

Production of biobutanol from white grape pomace by
Clostridium saccharobutylicum using submerged fermentation.

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A thesis submitted to
Auckland University of Technology
in partial fulfilment of the requirements for the degree
of
Master of Applied Science (MAppSc)

2010

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed:

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Acknowledgement

I would like to show my gratitude to my supervisor, Noemi Gutierrez-Maddox, whose encouragement, guidance and support from the beginning to the final stage enabled me to have a better understanding of the subject.

I am grateful to Barbara Breen and the owners of Oakura Bay Estate who provided me the white grape pomace and who gave words of encouragement.

I am grateful to Mark Duxburry, John Robertson, and Jamuna Shankar for assisting me with the analytical aspect involving the use of a Gas Chromatograph.

I owe my greatest gratitude to Brid Lorigan, Roger Whiting and Chris Yearsley for finding the resources I used to finish this project.

I am indebted to the laboratory staff of Auckland University of Technology, Dushi Bigwood, Saeedeh Sadooghy-Saraby, Christine Silverster, Percy Perera, Yan Wang, and Chris Whyburd whose help and assistance allowed me to finish this project on time.

Lastly, this thesis would not be possible without the support and encouragement of my family.

Abstract

The use of agricultural residues for the production of biofuel such as butanol is one potential alternative to fossil fuels. The abundance of white grape pomace in the wine industry in New Zealand makes grape pomace a potential substrate for the production of butanol using *Clostridium saccharobutylicum* (formerly known as *Clostridium acetobutylicum* P262).

Chardonnay grape pomace was fermented with *C. saccharobutylicum* using submerged fermentation. The concentration of reducing sugars was measured using the Dinitrosalicylic acid reagent while the pH was monitored using a pH meter. Gas Chromatography was used to measure acetone, butanol, ethanol, acetic acid and butyric acid. The growth of *Clostridium saccharobutylicum* was determined by viable plate count, which was converted to dry weight for evaluation of kinetic parameters. The kinetic parameters of the fermentation which included total ABE (acetone butanol ethanol) production, total solvent yield (solvents/substrate), total solvent productivity (g/L/h), reducing sugar utilisation (g/L/h), and specific growth rate (g/g/h) were determined.

The parameters necessary for a solventogenic fermentation of grape pomace (12.5 % wet weight/volume) by *C. saccharobutylicum* were first established. This study demonstrated that the parameters that enabled the production of solvents in grape pomace (12.5 % w/v) were the concentration of 11 % (v/v) inoculum, a concentration of 1 % (w/v) yeast extract, and the adjustment of pH 5.5 with potassium phosphate buffer (1M). The fermentation in white grape pomace with these parameters resulted in the production of ABE (acetone, butanol, ethanol) at 8.76 g/L, a total solvent yield of 0.30 g/g, and productivity of 0.21 g/L/h. The bioprocess and kinetic parameters were lower than a typical batch fermentation indicating that the presence of inhibitors could have hindered the fermentation of grape pomace. Copper and zinc are known to be found in grape pomace which could exert inhibitory effects on *C. saccharobutylicum*. Polyphenolic compounds are other potential growth inhibitors in grape pomace.

This study found that an alternative to Yeast Extract for the grape pomace fermentation involved the use of manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$). The addition of these mineral salts to grape pomace resulted in a total production of solvents 9.08 g/L, a solvent yield of 0.36 g/g, and a productivity of 0.19 g/L/h. The fermentation with the mineral salts produced a higher total solvent production and solvent yield (based on substrate) than those of a fermentation supplemented with yeast extract. Fermentation in the presence of these mineral salts was successful probably due to the presence of ferrous sulphate which might have repressed the growth inhibitory effect of copper and zinc in grape pomace.

The optimisation of the fermentation process which enabled the grape pomace to remain submerged led to an acidogenic fermentation due to oxygen being re-introduced to the substrate when the magnetic rod was activated. This shows that controlled agitation and controlled pH using a batch bioreactor should be applied instead.

The production of butanol by fermentation using grape pomace as substrate is promising, particularly if all toxic components in grape pomace could be removed.

Chapter 1

Introduction

1.1 Dependence on petroleum-based fuels

Over the last decade, the depletion of oil resources and concerns regarding both economic and environmental issues associated with petroleum-based fuels have renewed interests for the search of sustainable biofuel that are the product of renewable resources known as biomass (Antoni, Zverlov, & Schwarz, 2007). Today's society which is dependent on fossil fuels faces major issues associated with the increasing consumption of fossil fuels. According to Sperling and DeLuchi (1989), the oil resources are not distributed sustainably. They stated that the dependence on foreign petroleum supplier (energy security), indirect economic costs of energy import, global warming and air pollution are four major problems associated with this energy source:

1. In terms of energy security, most of countries that heavily rely on oil imports are at the mercy of supply disruption and restriction, price rises, cost of oil shipment, protection of oil supply (Sperling & DeLuchi, 1989).
2. From an economic perspective, being dependent on oil imports has indirect costs due to oil price volatility and rise of world oil price (Sperling & DeLuchi, 1989). For instance, the price volatility would impair the decision making in terms of investments (Sperling & DeLuchi, 1989). A world energy trade model developed by Stanford University and used by the US Department of Energy Policy Office showed that the use of alternative fuels of gasoline would not only reduce the worldwide oil demand but also the world oil barrel price (Sperling & DeLuchi, 1989). The increase of oil imports affects the national economy of the importing country because the funds used for importing oil could have been

allocated for both domestic goods and services (Sperling & DeLuchi, 1989).

3. The increasing emission of greenhouse gases such as carbon dioxide in the atmosphere from the burning of oil, coal, and natural gas is considered to be a major issue in terms of global warming (Sperling & DeLuchi, 1989). From an environmental perspective, the emissions of atmospheric greenhouse gases which lead to climate change have focused studies to the reduction of anthropogenic emissions of greenhouse gases, especially carbon dioxide from fossil fuels (Kheshgi, Prince, & Marland, 2000). Scientists believe that humans are contributing significantly to the climate change due to the emission of gases such as carbon dioxide, nitrous oxide and methane (Carroll & Somerville, 2009). In addition, burning fossil fuels which accounts for 85 percent of energy consumption is considered to be the main source of emission of carbon dioxide (Carroll & Somerville, 2009). More attention has been focused on this issue in comparison to energy security or the indirect economic aspect of importing oil because it supposedly has more adverse effect economically (Sperling & DeLuchi, 1989). One practical way to solve this issue is to reduce the emission of carbon dioxide by using lesser transport fuels of fossil fuel origin or increase the use of alternative fuels from renewable biomass (Sperling & DeLuchi, 1989). Using alternative biofuels makes transport fuels a potential target for the reduction of greenhouse gas (Antoni et al., 2007). The difficulty of removing carbon dioxide emissions from the transport sector on a large scale makes the use of biomass to produce a substitute for transport fuel very appealing (Carroll & Somerville, 2009). According to the Energy Information Administration (2009), the environmental impacts associated with the use of fossil fuels, the increasing oil price projected in the next decade, and proposals of governments to use renewable resources are incentives that urge the use of renewable energy in the future.

4. The emission of air pollutants from the combustion of transport fuels, refinery or fuel stations is known to contaminate the environment such as aquifer and coastal areas and to contribute to air pollution causing both acute and long term illnesses. As a result several measures to reduce the emission of greenhouse gas and air pollution would encourage the use of alternative fuels or cleaner transport fuels (Sperling & DeLuchi, 1989).

Countries which have the ability to produce biofuels from locally produced biomass would not only decrease their dependence on this limited resource but also the emission of greenhouse gas since biofuels are considered as a neutral energy (Antoni et al., 2007). New Zealand being a large producer of grapes for the production of wine would have the opportunity to become less dependent on fossil fuels and reduce its emission of greenhouse gases with the prospect of producing a biofuel by converting the biomass wastes of wine industry known as grape pomace using the anaerobic bacteria, *Clostridium saccharobutylicum*.

1.2 Biofuels

Any transportation fuel originating from a biological source, which can either be in a liquid or gaseous form such as ethanol, biodiesels, or as biogas or hydrogen, are known as biofuels (Fulton, 2004). There are several types of biofuels under study and they ranged from bioethanol, biodiesel and biobutanol. According to Antoni et al. (2007), bioethanol and biodiesel are the two main transport fuels produced on an industrial scale. Alcohol fuels such as methanol, ethanol, butanol are able to not only expand the use of diesel or gasoline, but are good additives in such fuels having oxygenisers, liquefiers or anti-knocking agent properties (Antoni et al., 2007).

Bioethanol currently accounts for 90 % of the biofuel produced around the world from renewable resources (Antoni et al., 2007; Gray, Zhao, & Emptage, 2006; Qureshi et al., 2008). Despite the popularity of using ethanol as a motor fuel,

there are several disadvantages associated with this biofuel in comparison with butanol. According to Enguítanos, Soria, Kavalov, and Jensen (2002), bioethanol cannot be run purely on current gasoline engines without modification because the presence of metals such as zinc, brass, lead, aluminium have to be replaced due to the leaching action by ethanol. In addition, it is not economically feasible to run a market on an industrial large scale with pure bioethanol due to the disadvantages associated with this biofuel.

Bioethanol having less energy than gasoline due to its low cetane number and high octane number requires more ethanol to be produced in order to have equal quantity of energy of gasoline (Balat, Balat, & Öz, 2008; La Cava, 2008). In addition, this biofuel has a low vapour pressure hence affecting cold starts in cooler climate. Ethanol is also miscible with water, and as there is always some water present in fuel tanks, the ethanol will separate into the water phase if any phase separation occurs in cold weather (Balat et al., 2008).

One of several environmental issues associated with bioethanol is that the biomass used for its production such as corn grain requires not only land but also the use of nitrogen, phosphorous and pesticides which all have a negative effect on the environment (La Cava, 2008). Since corn grain is used as human food, the production of this biomass destined for ethanol would not satisfy the current demand (La Cava, 2008). On the other hand, butanol is a potential biofuel that not only overcomes the shortcomings of bioethanol but it can be produced using a biomass that does not compete with food resources.

