₆H₈O₇·Fe·NH₃ per liter of ddH₂O. The biomass was freeze dried through sublimation for 48 hours. The dry biomass was maintained at 4°C prior to pretreatment. The mixed microalgae feedstock was primary dominated by *Scenedesmus*, *Chlorella*, *Ankistrodesmus*, *Micromonas*, and *Chlamydomonas*, as previously described (Ellis et al., 2012).

Reagents

Reagent grade chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified.

Acid hydrolysis

Algae (3.5 gdw) diluted in 35 mL of ddH₂O was placed in 100 mL serum vials with crimp top, 52 mm diameter, and 95mm height. The ranges of temperature (25-30°C, 45-55°C, and 80-90°C) were achieved by the use of different hotplates. For room temperature, a magnetic stirrer at stirring level 7 was used; for 45 -55°C, a Fisher Scientific Isotemp Basic

stirring hotplate (Cat. 11-100-100SH) at 1200 RPM, and for 80-90°C a Fisher scientific stirring hotplate (Cat. 11-520-49SH). Stirring bars of 1 inch were used through the experiment. The temperature was monitored using a thermometer and controlled to be maintained within the desired temperature range. The acid concentrations used were 0.0 M, 0.35 M, 0.50 M, 0.70M, 1.00 M, and 1.50 M. Retention times were 40 min, 80 min, and 120 min. Ca (OH)₂ was used to neutralize the hydrolyzed medium. The medium was clarified by means of centrifugation (1500 g for 20 min) before and after neutralization. Samples were filtered (0.2 µg) prior to carbohydrate analysis. The experiment was conducted in duplicates. Figure 2-1 illustrates the steps of the acid hydrolysis experiment.

ABE fermentation

Batch fermentations were performed in 10 ml serum vials. The pH of the media was adjusted to 6.5±0.5 prior to fermentation. The head space of the serum vials was flushed with nitrogen gas prior to the start of fermentation. The fermentation was initiated by inoculating with a 10% (*v/v*) actively proliferating (mid-log phase or 24 h vegetative growth) culture of cells in RCM media. All experiments were conducted at a constant temperature of 30 °C.

Analytical methods

Sugars were quantified by use of High-performance liquid chromatography (HPLC, LC-10AT Shimadzu) along with a CTO-10A Shimadzu column oven equipped with a carbohydrate guard column 802G BP-100H+ and an analytical column 802 BP-100H+, both manufactured by Benson Polymeric. The mobile phase used was 100% ddH₂O. The samples were injected at a flow rate of 0.4 mL/min by SIL-10A auto injector and detected by an Evaporative Light Scattering Detector (ELSD-LT II), both manufactured by Shimadzu.

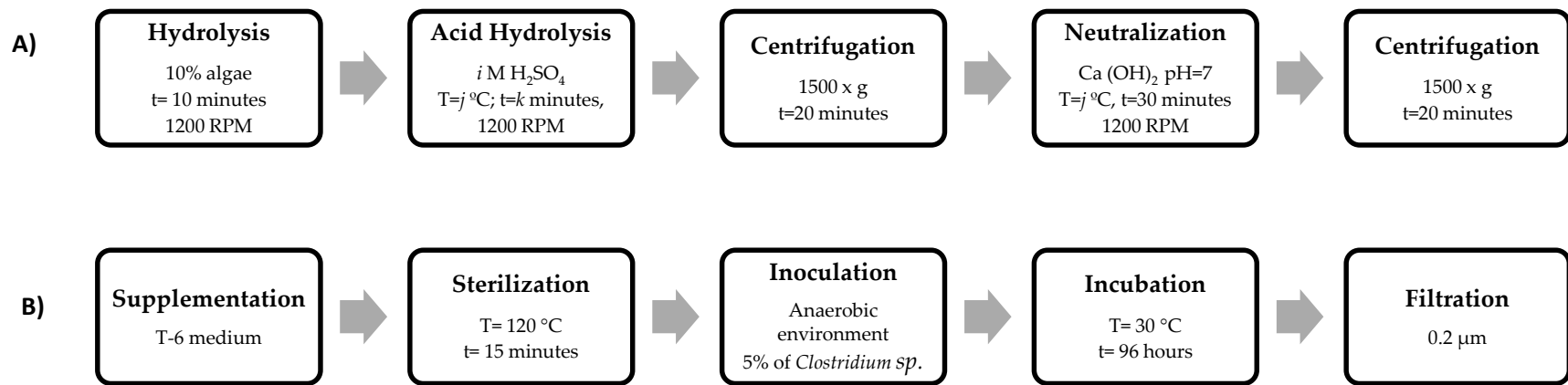


Figure 2-1. Flow diagram for dilute acid hydrolysis. The factors for the experiment are: acid concentration (i), temperature of reaction (j), and retention time (k). A) Operations for algae hydrolysis prior to sugar determination using HPLC. B) Operations required for fermenting the hydrolyzed algae medium before ABE determination using GC.

Standard curves for maltose, glucose, and xylose were generated with $R^2 > 0.99$. The peak elution time for maltose was 14.3 min, for glucose was 17.1 min, and for xylose was 18.3 min. Other peaks of interest were: galactose, 18.3 min; mannose, 18.2 min; maltotriose, 12.3 min; xylan, 10.5 min; formic acid, 10.4 min; and sulfates 10.6 min. Mannose, galactose, and xylose were essentially inseparable, as well as xylan, formic acid, and sulfates.

ABE concentration was evaluated using gas chromatography (7890B GC-System, Agilent Technologies, USA) equipped with a FID detector along with a Restek Stabiwax-DA, 30 m, 0.32 mmID, 0.25 μm df column. The inlet had an initial temperature of 30 °C for 1 min, ramped up at 5 °C/min up to 100 °C, and had a final ramp of 10 °C/min up to 250 °C. The column had a flow of 4 ml/min, pressure 15 psi, average velocity 54 cm/s, and holdup time 0.93 min. The initial oven temperature was 30 °C for 1 min, and then ramped up 5 °C/min up to 100 °C (no hold time), then ramped up to 20 °C/min up to 225 °C (no hold time), with a final ramp of ramp 120 °C/min up to 250 °C and hold for 2 min. All samples were clarified by centrifugation prior to analysis. Volumetric productivity was calculated as the concentration of solvents produced per hour (g/Lh).

Energy calculations

Energy calculations are based on a 10,000 L batch system at 50% capacity. The heat energy and the power of the mixing/stirring were calculated using the equations 2.1 and 2.2, respectively (see Table 2-1). The specific heat (cp) of the algae slurry is estimate to be 3.86 kJ $\text{kg}^{-1} \text{ } ^\circ\text{C}^{-1}$, calculations based on equation for cp estimations at temperatures above freezing from Earle (1983). The 10,000 L bioreactor used for the calculations is assumed to have a diameter of 75 inches and 135 inches height, based on the height to diameter ratio ($H/D \approx 1.8$) at bench scale experiment. Similarly, the impeller diameter used for calculations is 40 inches,

in order to keep the Dimpeller to Dtank proportion used in bench scale (i.e. $D_{\text{tank}}/D_{\text{impeller}} \approx 0.5$).

The rotational impeller speed was calculated using the rotational centrifugal force (RCF) from the bench scale experiment at 70% efficiency of stirring plates, obtaining $RCF \approx 10g$. The power number (N_p) of impeller is based on the Reynolds number ($N_{re} \approx 1.5 \times 10^5$), where the viscosity of the hydrolyzed slurry of 100 kg/m^3 mixed microalgae is assumed to be equal to *Chlorella vulgaris* at 80 kg/m^3 (i.e. $0.016 \text{ Pa}\cdot\text{s}$) (Coker, 2011). The estimation of N_p is attained through the Power number-Reynolds number correlation graph, assuming 3-blade hydrofoil impeller with wide blades is used (Smith, 2011). Calculations on the specific gravity of the algal slurry resulted in 1.16 when the acid portion is assumed to be the average molarity of 1.25M sulfuric acid. Previous calculations of specific gravity on *Chlorella*, with a moisture content of 75% (w/w), resulted in 1.1, which is similar to the value obtained in our study for the hydrolyzed mixed culture algae slurry (Boersma et al., 1978).

Cost analysis

Estimates of pretreatment costs were calculated to determine the cost effects of the factors including acid concentration, retention time, and temperature. The cost of the energy is assumed to be USD $\$0.12/\text{kWh}$, which is equivalent to USD $\$0.03/\text{MJ}$ based on the US Energy Information Administrator website (www.eia.gov). Costs of sulfuric acid and calcium hydroxide were assumed to be USD $\$0.26/\text{kg}$ and USD $\$0.20/\text{kg}$ respectively, based on the costs shown at Alibaba global trader website (www.alibaba.com). For details on costs calculations see sections C-3 and C-4 from Appendix C.

Table 2-1. Equations for cost analysis of wastewater microalgae acid hydrolysis. Equation 2.1 quantifies the cost due to temperature of reaction (°C) while equation 2.2 accounts for the cost due to retention time (min) by quantifying the power required for mixing.

Eq. 2.1	$Q = m c_p \Delta T$	<p>Q Energy (kJ) need to raise the temperature of algae slurry from room temperature (25°C) to optimal temperature.</p> <p>m Mass of algae slurry (kg)</p> <p>c_p Specific heat capacity of slurry= 3.86 kJ/kg°C</p> <p>ΔT Rise in temperature of the algae slurry (°C)</p>
<hr/>		
Eq. 2.2 ^a	$SHP = \frac{N_p N^3 D^5 S.G}{1.53 \times 10^{13}}$	<p>SHP Shaft horsepower (HP)</p> <p>N_p^b Power number of impeller. For a 3-blade hydrofoil impeller, wide blades, $N_p=0.60$</p> <p>N Impeller speed (RPM). N=130 RPM</p> <p>D Diameter of the impeller (in). D=40</p> <p>S.G Specific gravity of solution. S.G=1.16</p> <p>1.53×10^{13} Conversion factor</p>

^a Equation based on Principles of fluid mixing by Brawn mixer, Inc. (2003).

^b Based on Reynold's number ($N_{re}=ND^2Q/\mu$), $N_{re} \approx 1.5 \times 10^5$; and estimated from N_{re} - N_p correlation graph (Smith, 2011).

Statistical analysis

The sugar yields of the 54 treatment combinations in duplicates (resulting from 6 levels of acid concentration, 3 levels of retention time, and 3 levels of temperature) were analyzed as a complete factorial design ($\alpha=0.05$) using the Statistical Analysis Software (SAS). Similarly, the concentrations of butanol (g/L) were analyzed as unreplicated full factorial structure, which only accounts for the effect of acid concentration and retention time on butanol. Butanol concentration is an indicator of the parameters combination success regarding ABE fermentation. The assumptions of the analysis are normality and independence of variance. The comparison between the butanol costs was performed using t-test statistics from Graphpad website (<http://www.graphpad.com/quickcalcs/>).

2.4. Results and discussions

Effect of temperature, retention time, and acid concentration on sugar yield

The sugar yield of pretreated samples at a temperature range of 80-90°C is significantly higher than the samples pretreated at lower ranges ($p\text{-value}<0.05$). The interaction plot of the factors time and temperature is shown in Figure 2-2. According to these results, the optimal temperature range to be used in the pretreatment of wastewater algae biomass is 80-90°C. In contrast, room temperature is associated to the lowest sugar yield. Previous studies on acid hydrolysis used temperatures ranging from 110-130°C (Setyaningsih et al., 2012; Wang et al., 2011; Yazdani et al., 2011). However, calculations (see Eq. 2.1) show that the use of temperatures from 120-130°C instead of 80-90°C results in a heat energy cost increase of 46-90%. On a 10,000L batch system at 50% capacity, heating costs for temperature pretreatment at 45-55°C, 80-90°C, and 120-130°C are USD\$18.6, USD\$44.3, and

USD\$73.9, respectively. Studies have been conducted to evaluate high thermal pretreatment (i.e. 170°C) at retention times from 5-40 min revealing an increase of cellulose when the substrate (i.e. poplar) was pretreated from 20-30 min (Ma et al., 2014). Since the sterilization is a required process that occurs at 120°C (see Figure 2-1), the evaluation of high thermal pretreatment of wastewater algae at 170°C for 20-30min might be useful to eliminate the need of acid pretreatment and to reduce the operations of the pretreatment process by merging the saccharification and sterilization of the substrate medium.

The analysis of variance (ANOVA) indicates that the effect of reaction time on sugar yield is not significant (p -value > 0.05). Table 2-2 demonstrates that there is not significant effect of retention time at each level of acid concentration on sugar yields. For sulfuric acid concentrations 0.0 M, 0.35 M, 0.50 M, 0.70 M, and 1.50 M there is no significant difference between the retention time levels (40 min, 80 min, and 120 min). However, when 1.0 M of H₂SO₄ is used, 120 min of retention time (group A) is significantly higher than 40 and 80 min (group B), with a sugar yield of 166.1 g/Kg of dry algae at retention time 120 min and 65.2 -71.16 g/Kg at a retention time of 40 min and 80 min. The results obtained in this study are similar to those obtained by Yazdani et al. (2011), where the effect of retention time depended on the acid concentration. Acid hydrolysis concentrations from 0.05M to 0.70 M were evaluated, resulting in an increasing of glucose at 0.35M and 0.70M H₂SO₄ respectively. However, other sugars analyzed behaved differently, for instance, xylose showed positive correlation with retention time at 0.70M H₂SO₄, and galactose showed no correlation with retention time at any of the acid concentrations analyzed (Yazdani et al., 2011). Thus, the effect of retention time on the sugar yield not only depends on acid concentration, but also in the type of sugar.

The effect of sulfuric acid concentration on sugar yield is significant. The results presented in Table 2-2 show that the amount of sugar obtained after acid hydrolysis increases with the acid concentration. Group A yields the highest sugar concentration with values from 118.4-182.1 g of sugars/kg of dry algae hydrolyzed. Group A is associated with the highest concentration of sulfuric acid (1.5 M and 1.0 M) while group C is associated with concentrations of H₂SO₄ ranging from 0.0 M to 1.0 M, with sugar concentrations of 15.7-84.5 g/Kg.

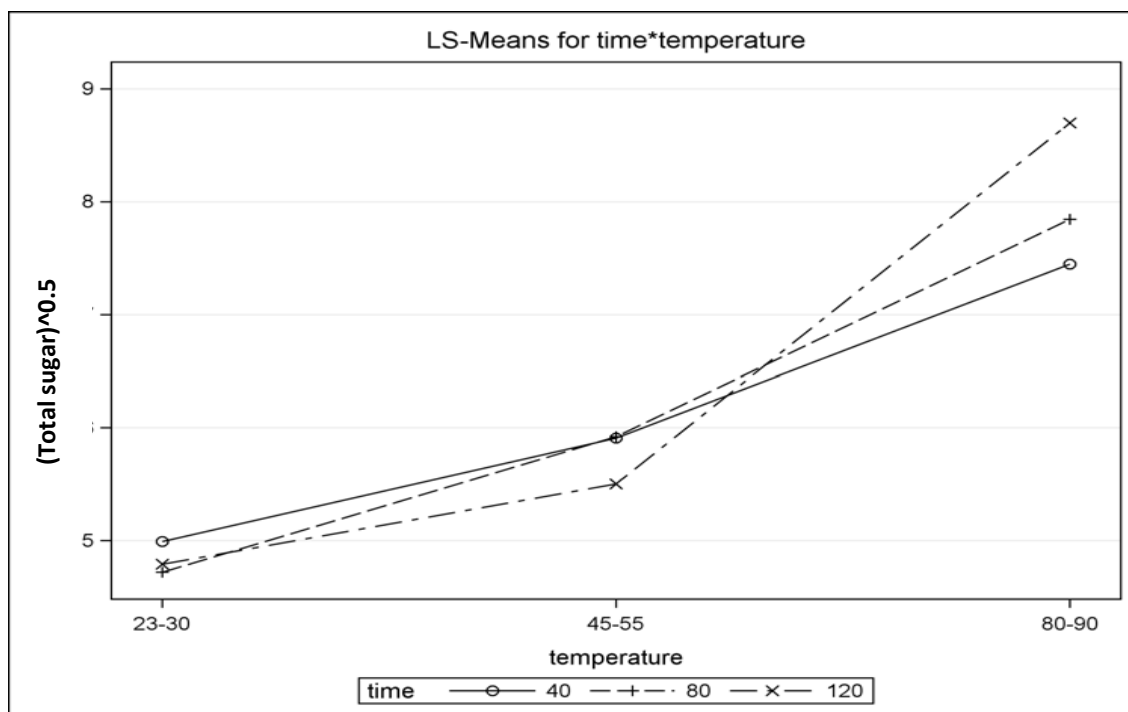


Figure 2-2. Interaction plot of sugar yield in terms of temperature (°C) and retention time (min). Y axis is the transformation of sugar yield for statistical analysis, $T_sugars = (\text{Sugar})^{0.5}$, measured in g/Kg of dry algae. At 80-90 °C, the sugar yield is significantly higher than 23-30°C and 45-55°C. The effect of retention time is not significant. Figure extracted from SAS results with R-squared of the model equal to 0.92.

Table 2-2. Comparison of sugar yield for combinations of acid concentration and retention time at 80-90°C. Groups with the same letters are not significantly different. The highest sugar yield (group A) occurs when the substrate is pretreated with sulfuric acid is 1.5 M for 40 to 120 min and at 1.0 M for 120 min. The lowest sugar concentration is obtained when no acid is added. Sugar values are grams of total sugar per kilogram of dry algae (g/kg). Table based on SAS results with R square of 0.94.

Pretreatment conditions		Sugars (g/Kg) ^a				Total	Statistical Grouping
Sulfuric acid (M)	Retention time (min)	Maltose	Glucose	Xylose/ galactose			
1.50	80	10.2	103.1	68.8	182.1	A	
1.50	120	ND	99.6	74.4	174.0	A	
1.00	120	20.9	81.5	63.7	166.1	A	
1.50	40	10.9	54.4	53.1	118.4	A B	
0.70	120	9.5	34.0	41.0	84.5	C B	
1.00	40	14.8	19.3	37.1	71.2	C B	
1.00	80	6.8	22.6	35.8	65.2	C B	
0.70	40	11.5	16.7	34.7	62.9	C B	
0.70	80	7.8	16.1	32.6	56.5	C B	
0.50	120	6.3	14.4	30.6	51.3	C B	
0.50	80	3.8	13.8	31.6	49.2	C B	
0.35	80	4.1	11.9	30.2	46.2	C	
0.50	40	ND	11.9	28.8	40.7	C	
0.35	40	ND	9.9	26.5	36.4	C	
0.35	120	ND	10.5	24.9	35.4	C	
0	40	13.6	12.2	ND	25.8	C	
0	80	11.0	8.5	ND	19.5	C	
0	120	11.8	3.9	ND	15.7	C	

^a Tukey grouping of sugar estimates based on least squares means ($\alpha=0.05$).

Table 2-3. ABE fermentation of optimal pretreated algae. ABE fermentation of algae pretreated by acid hydrolysis at a temperature range of 80-90 °C using the combinations of acid concentration and retention time associated with significantly higher sugar yields.

Sulfuric acid (M)	Retention time (min)	Sugar (g/kg) ^a	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L) ^b	ABE Concentration (g/L) ^c	ABE Volumetric productivity (g/Lh) ^d	Estimates costs per batch ^e			
								Acid/base (USD\$/L)	Energy (USD\$/L) ^f	Total Cost (USD\$/L) ^g	Butanol rate (USD\$/kg) ^h
1.00	40	71.2	0.72	2.86	0.43	4.01	0.024	0.037	0.0097	0.0467	16.32
1.00	80	65.2	0.84	3.28	0.42	4.54	0.022	0.037	0.0098	0.0468	14.27
1.00	120	166.1	0.96	3.74	0.53	5.23	0.021	0.037	0.0099	0.0469	12.54
1.50	40	118.4	0.83	3.05	ND	3.88	0.024	0.055	0.0097	0.0647	21.21
1.50	80	182.1	1.33	3.85	0.46	5.64	0.027	0.055	0.0098	0.0648	16.88
1.50	120	174.0	1.01	3.17	ND	4.18	0.017	0.055	0.0099	0.0649	20.47
Control ⁱ		-	0.12	0.59	0.10	0.81					

^a Sugar yield estimates are measured in grams of sugars per kilogram of dry algae hydrolyzed.

^b Ethanol concentrations with values out of the method detection limit are expressed as ND.

^c Total solvents (ABE) concentration is measured in grams of solvent per liter of wet algae (10% W/V).

^d The productivity is calculated assuming the pretreatment and fermentation times as total time.

^e Calculations based on a 10,000 L batch pretreatment system at 50% of its capacity. Values are in USD\$ per liter of wet algae (10% W/V).

^f Energy based on heating from room temperature to 90°C and mixing (see eq. 2.7.1 and eq. 2.7.2).

^g Total cost of batch per liter of wet algae (10% W/V) based on the acid /base and energy costs.

^h Rate of the pretreatment cost in USD per estimate kg of butanol produced.

ⁱ Inoculation of *c. saccharoperbutylacetonicum* in T-6 medium without sugar added.

Acetone-butanol-ethanol from wastewater algae

The results of the fermentation of sugars by *C. saccharoperbutylacetonicum* using parameter combinations associated with high sugar yield are shown in Table 2-3. There is no significant difference (p value < 0.05) between the butanol concentrations obtained from all the factorial combinations of sulfuric acid (1.0 M and 1.5 M) and retention time (40 min, 80 min, and 120 min), at 80-90°C in the acid hydrolysis of wastewater algae. Butanol concentrations at the optimal conditions range from 2.86 to 3.85 g/L, with a mean of 3.33 g/L. Even though there is no significant difference in the ABE concentration based on SAS analysis, the determination of the most suitable pretreatment combination requires taking into account the costs of the process in terms of acid, base, and energy consumed. The costs related to acid concentrations of 1.0 M and 1.5 M are 0.037 and 0.055 USD \$/L respectively. The costs associated with retention time are due to the energy consumed by mixing and stirring (see Table 2-1, Eq. 2.2). The costs of energy consumed due to heating from room temperature to 80-90°C and stirring for 40, 80, and 120 min are \$0.0092, \$0.0093, and \$0.0094 dollars per liter of wet algae (10% w/v) pretreated, respectively. According to these results (Table 2-3), the costs associated with acid and base consumption are statistically higher than the costs related to energy due to temperature (80-90°C) and retention time (agitation), with a p -value < 0.05 from t-test statistics. Pretreatment combinations where the acid concentration is lower would result in a more economical process.

Acid hydrolysis of wastewater algae using 1.0 M of H₂SO₄ at 80-90°C per 120 min is the most suitable combination with a sugar yield of 166.1 g/Kg of dry algae, 5.23 g/L of ABE, and 3.74 g/L of butanol concentration at a rate of USD \$12.54 per kg. The use of sterilization was considered when conducting these studies; however a previous report by our research

group revealed minimal sugar liberation when no pretreatment was conducted prior to sterilization in pH neutralized medium (Ellis et al., 2012). Previous ABE studies using wastewater microalgae under similar conditions, 1.0 M of sulfuric acid at 90°C for 30 min and had a lower ABE concentration of 2.74 g/L (Ellis et al., 2012). However, ABE yield ranging from 10-20 g/L is generally attained when using enzyme digestion to pretreat wastewater algae as well as cassava starch and degermed corn (Ezeji et al., 2007; Thang et al., 2010). These data presented here create a baseline for evaluating the cost effectiveness of wastewater microalgae pretreatment for ABE fermentation.

2.5. Conclusions

Dilute acid hydrolysis of microalgae is affected by temperature, retention time, and acid concentration. The amount of sugars liberated after acid hydrolysis increases proportionately with the acid concentration. Similarly, the temperature range of 80-90°C was found to be associated with the highest sugar yields. The effect of retention time on sugar yield depends on the acid concentration used in the pretreatment process. The combination of acid concentration, retention time, and temperature that yields the highest ABE concentration while controlling the costs of the acid hydrolysis process were found to be 1.0 M sulfuric acid for 120 min at 80-90°C. These parameters provided a sugar yield of 166.1 g/Kg dry algae, 5.23 g/L of ABE, and 3.74 g/L of butanol concentration at a rate of USD \$12.54 per kg dry algae.

CHAPTER 3

**CHEESE WHEY AS SUPPLEMENT AND CO-SUBSTRATE FOR ACETONE,
BUTANOL, AND ETHANOL (ABE) FERMENTATION
FROM WASTEWATER MICROALGAE**

3.1. Abstract

The effect of cheese whey as a supplement and substrate was determined for acetone, butanol, and ethanol (ABE) fermentation from wastewater microalgae by *Clostridium saccharoperbutylacetonicum* N1-4. Three media constituents, potassium phosphate, magnesium sulfate, and ferrous sulfate were evaluated to assess their need as supplements in the medium to be inoculated, when 50 g/L of cheese whey was present. The use of cheese whey resulted in a 380% higher ABE production than under standard conditions. Mean values of 11.4 g/L of ABE and 8.5 g/L of butanol were obtained when 10% acid hydrolyzed wastewater algae and 50 g/L of cheese whey was fermented by *C. saccharoperbutylacetonicum* N1-4. A cost reduction of USD\$0.33 per gallon of butanol generated was attained using TYA medium as an alternative to T6 medium, with no reduction in solvent concentrations from ABE fermentation.