1.3 Biomass for biofuel

Biomass, which is also known as biological matter, is generally from agricultural crops that have low or no profits from industrial processes or from crops grown for fuels (Claassen et al., 1999; Sperling & DeLuchi, 1989). The production of transportation fuels from biomass origin is not new since it has been manufactured in the nineteenth century (Sperling & DeLuchi, 1989). Antoni et al. (2007) reviewed the history of transport fuels from biomass. The review stated that throughout history, mankind has relied so much on renewable resources that it led to the development of energy resources which intertwine with the technological revolution. In addition, the most common product from renewable resource is the production of alcohols based on the fermentation of sugars, while others used plant oils. Alcohols were already considered as a biofuel at the beginning of the nineteenth century. In the 1860s, the biofuel ethanol was used when engine ignition was invented. Ethanol being considered as an anti-knocking additive in combustion engine was already mixed with gasoline in internal combustion engines between 1925 and 1945. The price of gasoline was so low in 1940 making ethanol so uncompetitive that it halted its production. The use of biofuels was still going on during the Second World War but after the war, gasoline became the main transport fuel. New interest in ethanol occurred in the 1970s in one of the largest producers in the world, Brazil.

Significant bioethanol production from biomass started in the seventies in both Brazil and United States using the fermentation of sugar cane and corn, respectively (Sperling & DeLuchi, 1989). The main crops used solely for energy production are sugar cane and corn (Claassen et al., 1999). The deforestation and increasing use of land required for the cultivation of single crop raised environmental concerns (Antoni et al., 2007). The same issues occurred around the world and the use of food crops for biofuel rather than food has hindered the development of the biofuel industry (Antoni et al., 2007).

On the other hand, there are other biomass that originate from agricultural or industrial wastes that do not make any profit (Sperling & DeLuchi, 1989). European countries and the United States have wheat straw, corn stalks, soybean residues as by-products of agricultural crops that are potential

renewable resources that can be used for the production of biofuels (Claassen et al., 1999). Grape pomace is a good potential renewable resource for biofuel production such as biobutanol because it is produced in large quantities and it does not currently have any value.

1.4 Butanol

Butanol is a product of an anaerobic fermentation, which was discovered by Pasteur in 1862 (Dürre, 2008). Although the annual production of butanol by chemical synthesis is between 10 to 12 billion pounds according to Lee et al. (2008), half of the production is used as butyl acetate and methacrylate, components of lacquers, enamels or latex surface coating. It is a colourless, miscible, flammable liquid that has a variety of industrial uses ranging from adhesives, fibres, plastics, textiles, oil additive to name a few but one of the most forthcoming application is the use of biobutanol as a biofuel.(Dürre, 2008). Butanol has several advantages in comparison to ethanol based on the reviews of Shapovalov and Ashkinazi (2008) and Dürre (2007) on butanol fermentation:

1. Butanol contains around 25 % more energy than ethanol, 29.2 MJ/L of caloric value for butanol whereas ethanol has 21.2 MJ/L and gasoline has 32.5 MJ/L (Dürre, 2007).
2. Butanol is safer to ethanol because it is less volatile than ethanol and gasoline due to its vapour pressure.
3. Butanol is less corrosive than ethanol and it would not require any change in the current infrastructure consisting of tanks, pipelines, pumps, and filling stations (Dürre, 2007).
4. The low miscibility of butanol in the presence of water allows it to be mixed with gasoline at higher concentrations than current biofuels, while ethanol used as an additive should not be less than 85 % in current motor engines.

5. Butanol would prevent the need of adding new infrastructure when using hydrogen as an alternative to gasoline.
6. During the combustion of butanol, there is no release of sulphur and nitrogen oxides which are present in current fossil fuels, hence the ecological benefits are advantageous.

With the above advantages in addition to the wide substrate range available for butanol production by fermentation, biobutanol seems to hold high potential as alternative fuel. Its use would prevent the rapid depletion and dependence on fossil fuels around the world, thus decreasing the emission of greenhouse gases (Dürre, 2008; Shapovalov & Ashkinazi, 2008). Throughout history, biobutanol has been produced at an industrial scale by using the process known as acetone-butanol-ethanol (ABE) fermentation (Jones & Woods, 1986; Shapovalov & Ashkinazi, 2008).

1.5 Industrial ABE fermentation

Butanol production with ABE fermentation is one of the oldest fermentation process used for the production of chemicals at an industrial scale (Qureshi et al., 2008). The first industrial production of butanol was in 1916 (Shapovalov & Ashkinazi, 2008). According to Qureshi and Ezeji (2008), this fermentation is second to ethanol based on history since commercial plants operated during both World War I and II for the production of acetone and butanol. In the 1950s, the petrochemical industries took over the butanol fermentation making it uncompetitive which led to the closure of the butanol plants (Qureshi et al., 2008). The current high price of substrate would not make butanol production successful in comparison to the low costs of molasses and corn in the 1950s which were both readily and economically available (Qureshi et al., 2008). There are several drawbacks that prevent ABE fermentation to be economically competitive when comparing with other biofuels (Dürre, 1998):

- The cost of the substrate is high.
- The concentrations of solvents are generally low due to their toxicity.
- The cost of recovery is expensive due to the low concentration of product.

Studies indicated that butanol fermentation is becoming more economical based on process technology, the constant improvement for each process involved during the production of butanol, and the ongoing research and development (Zverlov, Berezina, Velikodvorskaya, & Schwarz, 2006). In terms of recovery techniques, there have been some improvements for the recovery process of butanol from ABE fermentation and technologies ranging from simultaneous fermentation and product recovery techniques such as adsorption, liquid-liquid extraction, perstraction, reverse osmosis, pervaporation and gas stripping were developed to make butanol an economical product (Zheng et al., 2009). In addition, the availability of low cost substrates which could be used for the production of butanol make fermentation at an industrial scale successful (Zheng et al., 2009). This next generation biofuel can be

produced fermentatively using renewable resources by a very few bacteria that produce butanol as the main product, most of which are clostridia (Dürre, 2008).

1.6 *Clostridium saccharobutylicum*

Clostridium saccharobutylicum (formally known as *Clostridium acetobutylicum*) is a Gram-positive, spore-forming, and an obligate anaerobe bacterium that is able to perform the conversion from sugars to solvents that is referred as the acetone-butanol-ethanol (ABE) fermentation (Zheng et al., 2009) (Figure 1.2). According to Shinto et al. (2008), ABE-producing clostridia are capable of producing acetone, butanol and ethanol solvent from different types of biomass such as domestic waste, palm oil waste and agricultural crops.

The ABE fermentation based on renewable carbohydrates used to be the largest biotechnological process after ethanol fermentation by yeast (Grube, Gapes, & Schuster, 2002; Zverlov et al., 2006). There are three main types of fermentation products from *C. saccharobutylicum*, which include solvents (acetone, ethanol, and butanol), organic acids (acetic acid, butyric acid and lactic acid), and gases (carbon dioxide and hydrogen) (Qureshi et al., 2008; Zheng et al., 2009). The ABE fermentation using *C. saccharobutylicum* generally produces the typical Weizmann fermentation ratio of 3:6:1 (acetone-butanol-ethanol) (Jones & Woods, 1986).

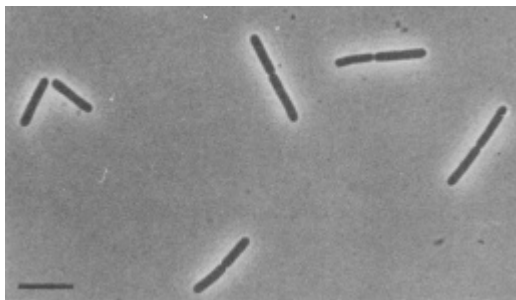


Figure 1.1. Photomicrograph of actively growing rods of *C. saccharobutylicum* in ABE fermentation. Bar, 10 μm (Jones et al., 1982).

ABE fermentation with *C. saccharobutylicum* is characterised by two distinct phases (Maddox et al., 2000). During the first phase also known as the acidogenic phase, the pH value of the fermentation medium decreases due to the conversion of sugars into acids while in the second phase known as the solventogenic phase, consists of sugars and acids are converted to solvents (Maddox et al., 2000). This major metabolic shift prevents the low pH to reach lethal levels and it is represented by the conversion of butyrate and acetate into solvent which increases the external pH (Dürre, 2008). Acetone, butanol and ethanol first undergo the same metabolic pathway from glucose to acetylCoA and then into separate and different pathways (Zheng et al., 2009). Figure 1.2 shows the solvent production pathway where each reaction is catalyzed by an enzyme (Häggström, 1985).