3.2. Introduction

The production of ABE from wastewater algae is a novel approach that takes advantage of the domestic bioremediation of wastewater streams through the harvesting of algae in order to minimize derived costs of production due to substrate expenses. The achievability of ABE fermentation by *Clostridium saccharoperbutylacetonicum* using

wastewater microalgae from the Logan City Wastewater Lagoon System (LCWLS) has been previously described (Ellis et al., 2012). Optimizations of wastewater microalgae saccharification has been studied resulting in increasing ABE concentration when acid hydrolysis is used for the release of sugars. These studies show concentrations of 5.23 g/L of total ABE, and 3.74 g/L of butanol obtained from wastewater algae hydrolyzed with sulfuric acid at a concentration of 1.0 M at 80-90°C for 120 min (see Chapter 2). The COST of the pretreatment process associated with this approach was \$12.83 USD per kg of butanol, considering the costs related to substrate pretreatment (i.e. raw materials for acid hydrolysis and energy used prior to fermentation). Further improvement of the ABE fermentation process is needed in order to improve the economic performance of the process.

The fermentation of wastewater microalgae for ABE production can be improved by increasing the solvent yield and by reducing production costs. Some reductions in costs to be considered for ABE improvement are those associated with supplementation of the hydrolyzed wastewater algae medium prior to microbial inoculation. Wastewater algae does not provided all the nutrients for *C. saccharoperbutylacetonicum* to grow efficiently. Previous studies describe supplementation of extraneous sugars or enzymes to T6 medium for proficient cellular growth (Ellis et al., 2012). Finding ways to reduce the medium nutrients in terms of either quantity or composition while meeting the supplementation objectives would decrease the pretreatment costs and increase the feasibility of ABE fermentation from wastewater microalgae.

The use of cheese whey in the fermentation medium would increase the ABE yield and decrease the environmental impact due to this waste. The use of cheese whey to produce biofuels mitigates the environmental problems caused by the high organic matter

content (i.e. BOD= 30,000-50,000 ppm) when released to the environment (Ellis et al., 2014; Gonzalez, 1996). Cheese whey serves as a nutrient supplement and also as a co-substrate. Cheese whey contains 70% carbohydrates (i.e. primary lactose), which makes it a suitable substrate for ABE fermentation. Concentrations of cheese whey from 33 to 70 g/L have been used to produce ABE (Foda et al., 2010; Raganati et al., 2013). The composition of cheese whey powder includes nutrients (i.e. potassium, magnesium, iron, and chloride) that are needed for the growth of *C. saccharoperbutylacetonicum* N1-4 and fermentation (De Witt, 2001). Similarly, the neutralization process generates sulfates that, combined with the right concentration of cheese whey, would provide some of the compounds contained in the supplementary media T6. See Figures D-1 and D-2, Appendix D, for details regarding the composition of cheese whey. When the mineral content of cheese whey powder is compared with the nutrient requirements for wastewater microalgae medium prior to inoculation of the *Clostridia* spp, a medium containing 50 g of cheese whey as co-substrate would theoretically meet the need of 0.5 g potassium phosphate, 0.3 g magnesium sulfate, and 0.01 g ferrous sulfate per liter.

Furthermore, non-supplemented cheese whey has been reported to be used as a substrate producing butanol concentrations of 4.93 g/L (Raganati et al., 2013). Through the use of cheese whey, conservative expectations are the replacement of T-6 medium with TYA medium for ABE fermentation (see supplementation, section 3.3 for details on T-6 and TYA media). The implementation of cheese whey as supplement and co-substrate in the ABE production from wastewater algae is expected to be an economically and operationally beneficial approach. Reduction on the toxic effect of butanol has been reported when using cheese whey as substrate for ABE fermentation. As a result, the inhibition of sugar

consumption by the strain is reduced and the generation of butanol is enhanced over the generation of acetone (Jones and Woods, 1986; Qureshi and Maddox, 2005; Qureshi et al., 2013). The integration of wastewater microalgae and cheese whey powder as substrate for ABE production, which is a novel process that has not been described in the refereed literature, will reduce the costs rates and increase the yield of solvents. The purposes of this study was to demonstrate the feasibility of ABE production from wastewater microalgae and cheese whey through the evaluation of costs associated with supplementation and ABE productivity.

3.3. Materials and methods

Algae biomass

Mixed microalgae biomass from the LCWLS was grown in SE media containing 850 mg NaNO₃, 350 mg KH₂PO₄, 150 mg MgSO₄·7H₂O, 150 mg K₂HPO₄, 50 mg CaCl₂·2H₂O, 50 mg NaCl, and 15 mg C₆H₈O₇·Fe·NH₃ per liter of ddH₂O. The biomass was freeze dried through sublimation for 48 hours. The dry biomass was maintained at 4 °C prior to pretreatment.

Cheese whey powder

Swiss whey powder was supplied by Gossner Foods (<http://www.gossner.com>). The mineral content in 100 g of cheese whey are 650 mg of sodium, 450 mg of calcium, 100 mg of magnesium, 2100 mg of potassium, 650 mg of phosphorus, and 1500 mg of chloride. See Figure D-2, Appendix D, for the specification sheet of the cheese whey powder we used in this study.

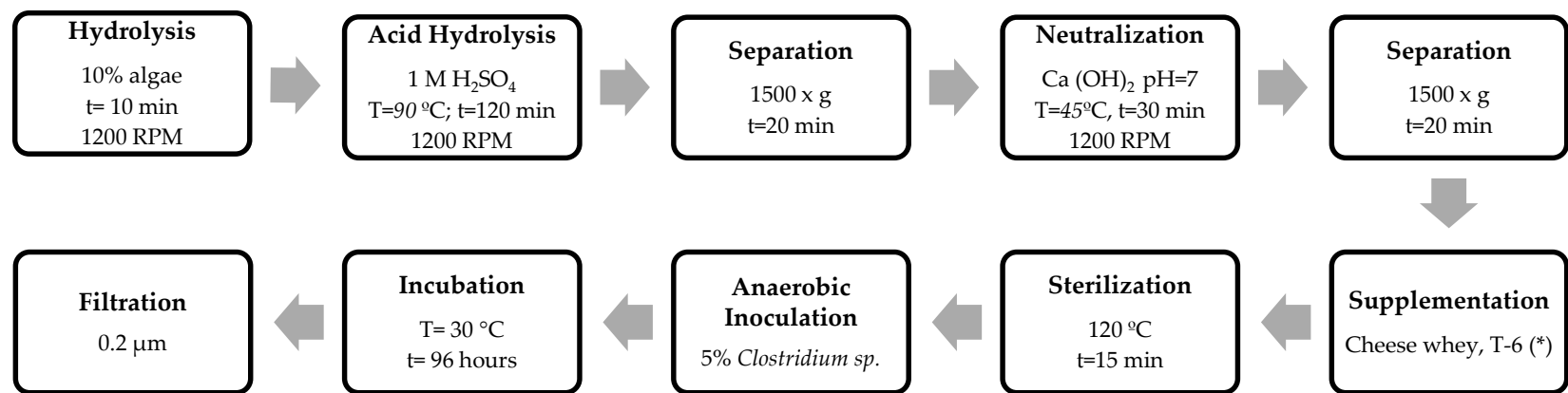


Figure 3-1. Flow diagram of ABE production from wastewater microalgae and cheese whey. * The process to be analyzed for determining the effect of cheese whey on the ABE generation is “supplementation”. Also, the effect of some T6 compounds on the wastewater microalgae and cheese whey medium was analyzed.

Acid hydrolysis of wastewater microalgae

Figure 3-1 illustrates the steps of the ABE production from wastewater algae and cheese whey. Wastewater microalgae was diluted in ddH₂O to reach 10% (*w/v*). Acid hydrolysis was performed by heating to a temperature range of 80-90 °C, adding sulfuric acid to a final working concentration of 1.00 M, and stirring for 120 min. Ca (OH)₂ was used to neutralize the hydrolyzed medium. The medium was clarified by means of centrifugation (1500 g for 20 min) before and after neutralization.

Supplementation

The hydrolyzed algae medium was supplemented by cheese whey and T6 according to statistical experiment designed. The media were stirred at 1200 RPM and heated at 80-90°C until the nutrients were dissolved. After the addition of cheese whey, the media were autoclaved at 120 °C for 15 min. The factorial experiment is based on T6 and TYA as supplementary media. TYA stands for tryptone, yeast, and ammonium acetate with concentrations of 6.0 g/L, 2.0 g/L, and 3.0 g/L respectively. T6 contains: 0.5 g/L potassium phosphate, 0.3 g/L magnesium sulfate, 0.01 g/L ferrous sulfate, and TYA ingredients. All media were supplemented with 0.5 g/L of cysteine hydrochloride (Ellis et al., 2012).

Fermentation

Batch fermentations were performed in 10 ml serum vials. The pH of the media was adjusted to 6.5±0.5 prior to fermentation. The head space of the serum vials was flushed with nitrogen gas prior to the start of fermentation. The fermentation was initiated by inoculating 10% (*v/v*) of actively proliferating (mid-log phase or 24 h vegetative growth) cells in RCM media. All experiments were conducted at a constant temperature of 30 °C.

Analytical methods

ABE concentration was evaluated using gas chromatography (7890B GC-System, Agilent Technologies, USA) equipped with a FID detector along with a Restek Stabiwax-DA, 30 m, 0.32 mmID, 0.25 μm df column. The inlet had an initial temperature of 30 °C for 1 min, ramped up at 5 °C/min up to 100 °C, and had a final ramp of 10 °C/min up to 250 °C. The column had a flow of 4 ml/min, pressure 15 psi, average velocity 54 cm/s, and holdup time 0.93 min. The initial oven temperature was 30 °C for 1 min, and then ramped up 5 °C/min up to 100 °C (no hold time), then ramped up to 20 °C/min up to 225 °C (no hold time), with a final ramp of 120 °C/min up to 250 °C and hold for 2 min. All samples were clarified by centrifugation prior to analysis. Volumetric productivity was calculated as the concentration of solvents produced per hour (g/Lh).

Cost analysis

Estimates of supplementation costs were calculated to determine the effects of the factors including cheese whey, potassium phosphate, magnesium sulfate, and ferrous sulfate on costs. The cost of cheese whey powder is assumed to be negligible since it is considered as waste by local factories. The costs of other nutrients were assumed to be USD \$1.8/kg for potassium phosphate, USD \$0.10/kg for magnesium sulfate, and USD \$0.20/kg for ferrous sulfate. All the costs are according to Alibaba global trader website (www.alibaba.com). For the cost analysis, the concentration of butanol is 8.5 g/L when cheese whey is present and 1.9 g/L when cheese whey is absent.

Experimental design

The experimental design for the supplementation process is a factorial structure consisting of four factors with two levels each, with a total of 16 measurement units. The factors and levels of the experiment are: (1) cheese whey, at 50 g/L and 0 g/L, (2) potassium phosphate, at 0.5 g/L and 0 g/L; (3) magnesium sulfate, at 0.3 g/L and 0 g/L; and (4) ferrous sulfate, at 0.01 g/L and 0 g/L. The concentration of ABE obtained at the end of the fermentation is the response of the experimental design analyzed using the Statistical Analysis Software (SAS) with $\alpha=0.05$. The assumptions of the analysis are normality and independence of variance. Some comparisons between values were performed using t-test statistics from Graphpad website (<http://www.graphpad.com/quickcalcs/>).

3.4. Results and discussions

Effect of nutrients on ABE production

The evaluation of the three compounds that differentiates T6 from TYA determines the reduction cost viability. The analysis of variance associated with the effects of the factors on the response are illustrated in Figure F-2, Appendix F. The effect of ferrous sulfate (FeSO_4) on ABE from wastewater algae is not significant, with $p > 0.05$. Consequently, adding 0.01g/L of FeSO_4 does not improve the ABE concentrations in the fermentation. Based on this result, we can state that the bacterial requirement for FeSO_4 is met in the hydrolyzed wastewater microalgae medium. Ferrous sulfate is used as a reducing iron powder for *Clostridia* spp. This material removes oxygen from the system by forming FeO_2 (rust) (Demain et al., 2006). The addition of 0.5 g/L cysteine hydrochloride to the media might have covered the need for FeSO_4 . The exclusion of this compound would only reduce the

production costs in a rate of USD\$0.0007 per gallon of butanol when cheese whey is used as supplement and co-substrate. Consequently, omitting FeSO_4 is not really effective for cost reductions.