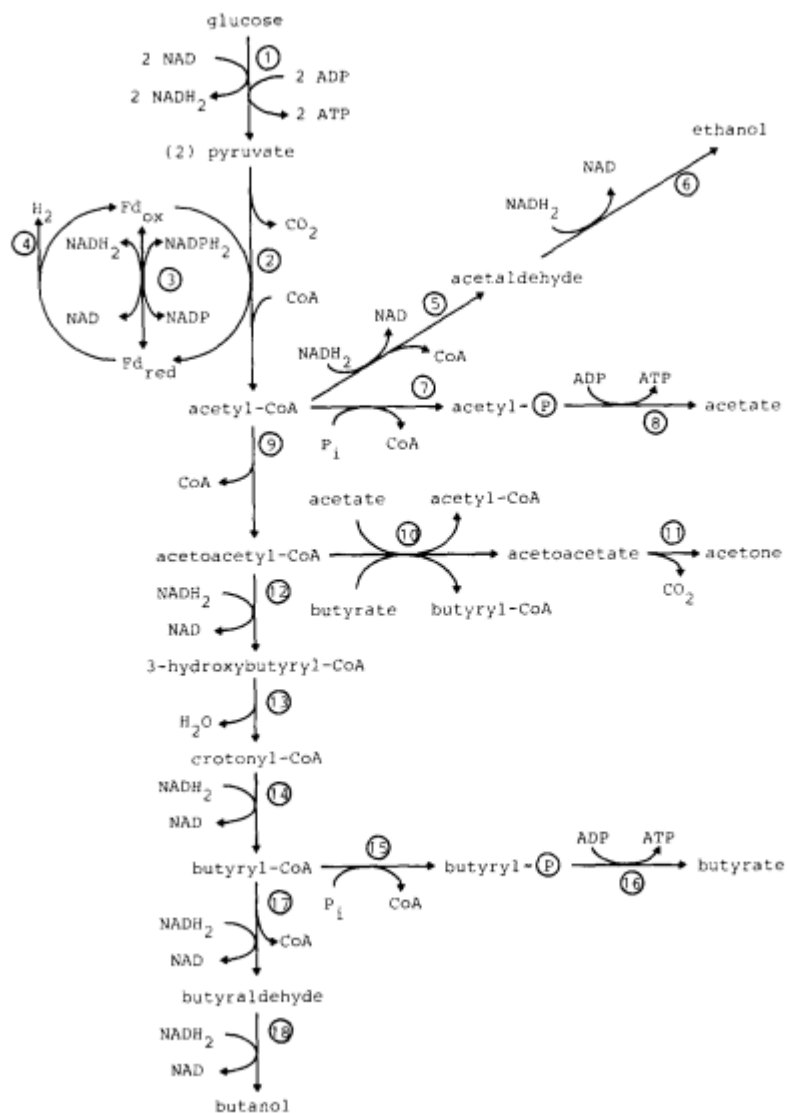


Figure 1.2. Energy metabolism in *C. saccharobutylicum* (Häggström, 1985).

Enzymes of the Embden-Meyerhof Pathway; 2, pyruvate-ferredoxin oxidoreductase; 3, NADH and NADPH-ferredoxin oxidoreductases; 4, hydrogenase; 5, acetaldehyde dehydrogenase; 6, ethanol dehydrogenase; 7, phosphate acetyltransferase; 8, acetate kinase; 9, thiolase; 10, acetoacetyl-CoA: acetate (butyrate) CoAtransferase; 11, acetoacetate decarboxylase; 12, 3-hydroxybutyryl-CoA dehydrogenase; 13, crotonase; 14, butyryl-CoA dehydrogenase; 15, phosphate butyryltransferase; 16, butyrate kinase; 17, butyraldehyde dehydrogenase; 18, butanol dehydrogenase.

1.7 Factors affecting growth and AB fermentation in *Clostridium saccharobutylicum*

The external pH is known to affect the outcome of ABE fermentation. The pH range that is optimum for ABE fermentation for *C. saccharobutylicum* is between 5.0 to 6.5. Any decrease below pH 4.5 will result in an inhibition of growth and consequently AB fermentation (Jones & Woods, 1986).

In terms of growth-limiting factors, the absence of certain nutrients also affects the outcome of the fermentation. According to Jones and Woods (1986), a reducing sugar concentration of less than 7 g/L in a batch fermentation system does not lead to a solventogenic fermentation. The review noted that the effect of nitrogen limitation was not yet well understood with some studies showing that nitrogen limited cultures obtained low concentrations of solvents while other reported that the solvents can be produced under nitrogen limitation. Batch culture systems with phosphate limitation have been demonstrated to produce solvents. In addition, Jones and Woods (1986) reported that both phosphate and sulphate had greater induction effects on the production of ABE than did ammonia or magnesium in a continuous system. In terms of sulphate and magnesium, solvents production was possible with both sulphate and magnesium limited system in a continuous process (Jones & Woods, 1986).

Iron also plays an important role in *C. saccharobutylicum*. According to Bard and Gunsalus (1950), iron has a significant function in the metabolism of the genus *Clostridium* because studies have demonstrated that iron has an influence on growth and in terms of glucose fermentation, where iron-deficient fermentation would cause a shift to lactate production.

1.8 Toxicity effects of copper and zinc on *Clostridium* species

Heavy metal ions such as copper and zinc are known to have inhibitory activity on microbial processes (Kuo & Genthner, 1996; Said & Lewis, 1991). According to Sevinç et al. (2009), the effect of heavy metals on microorganisms depends on the metal, concentration, and microorganism. In addition, environmental

factors such as the presence of other metals ions, buffer, pH or organic compounds can alter the interaction between metals ions and microorganisms. In their study, copper has the greatest inhibitory effect on sulphate reducing bacteria in comparison to zinc.

Copper has such an inhibitory effect on microorganisms that studies have examined the use of copper for the reduction of environmental contamination in hospital and food processing plant (Casey et al., 2009; Faúndez, Troncoso, Navarrete, & Figueroa, 2004). Santo, Taudte, Nies, and Grass (2008) reported that material surfaces containing copper can be used to fight bacterial contamination such as *Escherichia coli* due to its antimicrobial properties.

Copper and zinc are found in white grape pomace based on the analysis done by Spanghero et al. (2009). There have been a few studies done involving *Clostridium* species and the inhibitory effect of copper. Mato, Rodriguez, and Alatosava (2010) examined the effects of copper on several strains of *Clostridium tyrobutyricum* used in the production of Emmental cheese. They revealed that the inhibitory effects of copper (7.5 to 15 ppm) on the germination and sporulation were greater than the vegetative growth and sporulation of *C. tyrobutyricum* and that the inhibitory effects of copper on germination, vegetative growth and sporulation processes depended on the strain.

Markwiese and Colberg (2000) showed that copper toxicity can affect the anaerobic carbon oxidation. In addition, their study revealed that fermentative bacterium such as *Clostridium* spp has higher copper complexation capacity than iron reducing bacteria. They stated that Gram-positive bacteria readily bind with metals than Gram-negative bacteria. Weaver, Michels, and Keevil (2008) examined the survival of *Clostridium difficile* on copper and steel. They demonstrated that the survival of vegetative cells and spores is lesser on metal surfaces made of copper alloy containing more than 70% copper content in comparison to steel surfaces.

They stated that the production of hydroperoxide radicals produced by copper damage the cell membrane of the vegetative cells. Hence, the metabolism of bacterial cells is inactivated due to the interference of copper with electron transport pathways. However, in fermentative bacteria, chemiosmosis across

the cell membrane could be the process affected by copper damage. According to Mato, Rodriguez, and Alatossava (2010) this metal affects sporulation and germination because the molecules such as spore membrane, coat proteins which are involved in both processes are rich in cystein residues and might interact with copper.

Although studies involving the antimicrobial properties of zinc have not been investigated with *C. saccharobutylicum*, Yamamoto (2001) reported that zinc oxide exerts inhibitory effects on both Gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria such as *E. coli*. The inhibitory activity of zinc oxide could be similar to that of copper in generating hydrogen peroxide (H₂O₂) which could be toxic to clostridia.

1.9 Substrates used for the production of ABE

The review performed by Jones and Woods (1986) reported that apart from the conventional molasses and maize, there have been several types of substrate which qualify as potential substrates for *Clostridium saccharobutylicum* due to its ability to consume different types of carbohydrates for the production of butanol.

Carbohydrates that are fully consumed are glucose, fructose, mannose, sucrose, lactose, and dextrin while galactose, xylose, arabinose, raffinose, inulin, melezitose, and mannitol are partially consumed (Jones & Woods, 1986). The review stated that Jerusalem artichoke, algal biomass, apple pomace, cheese whey, potatoes, rice, tapioca, which are noncellulosic substrates are potential substrates for *C. saccharobutylicum*.

Agricultural residues such as barley hydrolysate were used by Qureshi, Saha, Dien, Hector, and Cotta (2010a) while Qureshi et al. (2010b) used corn stover and switchgrass hydrolysates for the production of butanol using *Clostridium beijerinckii* P260. Those studies concluded that high concentration of butanol can be produced only when toxic compounds were inactivated.

1.10 Wine Grapes

Vitis vinifera L., also known as grape, is considered to be one of the largest fruit crops in the world and it is mainly used for the production of wine (Ruberto, Renda, Amico, & Tringali, 2008). According to the Food and Agriculture Organisation (FAO) (Food and Agriculture Organisation Statistical, 2010), there were more than 60 millions of tons produced in 2007. Eighty percent of the grapes is used for wine and juice and it is estimated that 10 million tons of grape pomace is produced annually worldwide shortly after the grape harvest (Zhou, Li, Zhang, Bai, & Zhao, 2009).

According to New Zealand Wine (2010), the export value of wine in 2009 in New Zealand reached \$992 million based on the production of 113 million litres of wine. New Zealand Wine (2010) estimated that 60 million litres of wine was sold in New Zealand, which is an increase of 29 percent from the previous year. In terms of vintage, around 285 thousand tonnes was picked in January 2010, which consisted of 228 thousand tonnes of white *vinifera*. Marlborough Sauvignon Blanc had the highest vintage with 161 thousand tonnes of harvest followed by Chardonnay which accounted for 27 thousand tonnes (New Zealand Wine, 2010). Among different winery styles, the international export of Sauvignon Blanc increased by 37 percent since 2008 whereas Chardonnay and Riesling fell 13 and 22 percent, respectively (New Zealand Wine, 2010). The amount of white vine is significant in this present study since it accounted for 228 thousand tonnes of grapes produced and the by-product of this vintage is the production of 64 thousand tonnes of industrial waste.

Like most fermentation processes, the winemaking process does not just produce wine but also by-products such as grape pomace, which is considered as an industrial wastes (Lu & Yeap Foo, 1999). The main waste of wine making industry is grape pomace (Ruberto et al., 2008).

1.11 Grape pomace

The first step of making wine is to extract the grape juice by crushing the grapes (Spanghero et al., 2009). After the juice extraction, the solid residues consisting of peel, seeds, and pulp residues that remain in the wine-making process are referred as grape pomace (Antonia & Jaime, 2008; de Pina & Hogg, 1999). Figure 1.1 shows grape pomace after the pressing stage. It is estimated that 18 to 20 kilograms per 100 kilograms of grapes represent grape pomace (Spanghero et al., 2009).