Similarly, the effect of magnesium sulfate (MgSO_4) is not significant according to the statistical results, with $p > 0.05$. Thus, MgSO_4 is provided by hydrolyzed wastewater microalgae. The neutralization process after acid hydrolysis generates calcium sulfate (CaSO_4) as a byproduct. Most of the CaSO_4 is removed from the medium, but some of this supplement is expected to remain. Calcium sulfate is a source of inorganic ions that stimulate bacterial growth. Therefore the use of MgSO_4 is unnecessary whether or not cheese whey is present. This decision would reduce production costs in a significant rate of USD\$0.01/gal butanol. Conversely, the effect of potassium phosphate (KH_2PO_4) on the ABE production depends on cheese whey concentration. When cheese whey is used as a supplement, the effect of KH_2PO_4 on ABE production is not significant. However, in the absence of cheese whey, the concentration of solvents increase more than 45% (Figure 3-2). The use of cheese whey as a supplement and co-substrate covers the requirement of 0.5 g/L of KH_2PO_4 . Eliminating 0.5 g/L of KH_2PO_4 from media would reduce USD\$0.32/gal of butanol produced if cheese whey is used as a supplement instead.

The results suggest the elimination of the three nutrients under study if cheese whey is used as supplement and co-substrate with a total costs reduction rate of USD\$0.33/gal of butanol produced. When cheese whey is not present, potassium phosphate must be present, and as a result the reduction would only be USD\$0.04/gal of butanol produced. The remaining ingredients of the T6 medium that were not analyzed in this study (i.e. tryptone, yeast extract, ammonium acetate, and cysteine hydrochloride) might also be studied to

determine if are needed when cheese whey is used as supplement and co-substrate. The elimination of T-6 as supplementary media could be evaluated in order to reduce costs.

Fermentation of ABE from cheese whey by *Clostridium acetobutylicum* DSM 792 was reported to generate 4.93 g/L of butanol using un-supplemented cheese whey as feedstock (Raganati et al., 2013).

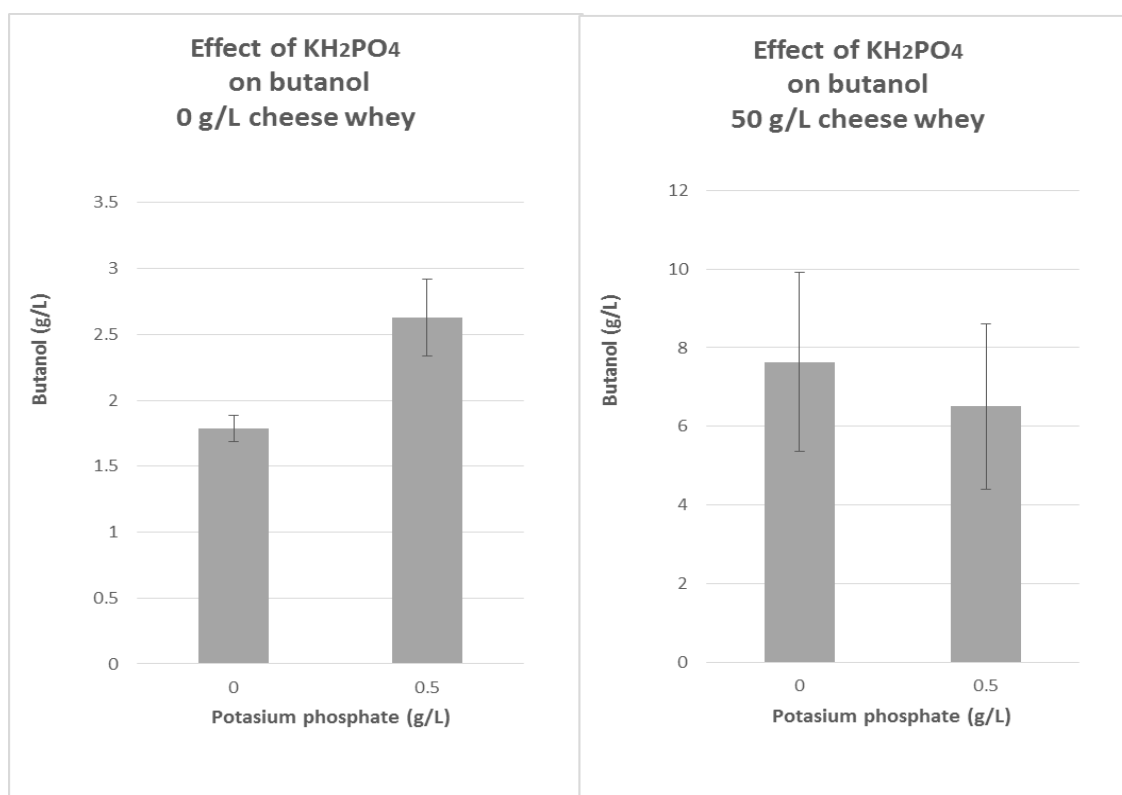


Figure 3-2. Effect of potassium phosphate (KH₂PO₄) on butanol concentration. When cheese whey is absent, the effect of KH₂PO₄ is statistically significant. However, when cheese whey is present, there is no significant difference on butanol concentration whether KH₂PO₄ is used or not. Figure is based on data from gas chromatography with calibration curve R²=0.99 (Appendix E). Error bars are the standard deviation.

Cheese whey as a supplement and co-substrate

The use of cheese whey as supplement for ABE fermentation eliminates the need for potassium phosphate, reducing USD\$0.32/gal of butanol generated (Figure 3-2). However, the effect of magnesium sulfate and ferrous sulfate on ABE generation does not depend on cheese whey presence according to statistical analysis. Additionally, Figure 3-3 illustrates that there is no significant difference between T6 and TYA media within each cheese whey concentration (i.e. 0 g/L and 50 g/L), which confirms that using TYA medium (absence of the nutrients under study) instead of T6 medium would result in similar concentrations of ABE.

In terms of ABE production, there is a significant statistical improvement on ABE concentration when cheese whey is present ($p < 0.05$). The Tukey-Kramer least square means estimates for butanol and ABE concentrations, when cheese whey is present, are 8.5 g/L and 11.4 g/L, respectively (Table 3-1). In the absence of cheese whey, the results show concentrations of 1.9 g/L of butanol and 2.3g/L of total ABE (Table 3-1). The use of cheese whey as a co-substrate increases the solvent concentrations more than 3 times their value when only wastewater algae is used. ABE concentrations obtained from the use of cheese whey are higher than previous results from wastewater microalgae pretreated with acid hydrolysis and enzymes (i.e. 9.74 g/L) (Ellis et al., 2012).

ABE efficiency might be further augmented by decreasing cheese whey concentration from 50 g/L to 20 g/L. Studies have shown that cheese whey concentrations over 20 g/L do not generate higher ABE concentrations (Napoli et al., 2009). Since hydrolyzed wastewater microalgae has shown to meet some of the nutritional requirements, a concentration of 50 g/L of cheese whey is higher than is needed. The use of 20 g/L of cheese

why as co-substrate for ABE fermentation from wastewater microalgae would meet the nutrient demand without reducing the concentrations of solvents.

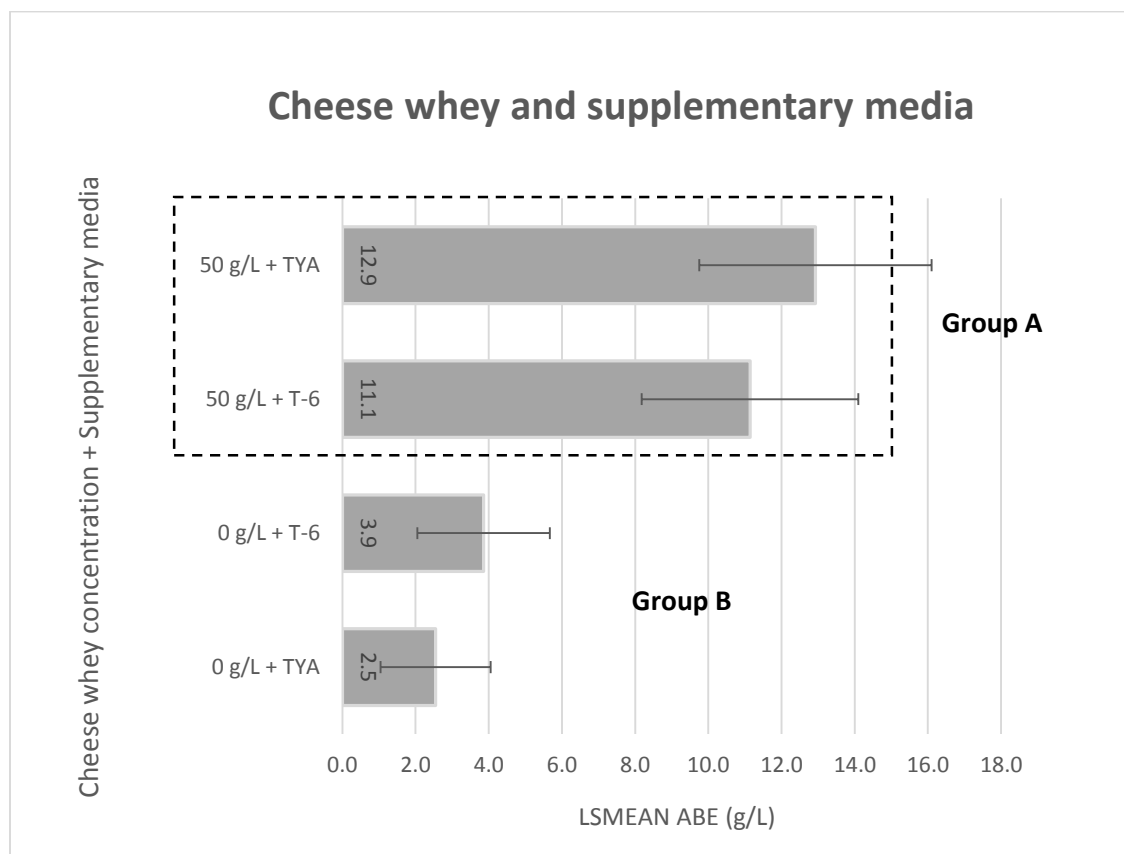


Figure 3-3. Comparison of ABE concentrations from cheese whey and supplementary media constituents. The highest amount of ABE is produced when 50 g/L of cheese whey is added as supplement and co-substrate (group A). The lowest ABE concentration is obtained when no cheese whey is added (Group B). ABE values are grams of total ABE per liter (g/L). Least squares means (LSMEAN) with the same letters (Group A or Group B) are not significantly different ($\alpha=0.05$) according to Tukey-Kramer grouping for cheese whey. Figure is based on SAS results with $R^2=0.88$ (see Figure F-3, Appendix F).

Table 3-1. Solvent concentrations for combinations of cheese whey, potassium phosphate, and magnesium sulfate. When cheese whey is present, butanol and ABE mean concentrations are 8.5 g/L and 11.4 g/L respectively. Table is based on SAS Tukey-Kramer least square means estimates for butanol and total ABE, with R² values 0.88 (see Figure F-3, Appendix F). Data obtained from ABE measurements is detailed in Table E-1, Appendix E).

Algae (% w/v)	Cheese whey (g/L)	Potassium Phosphate (g/L)	Magnesium sulfate (g/L)	Butanol (g/L) ^a	ABE (g/L) ^a	ABE Volumetric productivity (g/Lh) ^b
10	50	0	0.3	10.7	13.2	0.053
10	50	0	0	9.8	12.9	0.051
10	50	0.5	0.3	7.5	11.1	0.044
10	50	0.5	0	6.1	8.2	0.033
10	0	0.5	0.3	2.7	3.8	0.015
10	0	0	0	1.8	2.5	0.010
10	0	0	0.3	1.8	2.1	0.008
10	0	0.5	0	1.3	1.7	0.007
0	0	0.5	0.3	ND	ND	-

^a Butanol and total solvents (ABE) concentrations are the estimates values calculated by the Statistical Analysis Software (SAS) based on Table F-3, Appendix F.

^b The productivity of the solvents is calculated assuming the pretreatment and fermentation times as total time.

(*) Bold values are not significantly different within their own category according to Tukey-Kramer grouping of least square means of cheese whey with alpha=0.05.

The use of cheese whey as a supplement and co-substrate for ABE fermentation was expected to increase the butanol to acetone ratio. Figure 3-4 shows that the A:B:E ratio when no cheese whey is used as a supplement is 2:7.6:0.4, while the ratio in the presence of cheese whey is 2.4:7.1:0.5. Therefore, the ratio of butanol to acetone decreased with the addition of cheese whey by greater than 6%. These data contradict recent studies where lactose derived

substrates increased butanol to acetone ratios by 16% and 36% when *C. acetobutylicum* and *C. beijerinckii* were used respectively (Ujor et al., 2014). The use of cheese whey as a lactose derived feedstock for ABE fermentation by *C. saccharoperbutylacetonicum* did not increase the ratio of butanol to acetone in this study.