The increasing production of wine has lead to environmental issues regarding the disposal of grape pomace (Bates & Regulski, 1982). According to Ruberto, Renda, Amico, and Tringali (2008), there are two contradictory aspects of agro-industrial by-product. Firstly, their disposal causes ecological and economical issues since the large amount of accumulated grape pomace is an industrial pollutant that results in vinegar fly infestation or the development of pathogen which can affect the vineyard (Bates & Regulski, 1982). Secondly, this by-product of winemakers can be regarded as a renewable source which posses nutritional properties (Ruberto et al., 2008).



Figure 1.3. Chardonnay grape pomace after the pressing stage of winemaking.

Studies have been done which focus on exploiting the potential uses of grape pomace (Lu & Yeap Foo, 1999). Industrial applications of grape pomace range from animal feed, nutritive ingredients, production of citric acid, and the extraction of anthocyanins present in the grape skins as colorants (Lu & Yeap Foo, 1999). Grape pomace has low nutritive values when used as a feed for bovines because they are indigestible due to polyphenolic compounds such as tannin (Bates & Regulski, 1982). A high amount of tannin is found in grape pomace and their ability to bind to protein which prevent digestion in rumen make grape pomace an indigestible feed (Alipour & Rouzbehan, 2010). In addition, the high lignified fibre of grape seeds is one major limitation for their use as a feedstock because they remain undigested in bovines, while the presence of secondary compounds which are phenolics and anthocyanins appears to be detrimental on rumen fermentation (Spanghero et al., 2009). Since grape pomace has low nutritional value for ruminants, this residue has been limited to land applications (Spanghero et al., 2009). In addition to the use of grape pomace as animal feed, another common way to dispose it is to use it as a fertiliser (Ruberto et al., 2008). Instead of adding this waste directly to vineyard due to the presence of pathogens before using the compost as fertiliser and soil conditioner (Bates & Regulski, 1982).

Other studies on grape pomace were devoted to determine the benefit of health-products derived from by-product of grapes (Murthy, Singh, & Jayaprakasha, 2002). Grapes have high levels of polyphenolic compounds in skins, pulp, and seeds where small amounts are known to be transferred into wine during the wine making process (Guerrero, Torres, & Nuñez, 2008). Those polyphenolic compounds include phenolic acids, flavonoids, anthocyanins and proanthocyanidins (Lu & Yeap Foo, 1999). The health benefits of phenolic compounds have been actively investigated. Phenolic compounds have been demonstrated to exert antioxidant activity and food preserving ability. They act as free radical scavengers and prevent lipoprotein oxidation (Guerrero et al., 2008; Lu & Yeap Foo, 1999). Grape seeds are used for the production of oil, aromatic additives for lotions or nutraceuticals products after they are separated from skin and grape pulp (Spanghero et al., 2009).

Grape pomace is chemically complex because it consists of several compounds mainly, acids, aldehydes, esters, pectins, polyphenolic compounds, mineral

substances and sugars to name a few (Ruberto et al., 2008). The presence of micronutrients ranging from carotenoids, polyphenols, tocopherols, vitamins, oligoelements, which are beneficial for human health could be extracted from this increasing amount of residue making it an economical source of nutrients (Ruberto et al., 2008). However in this context, grape pomace can be used more wisely for the production of biofuel due to potential shortage of fossil fuel.

Grape pomace is known to contain reducing sugars, water, proteins, and ash based on the analysis performed by Bates and Regulski (1982). The carbohydrate content, moisture, phenol, lipids, nitrogen and mineral composition of grape pomace were analysed. The concentration of reducing sugars and the chemical composition differ depending on the grape variety. Hang & Woodams (2008) measured the glucose content of grape pomace with values between 7.81 to 10.81 % while Bates and Regulski (1982) had different grape varieties with concentrations varying from 12.5 g/L to 48.8 %. Based on previous studies, white grape pomace has, on average, a pH between 3.48 to 4.12 depending on the grape variety, making it acidic due to the content of malic, tartaric and citric acids in grapes (Hang, Lee, & Woodams, 1986; Hang & Woodams, 2008; Scalzo, Iannocari, & Summa, 2007; Soyer, Koca, & Karadeniz, 2003). Due to the nutrient content of grape pomace, this substrate was used to produce hydrolytic enzymes using *Aspergillus awamori* (Botella, Ory, Webb, Cantero, & Blandino, 2005). Based on the mineral composition of white grape pomace analysed by Spanghero et al. (2009), potassium is present in the pulp of grape pomace at a higher concentration (23.1 g/kg) than in the seeds (8 g/kg). The concentration of magnesium measured was slightly higher in seeds than in pulp of grape pomace with values of 1.3 g/kg compared to 1.1 g/kg, respectively. The presence of reducing sugars in high concentration, and of minerals and vitamins in grape pomace should permit the growth and solvent (ABE) production from *C. saccharobutylicum* in grape pomace.

The use of industrial wastes as substrate for the production of biofuel is not new. However, there have been few studies done in the production of biobutanol using grape pomace as substrate. A similar study was done by Voget, Mignone, and Ertola (1985) that involved the production of butanol from apple pomace using several strains of *C. acetobutylicum*. The study concluded that apple pomace is a potential substrate for the production of butanol (65 %)

from apple pomace. Hang et al. (1986) performed a solid-state fermentation on grape pomace using wine yeasts for the production of ethanol. The ethanol yield (based on substrate) of grape pomace fermentation with wine yeast were 0.81 for *Saccharomyces cerevisiae* Montrachet #522, *Saccharomyces bayanus* California Champagne # 505 and 0.82 for *Saccharomyces bayanus* Pasteur Champagne # 595 and *Saccharomyces cerevisiae* Epernay # 2. The high ethanol yield obtained in Hang et al. (1986) is typical of wine yeasts since ethanol is the main end-product of the anaerobic glycolytic pathway of yeasts (Lee, 2006). On the other hand, a solventogenic fermentation by *C. saccharobutylicum* yields ethanol which has the lowest ratio in comparison to butanol (1:6) (Jones & Woods, 1986).

Recently studies have focused on the use of agricultural residues for the production of butanol using *Clostridium* species. Qureshi et al. (2010a) used barley straw hydrolysate using batch fermentation. The study concluded that the removal of inhibitors in the substrate enabled the production of solvents with values typical of batch fermentation. The removal of the inhibitors in the barley straw hydrolysate resulted in a solvents production of (26.64 g/L) (Qureshi et al., 2010a). This study differs from the present study because a pre-treatment with enzyme is required for their lignocellulosic substrate while grape pomace, a noncellulosic substrate, will have high concentration of reducing sugars available for *C. saccharobutylicum*. Hang et al. (1986) who performed a solid-state fermentation in grape pomace with wine yeast did not perform any treatment on their substrate. Neither did Voget et al. (1985) applied any treatment to their substrate, apple pomace, for the production of butanol using submerged fermentation.

Only a few studies have been carried out on the utilisation of grape pomace for the production of butanol using *Clostridium saccharobutylicum*. This study was carried out to investigate if white grape pomace can be used for the production of butanol by *C. saccharobutylicum* using submerged fermentation. This study also aimed to establish the fermentation conditions which include inoculum concentration, yeast extract concentration, substrate, pH required to produce solvents from grape pomace. In addition, an alternative to the costly yeast extract and an optimised fermentation process of white grape pomace were determined.

Chapter 2

Material and Methods

2.1 Microorganism

The bacterial strain used in this study was the industrial strain *Clostridium saccharobutylicum* formerly known as *Clostridium acetobutylicum* P262 which was provided as a spore suspension from Dr. Noemi Gutierrez-Maddox, Auckland University of Technology, Auckland, New Zealand.

2.2 Fermentation substrate

Chardonnay grape pomace was a gift from Dr. Barbara Breen and Oakura Bay Estate, Waiheke Island. Ten kilograms (wet weight) of this grape pomace was collected immediately after the grapes were crushed. Figure 2.1 shows the Chardonnay grape before pressing. The grape pomace was divided into small lots of 40 grams in clean re-sealable Glad Zip Slide plastic bags (Glad Products, New Zealand) and stored in a freezer at minus 10°C.



Figure 2.1. Chardonnay grape from Oakura Bay Estate, Waiheke Island.

2.3 Culture media

Reinforced Clostridial Agar, RCA (BD Difco™, New Jersey, U.S.A.) was used to prepare slopes for the spore production of *C. saccharobutylicum* and for viable count from the fermentation experiments.

Cooked Meat Medium with Glucose, CMMG (Accumedia, Michigan, U.S.A.), used for the inoculum development was supplemented with glucose at 10 g/L.

Yeast Extract, Y.E. (BD Bacto™, New Jersey, U.S.A.), was added into the fermentation medium containing grape pomace. Two concentrations were used in this study, 0.5 % (w/v) (Maddox, 1980) and 1 % (w/v).

A Semi-Synthetic Medium, SSM, was prepared for the calibration curve between dry weight and viable count of *Clostridium saccharobutylicum*. Table 2.1 shows the composition of this medium (Gutierrez & Maddox, 1987):

Table 2.1: Composition of Semi-Synthetic Medium (Gutierrez & Maddox, 1987).

Ingredients	Concentration (g/L)
D-Glucose anhydrous	50.0
Yeast Extract	5.0
Ammonium acetate	2.0
L-Cysteine hydrochloride hydrate	0.5
NaCl	1.0
K ₂ HPO ₄	0.75
KH ₂ PO ₄	0.75
MgSO ₄	0.2
MnSO ₄ .7H ₂ O	0.01
FeSO ₄ .7H ₂ O	0.01
Deionised Water	1 L

Peptone water at 0.1 % g/L, used for viable counts, was prepared with Difco Bacto Petpone (BD Bacto™, New Jersey, U.S.A.).

2.4 Chemicals

All chemicals used for this study were of analytical grade. For the gas chromatograph, the standard solvents used for analysis were acetone, 2-butanol, butanol, ethanol, butyric acid, acetic acid. The sources of the chemical are listed in Table 2.2.