ABE fermentation from wastewater microalgae and cheese whey

Table 3-1 shows the results of ABE and butanol concentrations for cheese whey, potassium phosphate, and magnesium sulfate level combinations. The lower concentrations of solvents were obtained when wastewater microalgae was the sole substrate, with estimates as low as 1.3 g/L of butanol and 1.7 g/L of ABE. The solvent concentrations when no cheese whey is added and T6 is the supplementary medium are 2.7 g/L of butanol and 3.8 g/L of ABE. In contrast, the maximum butanol and ABE concentration estimates are 10.7 g/L and 13.2 g/L respectively, when cheese whey is co-substrate ($\alpha=0.05$). The results obtained in the current study are higher than previous results obtained when xylanase and cellulase enzymes were supplemented to the acid pretreated wastewater algae media, with an ABE concentration of 9.74 g/L (Ellis et al., 2012). However, the maximum volumetric productivity of ABE in the current study is only 0.053 g/L·h, while in the former study the volumetric productivity is 0.102 g/L·h. Similarly, high productivity is obtained when enzymatic hydrolysis is used for the pretreatment of corncobs (0.54 g/L·h), with an ABE concentration of 19.4 g/L (Gao and Rehm, 2014). According to Gao and Rehm (2014), this high concentration of solvents is due to the enzymatic hydrolysis that generated fermentable substrates. Conversely, in our present study, values as high as 18.6 g/L of ABE were

measured when cheese whey was present and no enzymes were involved (see Table E-1, Appendix E, for data measured).

The solvent concentrations obtained in this bench scale study are standard values when compared with the maximum amounts of ABE concentration produced from other substrates by *Clostridia* spp. within 'batch reactors', which is 20-30 g/L (Qureshi et al., 2013). In terms of productivity, enzymatic digestion of algae as opposed to acid hydrolysis might be considered, but additional optimization and reduction costs of that approach should be evaluated to be more cost competitive. Other alternatives must be studied in order to further increase the productivity of ABE fermentation, such as genetically engineering bacteria to produce ABE continuously while collecting ABE to avoid toxicity to the bacterial strain (Qureshi et al., 2013).

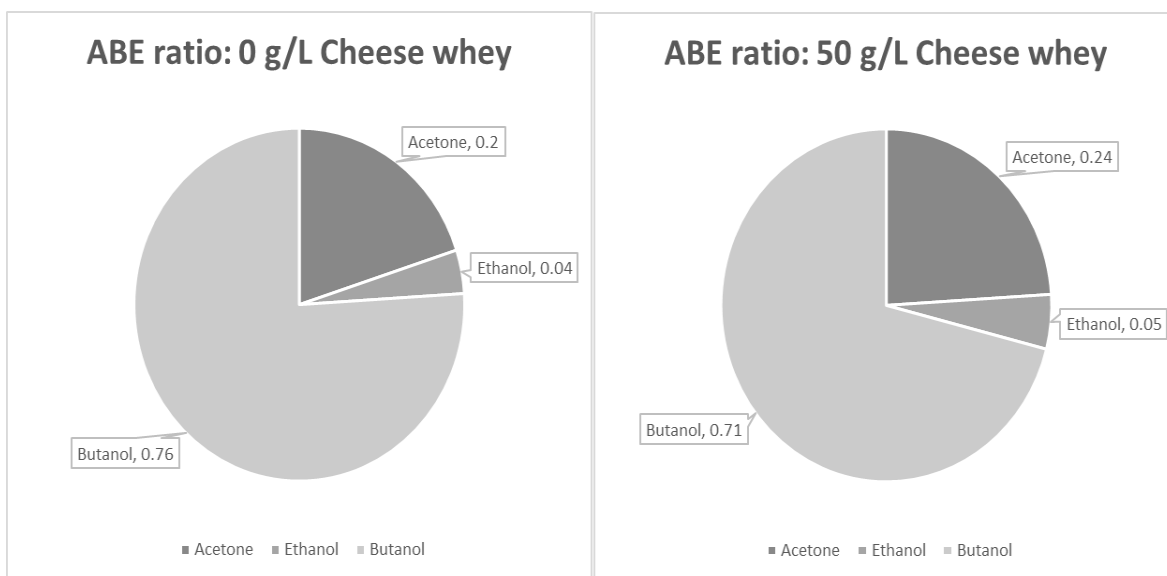


Figure 3-4. Comparison of A:B:E ratios from fermentation with and without cheese whey presence. Butanol to acetone ratio is higher when there is not cheese whey present.

3.5. Conclusions

ABE fermentation from wastewater microalgae by *Clostridium saccharoperbutylacetonicum* improves in terms of solvent concentration and reduction costs when cheese whey is added as supplement and co-substrate. The use of cheese whey as supplement for ABE fermentation prevents the need for potassium phosphate. Even though the effect of magnesium sulfate and ferrous sulfate on ABE generation does not depend on cheese whey, the need for these two nutrients is covered by the acid hydrolyzed wastewater microalgae. The total cost is reduced by USD\$0.32 for one gallon of butanol generated when TYA medium supplements 10% of hydrolyzed algae and 50 g/L of cheese whey for ABE fermentation with mean concentrations of 11.4 g/L of ABE and 8.5 g/L of butanol. In the absence of cheese whey, mean values of 2.3g/L of ABE and 1.9 g/L of butanol were produced.

CHAPTER 4

ENGINEERING SIGNIFICANCE AND CONCLUSIONS

Increasing global energy consumption has lead nations and research oriented institutions to focus on developing sustainable technologies in order to meet some of the energy demand worldwide. Butanol is an environmentally safe and high performing fuel that can successfully mitigate the liquid fuel demand and compete with commercial fuels if production costs are reduced and ABE yields increased. This thesis addressed the optimization of wastewater microalgae pretreatment, which is one of the costly processes that are needed for ABE fermentation, with the purpose of enhancing ABE productivity. The first part of the research covered the optimization of the substrate saccharification through acid hydrolysis. The second part addressed the use of a co-substrate to reduce the need for supplements and to increase ABE generation.

The studies conducted regarding the acid hydrolysis of the wastewater microalgae determined the optimal conditions for sugar liberation for subsequent fermentation to acetone, butanol, and ethanol (ABE) by *Clostridium saccharoperbutylacetonicum* N1-4. The best hydrolysis conditions were determined by evaluating the effect of acid concentration, retention time, and temperature on sugar and ABE yield, as well as the associated costs. The effect of temperature was directly proportional to solvent yield, obtaining the highest ABE concentrations at the highest temperature (i.e. 80-90°C). The effect of the acid concentrations on solvent yield was found to depend on the retention time. The acid hydrolysis conditions that yielded the highest ABE concentration, while controlling the costs of the process, were

found to be 1.0 M sulfuric acid for 120 min at 80-90°C. These parameters provided a sugar yield of 166.1 g/Kg dry algae, 5.23 g/L of ABE, and 3.74 g/L of butanol concentration at a cost of USD \$12.83 per kg dry algae.

In the second part of this research, an increase of solvent concentration and a reduction of pretreatment cost was attained through the implementation of cheese whey as a co-substrate for ABE fermentation from wastewater microalgae by *C.*

saccharoperbutylacetonicum N1-4. The effect of 50 g/L cheese whey as a supplement was determined through evaluating the requirement of three media constituents, potassium phosphate, magnesium sulfate, and ferrous sulfate, in the presence of cheese whey. The use of cheese whey as supplement for ABE fermentation eliminates the need for potassium phosphate. The need for magnesium sulfate and ferrous sulfate on ABE generation was met by the medium formed by 50 g/L cheese whey and acid hydrolyzed wastewater microalgae. An ABE increase of more than 100% resulted from the use of 50 g/L of cheese whey when compared with the results obtained in the first part of the research. Concentrations of 11.4 g/L of ABE and 8.5 g/L of butanol were obtained when 10% acid hydrolyzed wastewater algae and 50 g/L of butanol medium was fermented by *C. saccharoperbutylacetonicum* N1-4. Also, a cost reduction of USD\$0.33/gal of butanol was achieved by using TYA medium as an alternative to T-6 medium, with no reduction on solvent concentrations from ABE fermentation. Figure 4-1 is the schematic of the large scale batch system that would result from the operations performed currently at bench scale.

Further reduction of costs and increase in ABE generation need to be considered for improving the competitiveness of ABE using the processes studied. The integration of the acid hydrolysis and the sterilization processes might be an opportunity of improvement for

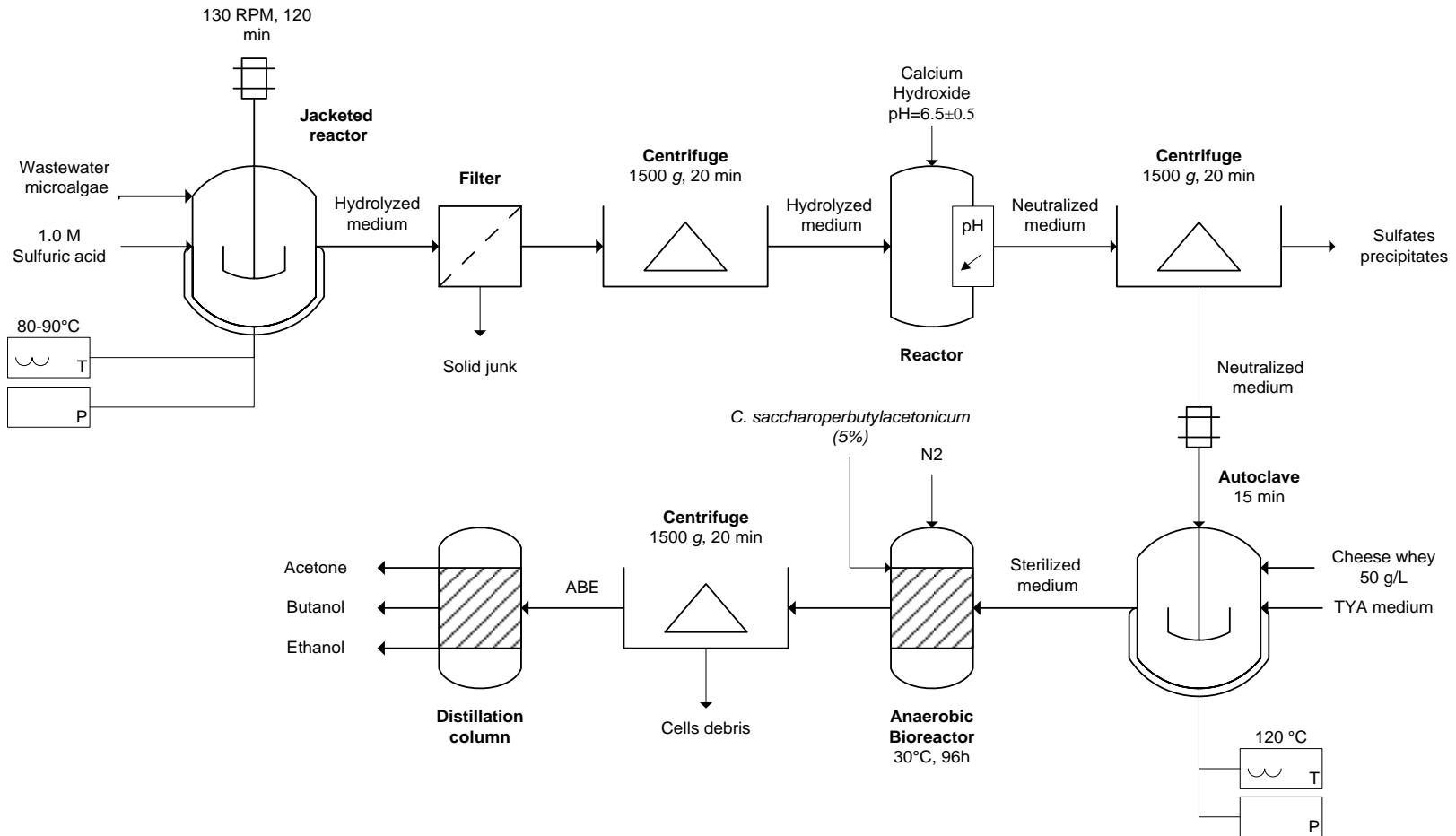


Figure 4-1. Schematic of a 5000-L batch system for ABE production from wastewater microalgae by *C. saccharoperbutylacetonicum*. Based on bench scale operations performed for experiments in this thesis project.

ABE fermentation. Acid hydrolysis is expected to increase sugar yield if performed at the temperature used for sterilization (i.e. 120°C). However, investments would be need on keeping the medium sterile from the unit where sterilization would be performed to the unit where inoculation would occur. The increase of temperature for acid hydrolysis might trigger reductions on acid concentrations that would decrease associated costs. Another approach to be considered in future research would be to genetically engineer bacteria for continuous ABE production while collecting solvents in order to avoid toxicity to the bacterial strain. The concentrations of ABE produced by *Clostridium* spp. are limited due to the harmful effect of the solvents on the bacteria. Extracting ABE from the medium where the bacteria are present would eliminate the solvents threat and enhance the production of ABE.