Table 2.2: List of chemicals used in this study and their manufacturers.

Chemicals	Manufacturer
Ammonium acetate	BDH AnalaR Chemicals Ltd Poole England
Ammonium chloride	Scharlau Chemie S.A. Barcelona, Spain
D-Glucose anhydrous	Biolab (Aust) Ltd Victoria, Australia
FeSO ₄ ·7H ₂ O	BDH AnalaR Chemicals Ltd Poole England
(NH ₄) ₂ ·SO ₄	
MgSO ₄ ·7H ₂ O	
MnSO ₄ ·4H ₂ O	
KH ₂ PO ₄	
K ₂ HPO ₄	
K ₂ HPO ₄ ·3H ₂ O	
L-Cysteine hydrochloride	
Acetone	BDH AnalaR VWR International Ltd Poole England
Butanol	
2-Butanol	
Ethanol	
Acetic acid	
Butyric acid	

2.5 Preparation of alkaline solution and buffers

2.5.1 Sodium hydroxide solution (1M)

Forty grams of sodium hydroxide were accurately weighed and dissolved into 1L deionised water using a volumetric flask. This alkaline solution was then placed in a clean 1 L bottle.

2.5.2 Sodium carbonate-bicarbonate buffer (1M)

Sodium bicarbonate at 10.6 grams of and sodium carbonate at 8.4 grams were accurately weighed and dissolved in 200 mL deionised water. The solution was adjusted to pH 5.65 and then added into a clean 200 mL Duran bottle.

2.5.3 Potassium phosphate buffer (1M)

Monosodium phosphate monohydrate (5.84 grams) and disodium phosphate, heptahydrate (15.5 grams) were dissolved in 1 L deionised water. The buffer solution was adjusted to pH 7.00 and then placed in a clean 1 L bottle.

2.6 Anaerobic incubation

Clostridium saccharobutylicum was incubated in a large anaerobic jar (Becton, Dickson and Company GasPak™150 Anaerobic System).

Three disposable sachets of anaerobic GasPak (Becton, Dickson and Company GasPak™150 Anaerobic System) were used to establish anaerobic condition in the jar (Becton Dickson and Company, 2010). In addition, a catalyst made of palladium enclosed in a wire gauze was placed in the anaerobic jar. The palladium catalyst was re-generated either by flaming in a Bunsen burner or heating in an oven at 42°C overnight.

2.6.1 Heat shocking

Spore stock cultures were revived by heat shocking an aliquot of 0.2 mL in 20 mL CMMG at 75°C for 2 minutes, followed by cooling in iced water for 1 minute.

2.7 Spore production and culture maintenance of *Clostridium saccharobutylicum*.

The spore stock of *C. saccharobutylicum* was revived by inoculating 0.2 mL into 20 mL CMMG, followed by heat shocking at 75°C for 2 minutes. The heat-shocked culture was immediately cooled on ice for 1 minute. The culture was then incubated in an anaerobic jar at 37°C for 19 hours by which highly motile cells were present.

Motile cells were transferred into a 100 mL slope of Reinforced *Clostridium* Agar which was incubated anaerobically at 37°C for 7 days. The formation of spores was determined using malachite green which was a spore staining technique developed by Bartholomew and Mittwer (1950). Spore suspension was prepared using sterile deionised water which was then dispensed aseptically at 2 mL into sterile screw-capped bottles. These are the spore stocks which were stored at a temperature of 4°C.

2.8 Preparation of fermentation substrate

2.8.1 Preparation of grape pomace substrate

The grape pomace stored in minus 10°C was thawed at ambient temperature and suspended in 200 mL deionised water at a final concentration of 12.5 % (dry weight/volume) using a clean 200 mL Duran bottle. 160 mL of distilled water was added, followed by 20 mL of potassium phosphate buffer (1M).

The suspension was then sterilized in an autoclave at 121°C for 15 minutes.

2.9 Fermentation

2.9.1 Inoculation of grape pomace

A freshly motile culture of *C. saccharobutylicum* grown in CMMG was used to inoculate the freshly autoclaved substrate of grape pomace. An inoculum concentration of either 5.5 % or 11 % was used.

2.9.2 Incubation of fermentation medium

The fermentation medium was incubated at 37°C anaerobically for 24 hours.

2.10 Sampling

Sampling from the fermentation medium for reducing sugars, solvent and acid analysis.

One milliliter was taken from the fermentation medium for the DNS analysis while nine milliliter was taken for the measurement of pH and GC analysis.

2.11 pH Measurement

The pH measurements were performed using a pH meter (PHM201, Radiometer analytical S.A. Meterlab, France). Before each measurement, the pH meter was calibrated using two buffer solutions (LabChem, Ajax FineChem Pty Ltd, Auckland, New Zealand), pH 4 and pH 7.

2.12 Viable count

The viability of *C. acetobutylicum* was determined by plate count. A sample of one milliliter was taken aseptically from the fermentation medium and was taken to a series of ten-fold dilutions. The bacteria were plated in Reinforced Clostridial Agar (BD Difco) at 17 grams per litre to prevent over-gassing and over-spreading of colonies. Agar Technical (Difco) per liter of distilled water. The plates were incubated anaerobically until colonies were visible. The viable count was calculated as colony forming units/mL (c.f.u./mL).

2.13 Sterilisation of glassware and plasticware

Glassware and plastic pipette tips were sterilized by autoclaving at 121°C for 15 minutes.

2.14 Determination of Cell Biomass in Dry Weight

Due to the nature of the substrate, the cell biomass in dry weight was determined using a calibration curve which was constructed between the viable count from grape pomace and cell dry weight from Semi-Synthetic Medium.

2.15 Dry weight determination for biomass

The method used to determine biomass in dry weight was based on a method by Yang, Tsai, and Tsao (1994).

Two milliliter of CCMG containing highly motile *C. saccharobutylicum* was transferred to 200 mL of Semi-Synthetic Medium, SSM.

After inoculation, which represented time 0, one milliliter of SSM was taken for plate count and another 20 mL of sample was taken from the SSM for determining the dry weight of cell biomass.

The RCA and the SSM cultures were placed in separate anaerobic jar for overnight incubation at 37°C. Then, the samples were taken for plate count and cell dry weight and this step was repeated over a period of 72 hours.

This 20 mL SSM sample was centrifuged for 8 minutes at 3500 RPM. The sample was rinsed three times with deionised water and it was placed in an oven at 90°C overnight. Finally the sample was weighed using an analytical balance (Uniblock AUY220, Shimadzu).

A line of best fit was obtained with a graph constructed between log of c.f.u./mL and dry weight. This was used as a calibration curve to convert viable counts to cell dry weight.

2.16 Reducing sugars concentration

The concentration of reducing sugars present in the substrate was measured by the Dinitrosalicylic acid reagent (DNS) method, which is a colorimetric detection of reducing sugars in solution (Dutta, 2008; Frost, 2004).

2.16.1 Preparation of Dinitrosalicylic acid reagent (Dutta, 2008):

The DNS reagent was prepared by dissolving 10.6 grams of 3,5-dinitrosalicylic acid reagent and 19.8 grams of sodium hydroxide (NaOH) into 1416 mL deionised water. Then, 306 grams of potassium sodium tartrate (Rochelle salts), 8.132 grams of melted phenol, and 8.3 grams of sodium metabisulfite were added into the previous solution with stirring. The list of ingredients is listed in Table 2.3.

A ± 6.5 % error was estimated.

Table 2.3: Ingredients of Dinitrosalicylic acid reagent.

Components	Concentration (g for 1416 mL)
3,5-dinitrosalicylic acid reagent (BDH Chemicals Ltd Poole England)	10.6
Sodium hydroxide (NaOH) (Scientific Supplies Ltd, East Tamaki, Auckland)	19.8
Potassium tartrate (Rochelle Salts) (Scientific Supplies Ltd, East Tamaki, Auckland)	0.306
Phenol (BDH AnalaR Chemicals Ltd Poole England)	8.132
Sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$)	8.3
Deionised water	1416 mL

2.17 DNS method

Each fermentation sample was assayed in triplicates. One milliliter of sample was transferred into each test tube with a cap. DNS reagent (1 mL) was added to the tube followed by 2 mL of deionised water. The mixture was placed in a boiling water bath for 5 minutes. The tubes were cooled to room temperature before taking the final volume up to 10 mL with deionised water. The solutions were mixed using a vortex mixer before reading the absorbance. The absorbance was measured at 540 nm using Spectrophotometer (Ultrospec 2100 pro UV/Visible spectrophotometer, Amersham Bioscience, U.K.).

A standard curve for glucose was prepared using anhydrous glucose (Biolab Aust. Ltd, Clayton, Victoria) dissolved in deionised water. A range of concentrations of standard glucose solution was prepared which included, 0.03 g/L, 0.045 g/L, 0.09 g/L, 0.12 g/L, 0.15 g/L. These known concentrations were assayed in triplicates. In a test tube covered with a cap, 1 mL of each sample was mixed with 1 mL DNS reagent followed by 2 mL of deionised water. The solutions were placed in a boiling water bath for 5 minutes and then cooled at room temperature. A volume of 6 mL of deionised water was added to make a final volume of 10 mL. The absorbance of the samples was determined in a spectrophotometer (Ultrospec 2100 pro UV/Visible spectrophotometer, Amersham Bioscience, U.K.) at 540 nm.

2.18 Analysis of solvents and acids using a Gas Chromatograph

The fermentation products such as acids and solvents were quantitatively measured by gas chromatography (GC) using the GC-2010 Shimadzu (Shimadzu Corporation Kyoto, Japan) with a flame ionization detector. The column used was a Zebron ZB-Wax (30 meters column length x 0.32 mm internal diameter x 0.15 μ m df) with a polyethylene glycol (PEG) phase. The flow rate of the carrier gas (nitrogen) was 1.5 mL/min. The operation conditions of the GC was programmed using a oven profile of 40°C for 5 minutes then, increased to 145°C at a rate of 20°C per minute and held for 7 minutes. The

injection and detector temperatures were both set to 250°C. Samples were injected at a volume of 1 µL.

The determination of concentration of both the solvents and acids were performed using the GCSolution software (GCSolution Ver.2). It calculates the concentration using the measurement of peak height based on the comparison of a known amount of internal standard 2-Butanol added to the sample. The internal standard 2-Butanol (25 g/L) was diluted in orthophosphoric acid (20% v/v) and one milliliter was added to 1 mL of sample to be analysed. The standard solution of each analyte to be analysed were prepared at different concentration, 1 g/L, 5 g/L, 10 g/L, 20 g/L in 200 mL deionised water and a known amount of internal standard was added to each standard solution. The GCSolution software recorded the peaks representing each concentration of standard solutions and the software calculated the concentration of unknown amount of analytes based on a calibration curve of known concentrations of an analyte.

2.19 Statistical analysis

Statistical analysis of data was performed using Microsoft Office Excel 2007 and Minitab 15.

2.20 Calculations of bioprocess parameters and kinetic parameters

The overall productivity (q_p) was determined graphically by calculating the slope of the total solvent produced over time based on the following equation (Pirt, 1975):

$$q_p = dp / dt$$

Where q_p = total solvent productivity (g/L/h)

dp = increase in total solvents concentration (g/L)

dt = time interval (h)

The solvent yield ($Y_{p/s}$) = dp / ds .

$$Y_{p/s} = dp / ds$$

Where $Y_{p/s}$ = solvent yield (g/g)

dp = total solvent concentration (g/L)

ds = Reducing sugars utilised (g/L)

The reducing sugars utilization rate ($q_{p/s}$) was determined graphically by calculating the slope of substrate utilised over time based on the equation below (Pirt, 1975):

$$q_{p/s} = ds / dt$$

Where $q_{p/s}$ = rate of utilisation (g/L/h)

ds = concentration of substrate utilised (g/L)

dt = time interval (h)

The specific growth rate (μ) in h^{-1} was determined graphically by calculating the slope of exponential growth over time and multiplying the slope by 2.303 (Pirt, 1975):

$$\mu = dx / dt \cdot 1 / x$$

Where μ = specific growth rate (h^{-1})

dx = increase in biomass (g)

dt = time interval (h)

x = biomass concentration (g)

Chapter 3

Results

The initial experiments were performed to establish the necessary parameters required for both growth and solvent production in white grape pomace using *C. saccharobutylicum*. A potential alternative to yeast extract was then determined followed by the attempt to optimise the fermentation process.

3.1 Effect of initial pH of grape pomace

The initial pH of the fermentation substrate consisting of white grape pomace at 11 % (dry weight /volume), had an average pH of 4.40. To allow growth of *C. saccharobutylicum* in the substrate, the initial pH was adjusted to pH 5.5 by addition of alkaline solution or use of buffer solutions.

Sodium hydroxide (1 M) was added to the substrate until pH 5.5 was obtained. This method only increased the pH of the substrate but was not effective in maintaining the pH at 5.5 after autoclaving. Table 3.1 shows that using sodium hydroxide at 0.125 % without buffering resulted in a decrease in pH to pH 4.58 after autoclaving. Gas formation, which was indicative of active growth, was not observed even after 72 hours of incubation at 37°C in grape pomace where sodium hydroxide was used for pH adjustment. The absence of gas formation was accompanied by negligible decrease of pH and in reducing sugars concentration after 72 hours of incubation (Table 3.1).

Sodium carbonate (1M) was also used to adjust the pH of the fermentation medium to pH 5.66. However, after autoclaving the pH decreased from pH 5.66 to pH 5.23 due to the lack of buffering capacity in the substrate. Table 3.1 shows the absence of growth similar to those obtained with sodium hydroxide. The absence of growth was indicated by the absence of gas formation, absence

of marked decreased pH due to acid production, and by negligible utilisation of reducing sugars after 72 hours of incubation.

Since sodium hydroxide and sodium carbonate were not able to maintain a pH of 5.5, the following experiments involved the use of potassium phosphate buffer (1M) to adjust the pH of the fermentation medium to 6.10 before autoclaving to compensate for the typical drop in pH due to autoclaving. After autoclaving, the pH decreased to pH 5.50.

Table 3.1. pH and reducing sugars concentration in grape pomace with initial pH adjustment with sodium hydroxide solution (1 M) and sodium carbonate (1 M); 5.5 % inoculum.

Alkaline/Buffer and parameters	Fermentation (hours)	pH	Reducing sugars (g/L)	Growth
Sodium Hydroxide	0	4.58	29.21	Negative
(NaOH, 1M)	24	4.52	30.35	
5.5 % (v/v) inoculum	48	4.51	29.32	
Free of Y.E.	72	4.52	28.93	
Sodium Carbonate	0	5.23	28.16	Negative
(NaCO₃, 1M)	24	5.24	32.30	
5.5 % (v/v) inoculum	48	5.16	31.60	
0.5 % (w/v) Y.E.	72	5.18	31.79	

3.2 Effect of Yeast Extract and inoculation concentration on fermentation of white grape pomace

To obtain growth and solvent production in grape pomace, the inoculum level was increased from 5.5 % used in previous experiments to 11 %. To demonstrate the effect of Yeast Extract on solvents production from grape pomace, fermentation trials with and without Yeast Extract were performed. For trials without Yeast Extract, the substrate consisted of grape pomace (12.5 % w/v), inoculum at 11% v/v, potassium phosphate buffer (1M).

3.2.1 Effect of inoculum on the fermentation of white grape pomace

The concentration of inoculum was observed to influence growth and fermentation in grape pomace. Using an inoculum concentration of 5.5 % (v/v) for grape pomace buffered by phosphate buffer at around pH 5.5 and supplemented with a higher Y.E. concentration of 1 g/L, these conditions resulted in an increase in biomass concentration but an acidogenic fermentation.

A final biomass concentration of 0.69 g/L was produced after 48 hours of fermentation (Table 3.2). This was equivalent to a biomass production at 35 %. Further, the final biomass concentration obtained in this fermentation was four times higher than that obtained in grape pomace unsupplemented with Y.E. The utilisation of sugar was equivalent to 11.8 %. Table 3.2 and Figure 3.2b show that the pH of the fermentation decreased steadily over 48 hours indicating low production of acids and the lack of pH breakpoint. In addition, acetic acid and butyric acid were produced after 24 hours but there was no further increase in acid concentration (Table 3.2). Very low concentrations of ethanol, acetone and butanol were obtained after 48 hours of fermentation. These results demonstrate an acidogenic fermentation. Figure 3.1 shows an acidogenic grape pomace fermentation.



Figure 3.1. Acidogenic fermentation in grape pomace at a concentration of 12.5% (w/v), inoculated with 5.5 % (v/v) inoculum, and supplemented with Y.E. 1 % (w/v), pH 5.5 adjusted with potassium phosphate buffer (1M) over 24 hours of fermentation.

Table 3.2. Fermentation summary of 12.5 % (w/v) white grape pomace with 5.5 % (v/v) inoculum, 1 % (w/v) Y.E. and pH 5.5 adjusted with potassium phosphate buffer (1M) over 48 hours of fermentation.

Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.51	33.25	0	0	0	0	0	0.45
24	5.48	31.60	1.02	0.13	0.07	1.96	2.46	0.52
48	5.27	29.30	0.98	0.13	0	1.95	2.38	0.69

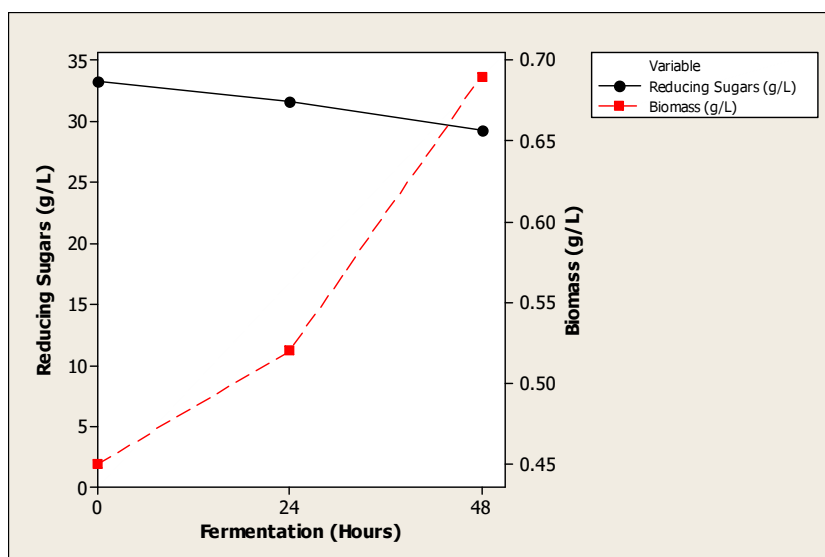


Figure 3.2a. Concentration of reducing sugars and biomass in white grape pomace (12.5 % w/v) inoculated at 5.5 % (v/v), supplemented with 1 % (w/v) Y.E. and pH 5.5 adjusted using potassium phosphate buffer (1M).

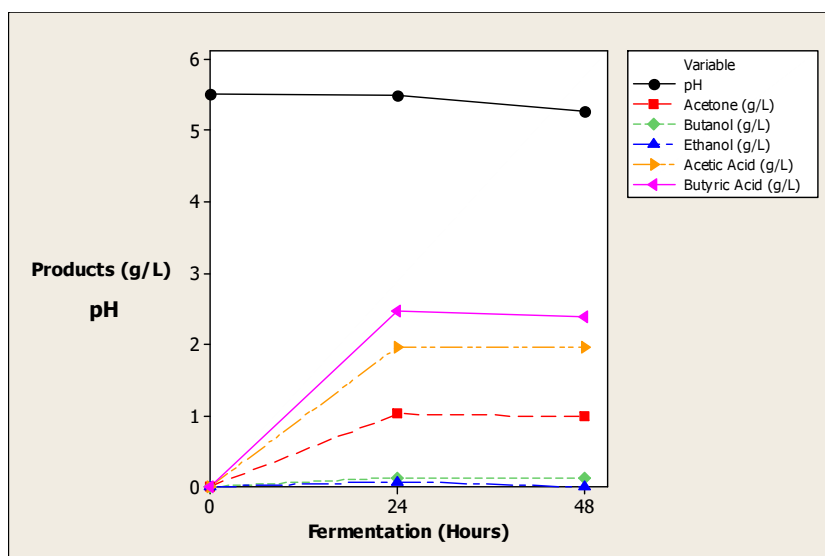


Figure 3.2b. Course of fermentation in white grape pomace (12.5 % w/v) using an inoculum concentration of 5.5 % (v/v), Y.E. at 1 % (w/v) and pH 5.5 adjusted with potassium phosphate buffer (1M) over 48 hours.