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APPENDICES

APPENDIX A

PREVIOUS WORK ON ABE FERMENTATION

Table A-1. Description of different approaches on ABE fermentation.

Scale/ Mode	Substrate		Bacteria Strain	Hydrolysis		Sterilization	pH	Nourishment	ABE yield (g/L)	Reference
	Material	%		Acid/Enzyme	Thermolysis					
Industrial	Maize mash	8.0-10.0	<i>Clostridium acetobutylicum</i>	NA	T= 130-135°C , 60-90 min		NA	NA	12-20	Jones and Woods, 1986
Industrial	Molasses	5.0-7.5	<i>Clostridium acetobutylicum</i>	NA	T=107-120°C , 15-60 min		NA	Organic/inorganic nitrogen, phosphorus and buffer.	18-22	Jones and Woods, 1986
Lab/ Batch	Wastewater microalgae	10	<i>Clostridium saccharoperbutyl acetonicum N1-4</i>	1 M H ₂ SO ₄ ; 5 M NaOH. 10 U xylanase, 100 U cellulase	T= 90°C 60 min	120°C , 15 min	6.5	T6 and 0.5 g/L cysteine hydrochloride	10	Ellis et al., 2012
Bench/ Batch	Cassava starch	4-7	<i>Clostridium saccharoperbutyl acetonicum N1-4</i>	Amylasee (95°C 2h) , Gluczym (58°C, 15 h)	T= 80°C 30 min	115°C , 15 min	6.2	T6	23	Thang et al., 2010
Bench/ Continuous	Degermed corn	5.5-6.0	<i>Clostridium beijerinckii BA101</i>	1M HCl, amylase glucoamylase;	100 °C, 3 h	121 °C, 15 min	6.0	Phosphate buffer and yeast extract	14	Ezeji et al., 2007
Lab/Batch	Corn fiber and xylose	7.5	<i>Clostridium acetobutylicum P260</i>	1 M NaOH/H ₂ SO ₄	70-90 °C	121 °C, 15 min.	6.1	Glucose, yeast extract, and stock solutions	25	Qureshi et al., 2004
Bench/ batch	Gelatinized sago starch	5	<i>Clostridium saccharobutylicum DSM 13864</i>	NA	70C°	121 °C, 20 min.	6.0	T6 Glycerol, 2HPO ₄ , MnSO ₄ .H ₂ O, NaCl, resazurin, cysteine, P-amino benzoic acid and biotin.	16	Liew et al., 2006
Bench/ Continuous									9	

Table A-2. Comparison of algal saccharification through dilute acid hydrolysis on different algal species.

Substrate	Acid (Moles/L)	Temperature (°C)	Reaction time (min)	Sugar yield			Reference
				Compound	g/Kg	% (w/w)	
Invasive Macroalga	0.1, 0.2, 0.50 , 1.0	105, 115, 125 , 128	30	glucose	4.3	-	Wang et al., 2011
Marine Macroalga	0.70 , 0.35, 0.05	121	30, 45, 60	Total sugars	30	-	Yazdani et al., 2011
Red Macroalga	0.15, 0.30 , 0.45	121	15, 30, 45 , 60	Reducing sugars	-	32.8	Setyaningsih et al., 2012
Red algae	0.45	130	15	Galactose	-	26.2	Khambhaty et al., 2012

*The values in bold are optimal according to the author.

APPENDIX B

THERMOLYSIS OF WASTEWATER MICROALGAE

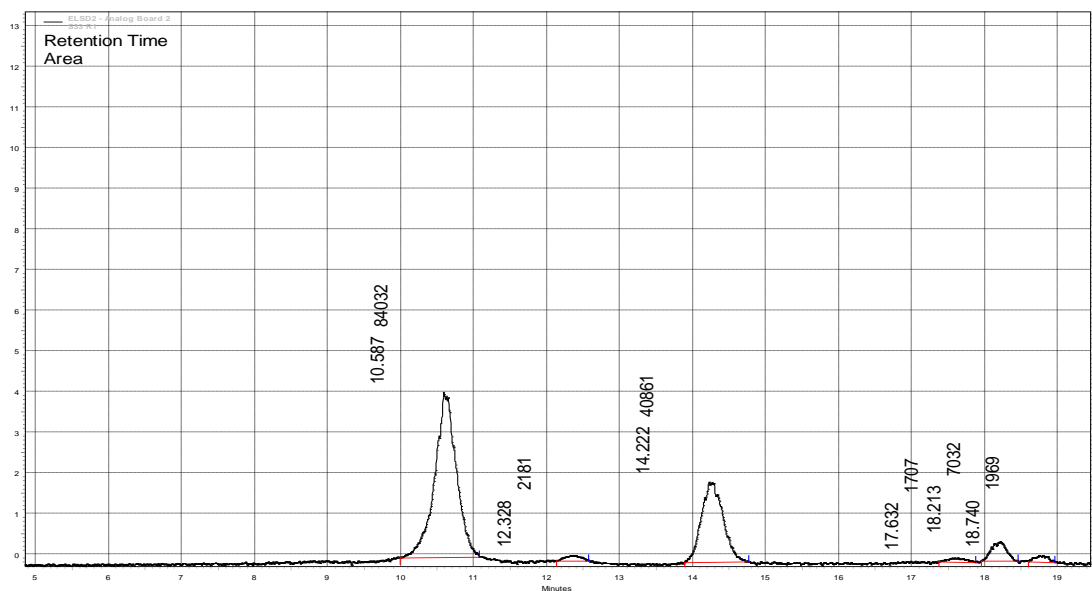


Figure B-1. HPLC chromatograph of 10% pretreated algae (thermolysis: 120 min, 85°C). A polysaccharide (xylan), is shown at 10.5 min; traces of a trisaccharide (maltotriose) at 12.3 min; a disaccharide (maltose) at 14.2 min; and some monosaccharides (glucose at 17.6 min, xylose at 18.2 min, and galactose at 18.7 min). Vertical axis is mVolts. Data from 04/04/13.

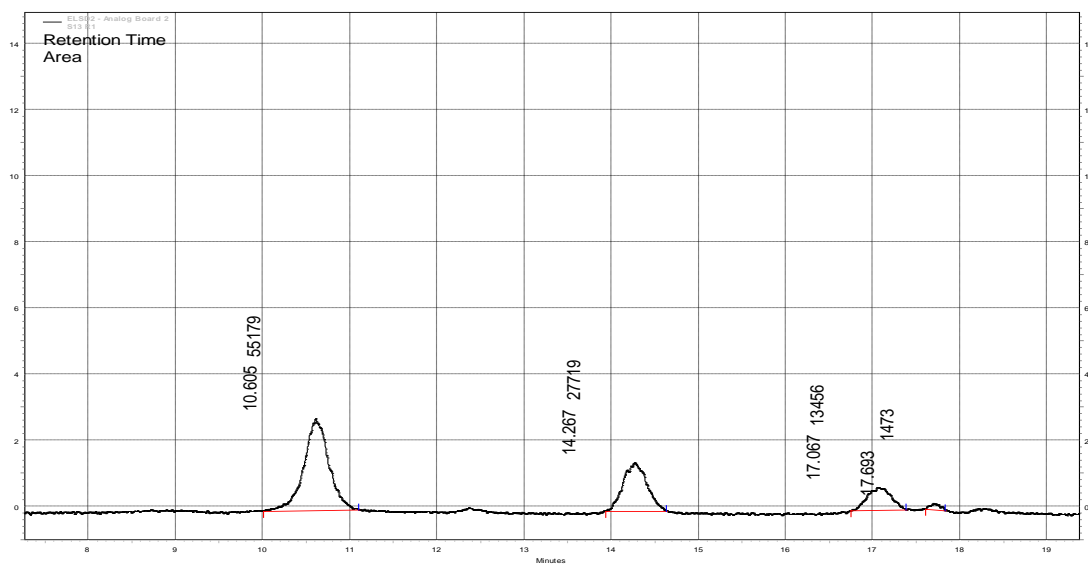


Figure B-2. Chromatograph of 10% pretreated algae (thermolysis: 30 min, 85°C). Xylan, maltose, and glucose are shown at 10.6 min, 14.2 min, and 17.0 min, respectively. Data from 04/04/13. Vertical axis is mVolts.

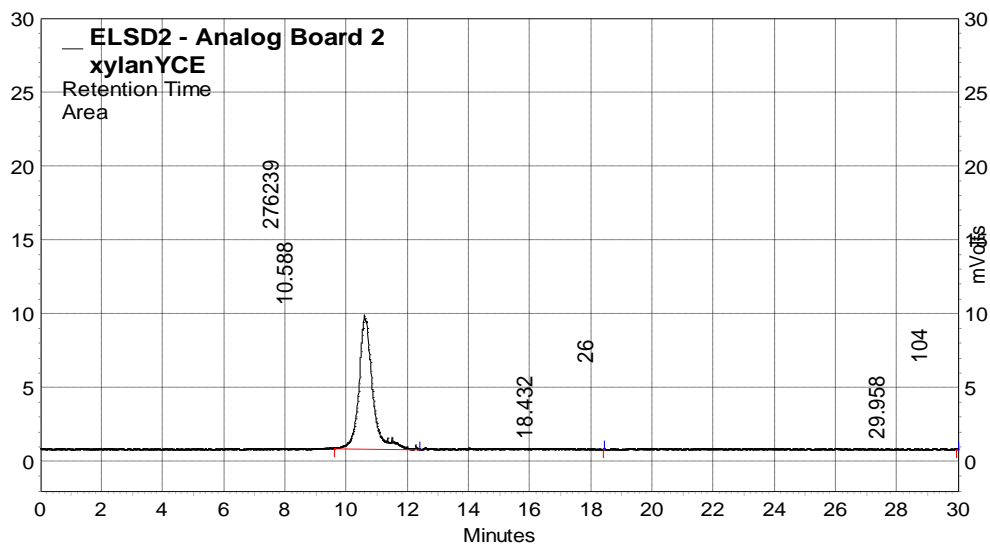


Figure B-3. HPLC chromatogram of 20g/L xylan standard at 10.5 min. Data from 6/22/13.

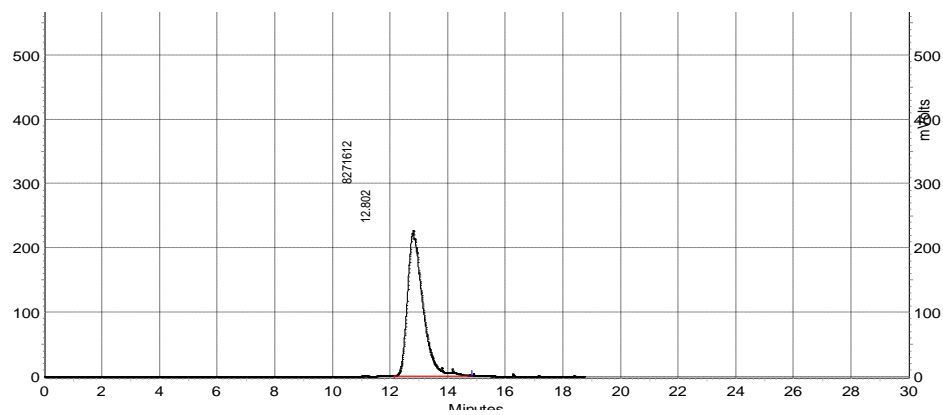


Figure B-4. Chromatogram of maltotriose standard at 12.8 min. Data from 6/22/13.

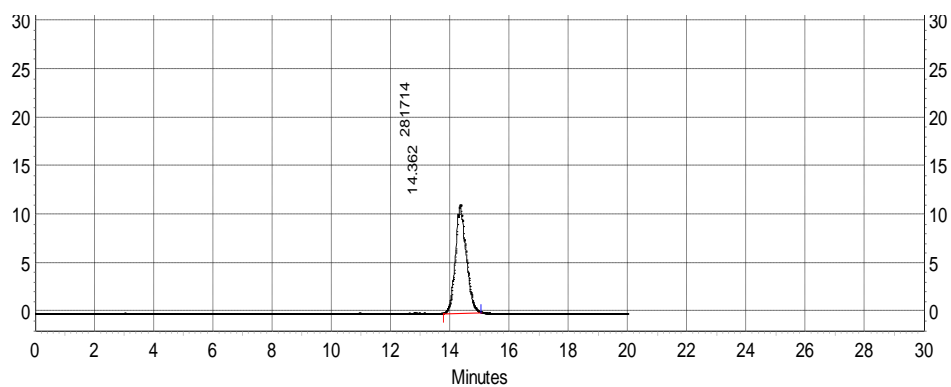


Figure B-5. Chromatogram of maltose standard at 14.3 min. Vertical axis is mVolts.

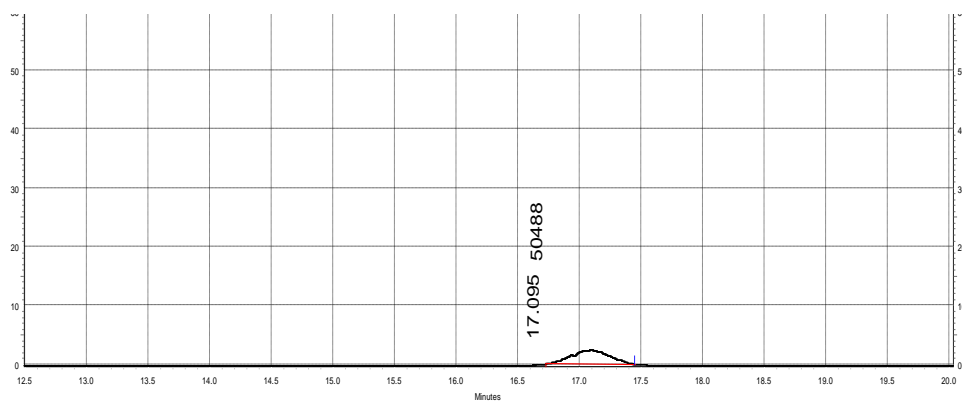


Figure B-6. Chromatogram of glucose standard at 17.0 min. Vertical axis is mVolts.

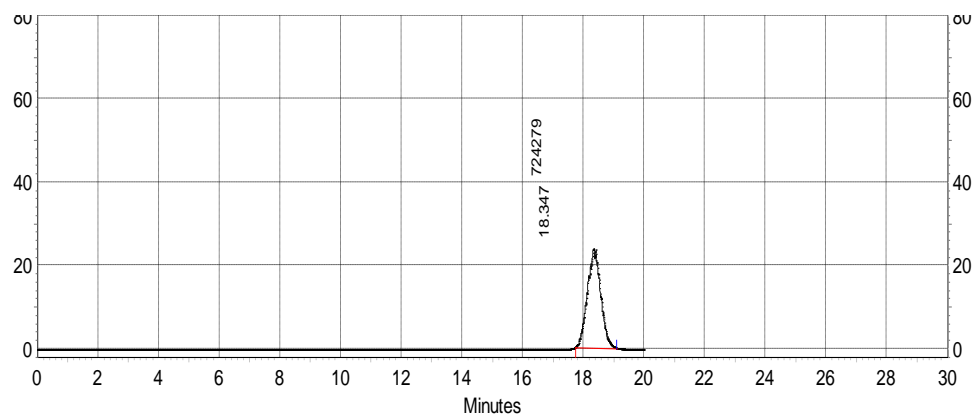


Figure B-7. Chromatogram of xylose standard at 18.3 min. Vertical axis is mVolts

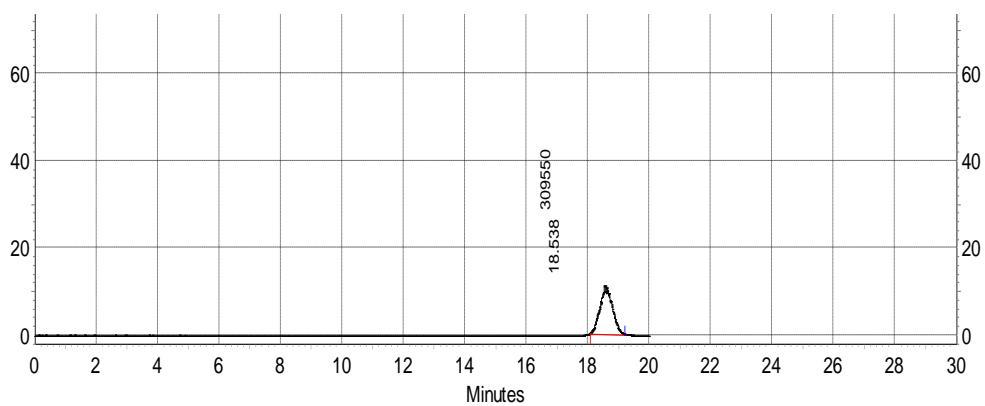


Figure B-8. Chromatogram of galactose standard at 18.6 min. Vertical axis is mVolts.

APPENDIX C
CALCULATIONS

C-1 Heat transfer calculations from Eq. 2.1

Specific heat (Cp) of hydrolyzed algae

$$C_p = 4.19p/100 + 0.84(100 - p)/100 \text{ (kJ kg}^{-1} \text{ }^\circ\text{C}^{-1} \text{ above freezing (Earle, 1983))}$$

p = percentage of water in the slurry =90%

$$C_p = 4.19 (0.9) + 0.84(0.1) \text{ kJ kg}^{-1} \text{ }^\circ\text{C}^{-1}$$

$$C_p = 3.86 \text{ kJ kg}^{-1} \text{ }^\circ\text{C}^{-1}$$

Specific gravity (SG) of hydrolyzed algae (10%w/v)

$$\rho_s = (m_s/V) + \rho_l$$

ρ_s = density of hydrolyzed algae slurry (kg/m³) =?

m_s = mass of dried algae in slurry per liter of liquid (kg/L) = 0.1 Kg/L

ρ_l = density of liquid portion- H₂O and H₂SO₄- (kg/m³) = 1056 (1.25M H₂SO₄)

V = volume conversion (m³/L) = 0.001

$$\rho_s = (0.1/0.001) + 1056$$

$$\rho_s = 1155 \text{ kg/m}^3$$

SG = Slurry density/ Water density

$$SG = 1155/1000$$

$$SG = 1.16$$

Heat energy (KJ/L)

Temperature=50°C

$$Q = 1.16(3.86)(50 - 25)$$

$$Q = 112.5 \text{ KJ/L}$$

Temperature=90°C

$$Q = 1.16(3.86)(90 - 25)$$

$$Q = 291.0 \text{ KJ/L}$$

Temperature=120°C

$$Q = 1.16(3.86)(90 - 25)$$

$$Q = 425.4 \text{ KJ/L}$$

C-2 Energy from mixing

$$SHP = \frac{N_p N^3 D^5 S.G}{1.53 \times 10^{13}}$$

SHP = Shaft horsepower (HP)

N_p = Power number of impeller. $N_p = 0.60$

N = Impeller speed (RPM). $N = 130$ RPM

D = Diameter of the impeller (in). $D = 40$

$S.G$ = Specific gravity of solution. $S.G = 1.16$

1.53×10^{13} = Conversion factor

Calculation of impeller speed from bench scale

$$RCF = (1.118 \times 10^{-5}) R (N)^2$$

RCF = Relative centrifugal force (g)

R = rotational radius (cm)

N = rotational speed (RPM)

Bench scale (assuming 70% efficiency)

$$RCF = (1.118 \times 10^{-5}) (1.27) (1200 (.7)) ^2$$

$$RCF = 10 \times g$$

Large scale

$$N = (RCF / (1.118 \times 10^{-5}))^{0.5}$$

$$N = (10 / (50.8) (1.118 \times 10^{-5}))^{0.5}$$

$$N = 130 \text{ rpm}$$

Power number (N_p)

$$N_{re} = (N (D^2) \rho) / \mu$$

N_{re} = Reynold number

D = Impeller diameter (m) = 1.0

N = rotational speed (rps) = 130/60

ρ = density (kg/m³) = 1100

μ = fluid viscosity (Pa s) = 0.016

$$N_{re} = (N D^2 \rho) / \mu$$

$$N_{re} = [(130/60)(1.0)(1100)] / 0.016$$

$$N_{re} = 148958$$

$N_{re} \approx 1.5 \times 10^5$, thus $N_p \approx 0.6$
(Smith, 2011).

$$SHP = [(0.6) (130)^3 (40)^5 (1.16)] / 1.53 \times 10^{13}$$

$$SHP = 10.2 \text{ hp}$$

Stirring energy (E_s)

$$E_s = [0.746 \text{ SHP (RT)}]/V$$

RT=80 minutes
 E_s = Energy due to stirring (KJ/L)

$$E_s = [0.746 (10.2) (80 \times 60)] / (5000L)$$

SHP = Shaft horsepower (HP)

$$E_s = 7.3 \text{ KJ/L}$$

RT = Retention time (s)

V = Batch volume (L)

RT=120 minutes

0.746 = Conversion factor (kw/hp)

$$E_s = [0.746 (9.7) (120 \times 60)] / (5000L)$$

RT = 40 minutes

$$E_s = 10.4 \text{ KJ/L}$$

$$E_s = [0.746 (10.2) (40 \times 60)] / (5000L)$$

$$E_s = 3.6 \text{ KJ/L}$$

C-3 Costs from acid and base

$$ABC_{\text{Cost}} = (V_a \rho_a P_a + M_b P_b) / V$$

 ABC_{Cost} = Cost of acid and base for pretreatment (USD\$/L)

 V_a = Volume of 18.5 M H_2SO_4 stock required for batch (L)

 ρ_a = Density of 18.5 M H_2SO_4 (Kg/L) = 1.84

 P_a = Price of acid (USD\$/kg) = 0.26

 M_b = Mass of $Ca(OH)_2$ (kg)

 P_b = Price of base (USD\$/kg) = 0.20

 V = Batch volume (L) = 5000

X mL of H_2SO_4 needed approximately X g of $Ca(OH)_2$. Thus, $M_b = V_a$

Mass of of Ca(OH)₂ (kg)

$$M_b = V_a = C \cdot V / Ca$$

V_a = Volume of stock required for batch (L) = ?

Ca = stock concentration of sulfuric acid (M) = 18.5

C = Required concentration in batch for pretreatment (M)

V = Batch volume (L) = 5000

1.0M H₂SO₄

$$V_a = (1)(5000)/18.5$$

V_a = 270.3 L = 270 kg of Ca(OH)₂

$$ABCost = (270.3)[(1.84)(0.26) + (0.20)]/5000$$

$$ABCost = 0.037 \text{ USD\$/L}$$

1.5M H₂SO₄

$$V_a = (1.5)(5000)/18.5$$

V_a = 405.4 L = 405.4 kg of Ca(OH)₂

$$ABCost = (405.4)[(1.84)(0.26) + (0.20)]/5000$$

$$ABCost = 0.055 \text{ USD\$/L}$$

C-4 Energy costs

$$QCost = (Q_h + Q_s) P_e$$

QCost = Cost of energy for pretreatment (USD\$/L)

Q_h = Energy due to heating to 90°C (KJ/L) = 291

Q_s = Energy due to stirring (KJ/L)

P_e = Price of energy (USD\$/KJ)

$$P_e = (\text{USD\$}0.12/\text{kwh})(\text{kwh}/3600 \text{ kJ})$$

$$P_e = 3.3 \times 10^{-5} \text{ USD\$/KJ}$$

RT=40min

$$QCost = (291 + 3.6)(3.3 \times 10^{-5})$$

$$QCost = 0.0097 \text{ USD\$/L}$$

RT=80min

$$QCost = (291 + 7.3)(3.3 \times 10^{-5})$$

$$QCost = 0.0098 \text{ USD\$/L}$$


RT=120min

$$QCost = (291 + 10.4)(3.3 \times 10^{-5})$$

$$QCost = 0.0099 \text{ USD\$/L}$$

APPENDIX D

NUTRITIONAL CONTENT OF CHEESE WHEY POWDER



ASEPTIC PLANT: 1105 N 1000 W
 CHEESE PLANT: 1051 N 1000 W
 LOGAN UT, 84321
 Phone: (435) 752-8365
 Fax: (435) 752-0185

GOSSNER FOODS SWISS WHEY POWDER

Guarantee of Analysis

GENERAL

Color.....	White to Light Cream
Flavor.....	Bland, Slightly Sweet
Titrateable Acidity.....	0.16% maximum
Scorched Particles (ADMI).....	B maximum

PROXIMATE

Fat.....	1.50% maximum
Protein.....	11.00% minimum
Carbohydrate.....	75.00% minimum
Ash.....	8.00% maximum
Moisture.....	5.00% maximum

MINERAL

Sodium.....	650 mg/ 100g
Calcium.....	450 mg/ 100g
Magnesium.....	100 mg/ 100g
Potassium.....	2100 mg/ 100g
Phosphorus.....	650 mg/ 100g
Chloride.....	1500 mg/ 100 g

Mineral. The above quoted values reflect averages. Actual values will differ by lot.

MICROBIOLOGY

Total Plate Count.....	≤30,000/g
Coliform.....	≤10/g
E. coli.....	<10/g
Salmonella.....	Negative

Figure D-1. Cheese whey specifications sheet. Provided by Gossner Food (Logan, Utah).