Using an inoculum concentration of 11 % resulted in a solventogenic fermentation. Figure 3.4 shows gas production 24 hours after inoculation. Acetone, butanol, and ethanol were produced at a higher concentration than those of acetic acid and butyric acid (Table 3.4).

A marked increase in biomass was obtained after 48 hours. The biomass concentration of (0.83 g/L) produced after 48 hours in this fermentation was higher (Figure 3.5a) than that obtained (0.69 g/L) using a lower inoculum concentration (Figure 3.2a).

The growth of *C. saccharobutylicum* in this substrate resulted in a marked by high utilisation of sugars equivalent to 89 % (Table 3.4). The production of active growth was indicated by vigorous gassing in the substrate (Figure 3.4). Acetic acid and butyric acid concentration increased after 24 hours but then decreased after 48 hours of fermentation (Figure 3.5b). This profile was reflected in the pH values which demonstrated a pH breakpoint after 48 hours due to the production of solvents. The butanol concentration obtained in this fermentation was 5.84 g/L which was markedly higher than from the fermentation using Yeast Extract and lower inoculum concentration.

3.2.2 Fermentation of grape pomace unsupplemented with yeast extract

The fermentation substrate consisting of grape pomace (12.5 % w/v), inoculum (11 % v/v), at a pH of 5.5 adjusted with potassium phosphate buffer (1M) but devoid of Yeast Extract produced nil biomass and resulted in the production of only acids, instead of solvents (Table 3.3).

Only 8 % of reducing sugars was consumed after 72 hours (Figure 3.3a). This small consumption of reducing sugars was reflected in the very low biomass concentration of 0.71 g/L (Table 3.3).

Results show a decrease in pH without pH breakpoint over 72 hours of fermentation (Figure 3.3b). This substrate was growth-supporting for *C. saccharobutylicum* but resulted in an acidogenic fermentation where the main products were acetic acid and butyric acid at 5.28 g/L and 2.77 g/L, respectively. A low concentration of ethanol was detected but acetone and butanol were absent (Table 3.3).

Figure 3.3b shows the fermentation profile in this medium where there was a significant production of acetic acid and butyric acid. Ethanol production started shortly after inoculation and peaked after 24 hour of fermentation. The concentration of ethanol remained constant until 72 hours of fermentation.

Table 3.3. Fermentation profile in grape pomace (12.5 % w/v), unsupplemented with Yeast Extract; (inoculum at 11 % (v/v), initial pH adjusted with phosphate buffer (1M).

Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.76	30.20	0	0	0	0	0	0.69
24	5.64	29.62	0	0	0.64	4.66	2.37	0.68
48	5.65	28.34	0	0	0.51	4.68	2.50	0.69
72	5.45	27.71	0	0	0.64	5.28	2.77	0.71

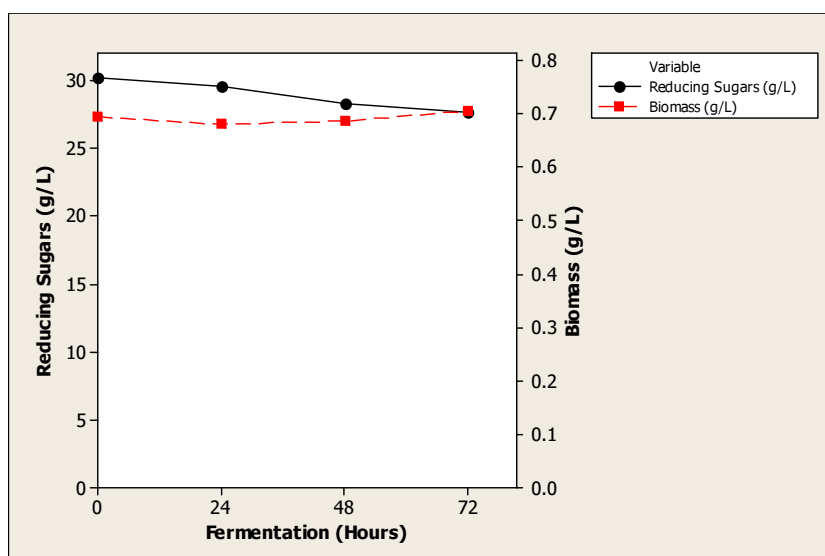


Figure 3.3a. Reducing sugars utilisation and biomass production in grape pomace unsupplemented with Yeast Extract; (11 % (v/v) inoculum and pH 5.5 adjusted with potassium phosphate buffer (1M)).

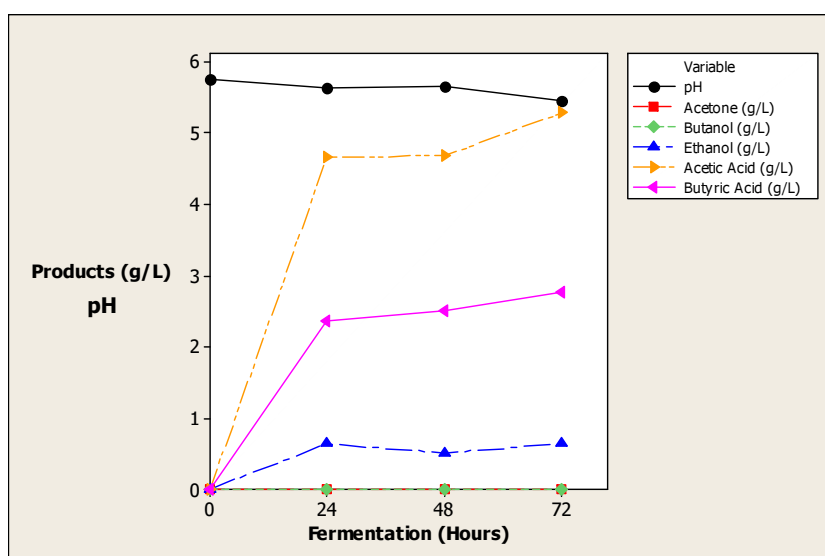


Figure 3.3b. Fermentation profile in grape pomace (12.5 % w/v), unsupplemented with Yeast Extract; (inoculum at 11 % (v/v), initial pH 5.5 adjusted with phosphate buffer (1M)).

3.2.3 Effect of Y.E. on the fermentation of grape pomace

Using grape pomace supplemented with Y.E. (1 %), the total solvents concentration of 9.6 g/L obtained using higher inoculum level was much higher than that obtained using lower inoculum level which led to a total solvent of 1.1 g/L. Table 3.5 shows the bioprocess and kinetic parameters obtained from the grape pomace inoculated with a high inoculum concentration. The fermentation resulted in a total solvent productivity of 0.29 g/L/h with a solvent yield of 0.32 g/g. The final biomass was 0.83 g/L and the sugar utilisation rate was 0.65 g/L/h. The increase in concentration of acids and the increase in pH value after 48 hours indicated the conversion of acids to solvents typical of solventogenic fermentation (Figure 3.5b). The ratio of the concentration of acetone, butanol and ethanol produced was 3:6:1 which is also the typical ratio obtained from a solventogenic fermentation (Jones & Woods, 1986). The specific growth rate was 0.35 g/g/h. Gas production, indicative of growth, occurred 24 hours after inoculation (Figure 3.4).



Figure 3.4. Gas production after 24 hours of fermentation in grape pomace using an inoculum concentration of 11 % (v/v), supplemented with 1 % (w/v) Y.E., and pH adjusted to pH 5.5 with potassium phosphate buffer (1M).

Table 3.4. Summary of fermentation of grape pomace using 11 % (v/v) inoculum, 1 % (w/v) Y.E. with pH 5.5 adjusted with potassium phosphate buffer (1M) over 48 hours of fermentation.

Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.56	34.00	0	0	0	0	0	0.54
24	4.70	19.45	1.39	1.21	0	2.74	3.60	0.75
48	5.49	3.76	3.75	5.84	0.05	1.64	2.79	0.83

Table 3.5. Solvent yield, productivity and glucose utilisation based on fermentation of grape pomace with 11% (v/v) inoculum, 1 % (w/v) Y.E., and pH 5.5 adjusted with potassium phosphate buffer (1M).

Total ABE produced after 48 hours of fermentation (g/L)	Solvent Yield (g/g) (a)	Total solvent productivity (g/L/h) (b)	Reducing sugars utilisation rate (g/L/h)	Specific growth rate (g/g/h)	pH breakpoint
9.64	0.32	0.29	0.65	0.35	4.70

a. Grams of ABE solvents produced per gram of reducing sugars utilised.

b. Grams of ABE solvents produced per hour.