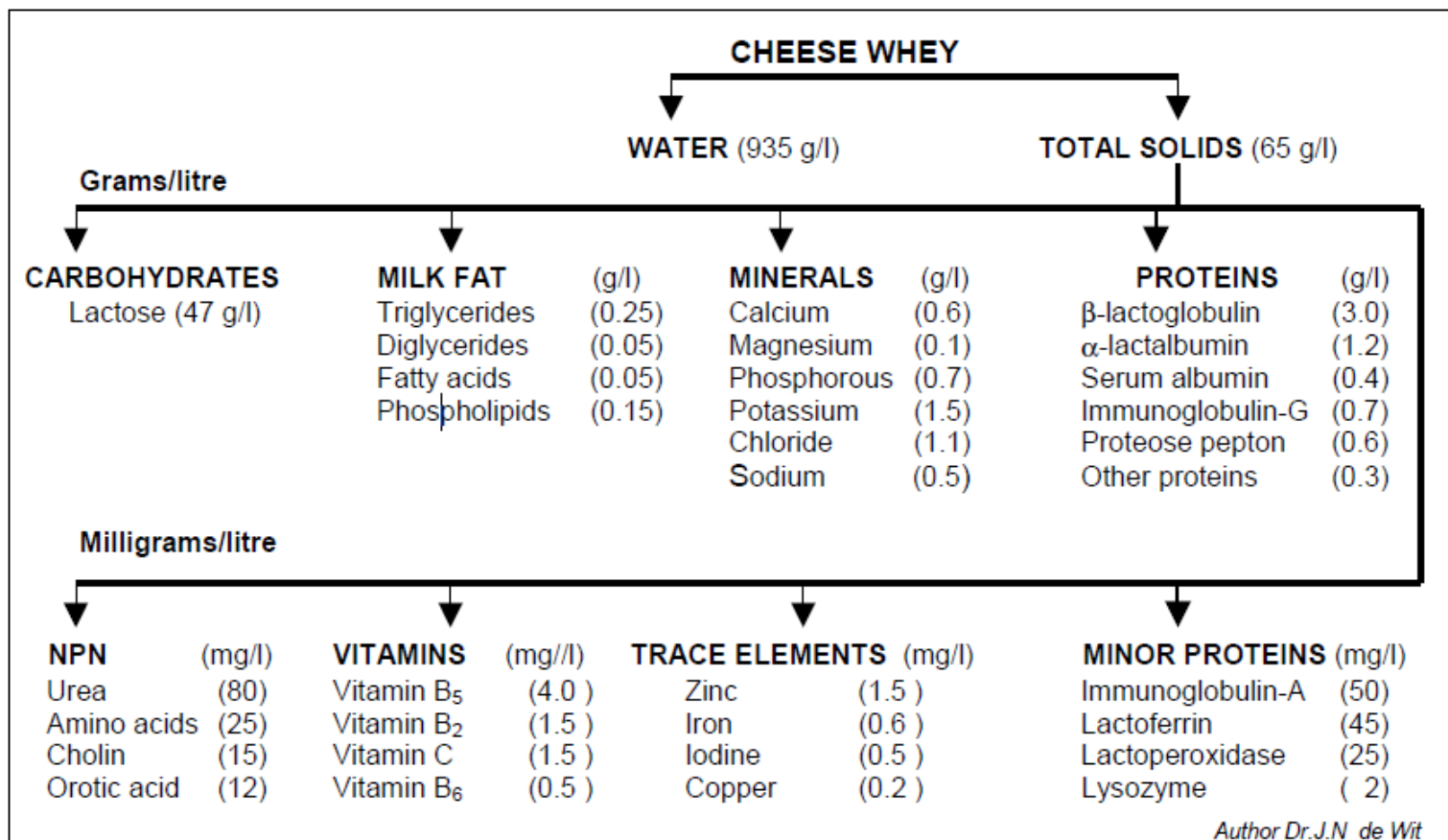


Figure D-2. Approximate composition of cheese whey Gouda (extracted from De Witt, 2001).

APPENDIX E
DATA OF CHEESE WHEY AS SUPPLEMENT
AND CO-SUBSTRATE EXPERIMENT

Table E-1. Results obtained from ABE measurements using Gas Chromatography.

Cheese	Potassium	Magnesium	Ferrous	Acetone	Ethanol	Butanol	ABE
Whey	Phosphate	Sulfate	Sulfate	(g/L)	(g/L)	(g/L)	(g/L)
No	No	No	No	0.93	0.01	1.64	2.58
No	No	No	Yes	0.42	0.06	2.04	2.52
No	No	Yes	No	ND	0.20	1.58	1.78
No	No	Yes	Yes	0.38	0.05	2.00	2.43
No	Yes	No	No	ND	0.01	0.14	ND
No	Yes	No	Yes	0.72	0.13	2.80	3.65
No	Yes	Yes	No	1.18	0.04	2.75	3.97
No	Yes	Yes	Yes	0.84	0.20	2.72	3.75
Yes	No	No	No	2.30	1.52	4.50	8.32
Yes	No	No	Yes	2.86	0.52	15.17	18.55
Yes	No	Yes	No	2.59	0.34	5.86	8.79
Yes	No	Yes	Yes	2.34	0.74	15.50	18.58
Yes	Yes	No	No	ND	ND	ND	ND
Yes	Yes	No	Yes	1.96	0.35	7.62	9.93
Yes	Yes	Yes	No	3.49	0.28	8.99	12.76
Yes	Yes	Yes	Yes	3.56	ND	6.07	9.63
No	Yes	Yes	Yes	ND	ND	ND	ND

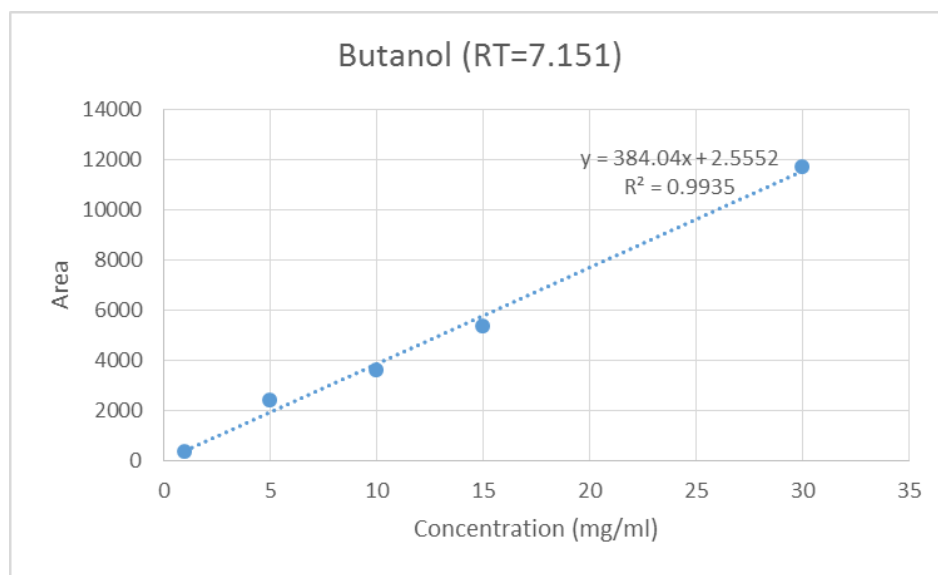


Figure E-1. Calibration curve of butanol in gas chromatography. Data from 7/20/2014.

APPENDIX F

STATISTICAL ANALYSIS RESULTS

Table F-1. Results of GML procedure from SAS. T_sugars is in g/Kg of dried algae

Class Level Information					
Class	Levels	Values			
block (replicates)	2	1 2			
acid	6	0 0.35 0.5 0.7 1 1.5			
time	3	40 80 120			
temperature	3	23-30 45-55 80-90			
Number of Observations					
Read	108				
Number of Observations					
Used	108				
Dependent Variable: T_Sugars (sugar^0.5)					
Source					
	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	54	586.33	10.86	11.78	<.0001
Error	53	48.86	0.92		
Corrected Total	107	635.19			
R-Square					
	Coeff Var	Root MSE	T_Sugars Mean		
	0.9231	15.4769	0.9601	6.2037	
Source					
	DF	Type III SS	Mean Square	F Value	Pr > F
block	1	0.63	0.63	0.68	0.4137
acid	5	251.24	50.25	54.51	<.0001
time	2	0.92	0.46	0.50	0.6091
acid*time	10	13.87	1.39	1.50	0.1638
temperature	2	189.66	94.83	102.87	<.0001
acid*temperature	10	89.72	8.97	9.73	<.0001
time*temperature	4	10.77	2.69	2.92	0.0294
acid*time*temperatur	20	29.52	1.48	1.60	0.0877

<i>Source</i>	<i>DF</i>	<i>Sum of Squares</i>	<i>Mean Square</i>	<i>F Value</i>	<i>Pr > F</i>
<i>Model</i>	18	96213.9683	5345.2205	17.52	<.0001
<i>Error</i>	17	5187.3636	305.1390		
<i>Corrected Total</i>	35	101401.3319			

<i>R-Square</i>	<i>Coeff Var</i>	<i>Root MSE</i>	<i>sugars Mean</i>
0.948843	24.16596	17.46823	72.28444

<i>Source</i>	<i>DF</i>	<i>Type I SS</i>	<i>Mean Square</i>	<i>F Value</i>	<i>Pr > F</i>
<i>block</i>	1	1545.80028	1545.80028	5.07	0.0379
<i>acid</i>	5	75794.66032	15158.93206	49.68	<.0001
<i>time</i>	2	5036.45057	2518.22529	8.25	0.0031
<i>acid*time</i>	10	13837.05709	1383.70571	4.53	0.0031

<i>Source</i>	<i>DF</i>	<i>Type III SS</i>	<i>Mean Square</i>	<i>F Value</i>	<i>Pr > F</i>
<i>block</i>	1	1545.80028	1545.80028	5.07	0.0379
<i>acid</i>	5	75794.66032	15158.93206	49.68	<.0001
<i>time</i>	2	5036.45057	2518.22529	8.25	0.0031
<i>acid*time</i>	10	13837.05709	1383.70571	4.53	0.0031

Figure F-1. GLM procedure for analyzing the effect of acid and retention time at 80-90 °C on sugar yield. Sugar is measured in g/Kg of dried biomass

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.49225508	0.06153189	17.25	0.0031
Error	5	0.01783354	0.00356671		
Corrected Total	13	0.51008862			

R-Square	Coeff Var	Root MSE	T_butanol Mean
0.965038	19.66473	0.059722	0.303701

Source	DF	Type III SS	Mean Square	F Value	Pr > F
FS	1	0.01743348	0.01743348	4.89	0.0780
CW	1	0.34235065	0.34235065	95.99	0.0002
PP	1	0.01800087	0.01800087	5.05	0.0746
CW*PP	1	0.03219506	0.03219506	9.03	0.0299
MS	1	0.00094479	0.00094479	0.26	0.6287
CW*MS	1	0.00039981	0.00039981	0.11	0.7514
PP*MS	1	0.00048706	0.00048706	0.14	0.7269
CW*PP*MS	1	0.00033991	0.00033991	0.10	0.7700

Figure F-2. GLM procedure for T_butanol (butanol⁻¹) in Cheese whey experiment.

Butanol is measured in g/L. Factors evaluated are ferrous sulfate (FS), cheese whey (CW), potassium phosphate (PP), and magnesium sulfate (MS).

cheese whey (CW), potassium phosphate (PP), and magnesium sulfate (MS).

*T*_{ABE}

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	15.90322801	2.27188972	7.40	0.0085
Error	7	2.15026572	0.30718082		
Corrected Total	14	18.05349374			

R-Square	Coeff Var	Root MSE	T _{ABE} Mean
0.880895	22.71058	0.554239	2.440444

Source	DF	Type III SS	Mean Square	F Value	Pr > F
FS	1	1.21383121	1.21383121	3.95	0.0872
CW	1	11.56646522	11.56646522	37.65	0.0005
PP	1	0.23364489	0.23364489	0.76	0.4121
CW*PP	1	0.30183933	0.30183933	0.98	0.3546
MS	1	0.34422545	0.34422545	1.12	0.3249
CW*MS	1	0.00164017	0.00164017	0.01	0.9438
PP*MS	1	0.47462174	0.47462174	1.55	0.2539

Figure F-3. GLM procedure for T_{ABE} (ABE^{0.5}). ABE is measured in g/L.

APPENDIX G
CO-AUTHORSHIP AUTHORIZATION

08/12/2014

To whom it may concern,

My name is Joshua T. Ellis. I am co-author on Yessica Castro manuscript titled, "**OPTIMIZATION OF WASTEWATER MICROALGAE SACCHARIFICATION FOR ACETONE, BUTANOL, AND ETHANOL FERMENTATION USING DILUTE ACID HYDROLYSIS**". This paper corresponds to chapter 2 of her MS thesis. Yessica was first author and the major contributor to the manuscript. As co-author, I give her permission to reprint the manuscript in its entirety in her MS thesis.

Sincerely,

A handwritten signature in black ink, appearing to read 'J. T. Ellis', is written over a horizontal line.

Joshua T. Ellis