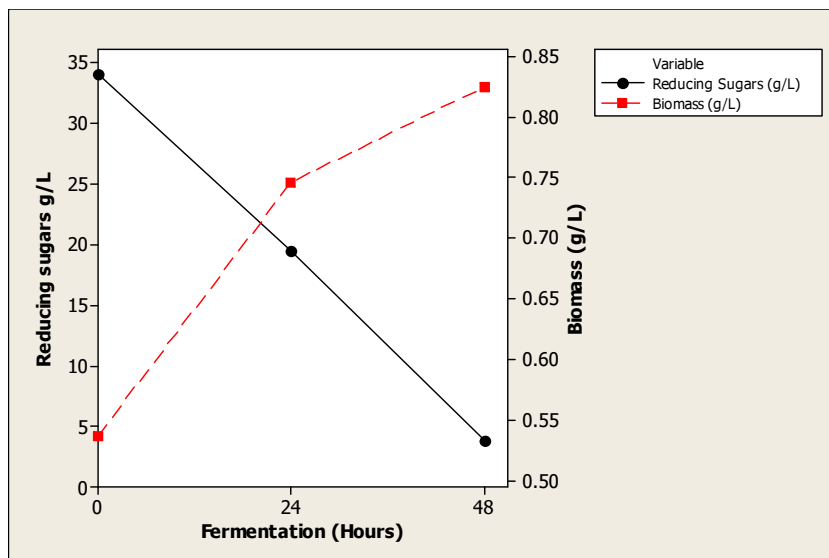


Figure 3.5a. Biomass production and reducing sugars consumption during fermentation of white grape pomace (12.5 % w/v); (11 % (v/v) inoculum, 1 % (w/v) Y.E., pH 5.5 adjusted with potassium phosphate buffer (1M)).

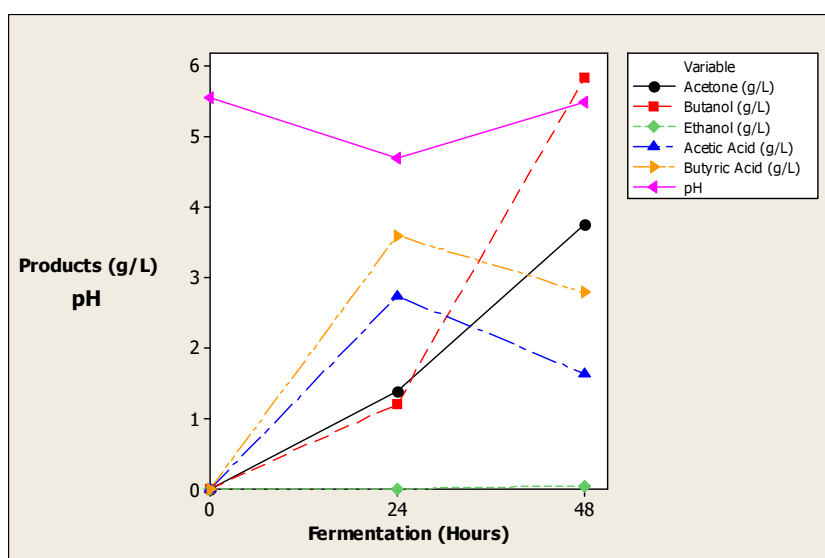


Figure 3.5b. Course of fermentation of white grape pomace (12.5% w/v); (11 % (v/v) inoculum, 1 % (w/v) Y.E., pH 5.5 adjusted with potassium phosphate buffer (1M)).

3.3 Solventogenic fermentation of white grape pomace

The parameters required for solvent production from grape pomace (12.5 % w/v) using *C. saccharobutylicum* were 11 % (v/v) inoculum, 1 % (w/v) Y.E., and pH adjusted to pH 5.5 with potassium phosphate buffer (1M). The results obtained are average of two fermentation trials.

The final biomass concentration of 0.85 g/L was produced after 72 hours of fermentation (Table 3.6). The use of these parameters resulted in a high production of gas after 24 hours of fermentation (Figure 3.6). Results indicated that 97 % of reducing sugars was consumed over a period of 72 hours and the sugar utilisation rate was 0.57 g/L/h (Figure 3.7a and Table 3.7).

Table 3.6 and Figure 3.7b indicated that acetic acid and butyric acid were produced after 24 hours. Over the following 48 hours, acetic acid concentration decreased steeply while butyric acid concentration increased gradually. This profile shows the presence of a pH breakpoint after 48 hours of fermentation based on the pH values (Table 3.6).

The final concentration of acetone, butanol, and ethanol were 2.38 g/L, 5.80 g/L, and 0.58 g/L, respectively (Table 3.6). The ratio of the acetone, butanol, and ethanol produced in this fermentation was 3:6:1 and it is identical to the Weizmann's fermentation (Jones & Woods, 1986).

Table 3.7 shows the bioprocess and kinetic parameters of the fermentation of white grape pomace. The solvent yield was 0.30 g/g while the total solvent productivity was 0.21 g/L/h. *C. saccharobutylicum* utilised the sugars at a rate of 0.57 g/L/h and produced a total solvent concentration of 8.76 g/L. The specific growth rate in this fermentation was 0.33 g/g/h.

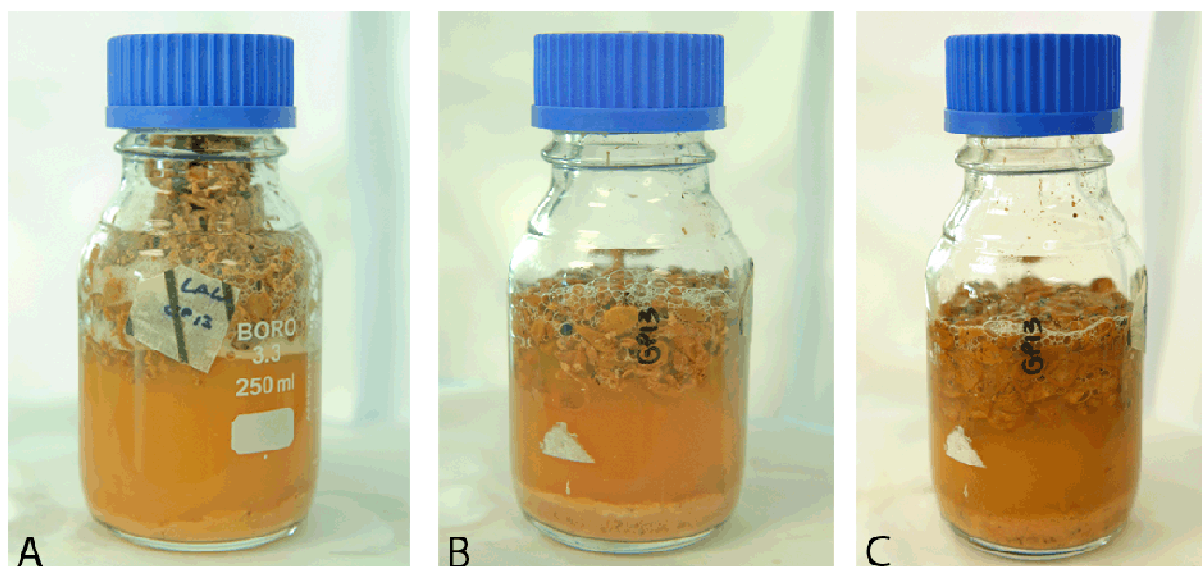


Figure 3.6. Photographs of fermentation of white grape pomace (12.5 % w/v) inoculated with *C. saccharobutylicum* at 11 % (v/v), supplemented with 1 % (w/v) Y.E. and adjusted to pH 5.5 using potassium phosphate buffer (1M) over 72 hours. (A) After 24 hours of fermentation, (B) after 48 hours of fermentation, and (C) after 72 hours of fermentation.

Table 3.6. Fermentation profile of grape pomace with 11 % (v/v) inoculum, 1% (w/v) Y.E. and pH adjusted to pH 5.5 using potassium phosphate buffer (1M).

Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.52	30.56	0	0	0	0	0	0.69
24	4.78	19.93	0.44	1.50	0.71	5.37	3.58	0.73
48	5.11	6.19	2.10	5.00	0.68	3.35	4.02	0.81
72	5.07	0.88	2.38	5.80	0.58	3.17	4.13	0.85

Table 3.7. Solvent yield, productivity and glucose utilisation based on the fermentation of white grape pomace (12.5 % w/v); 11 % (v/v) inoculum, 1 % (w/v) Y.E., and pH 5.5 adjusted with potassium phosphate buffer (1M).

Total ABE produced after 72 hours of fermentation (g/L)	Solvent Yield (g/g) (a)	Total solvent productivity (g/L/h) (b)	Reducing sugars utilisation rate (g/L/h)	Specific growth rate (g/g/h)	pH breakpoint
8.76	0.30	0.21	0.57	0.33	4.78

a. Grams of ABE solvents produced per gram of reducing sugars utilised.

b. Grams of ABE solvents produced per hour.

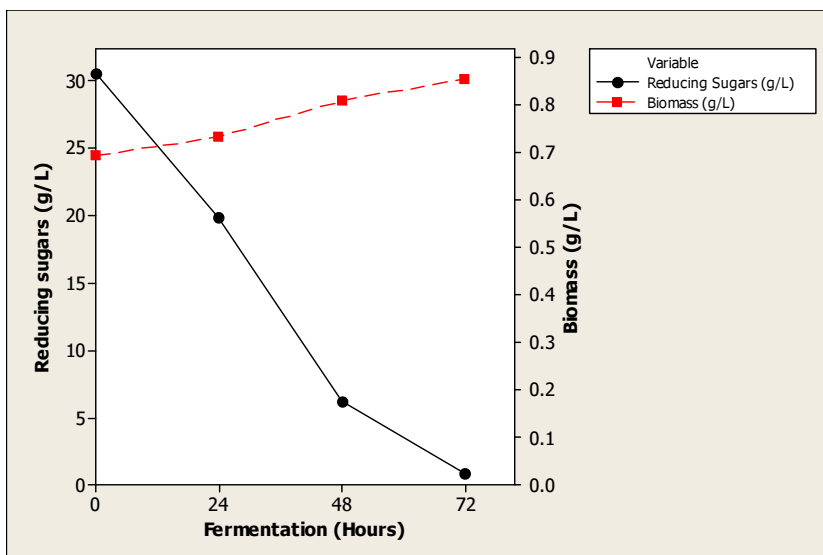


Figure 3.7a. Utilisation of reducing sugars and biomass production of white grape pomace (12.5 % w/v); (11 % (v/v) inoculum, 1 % (w/v) Y.E. and pH 5.5 adjusted with potassium phosphate buffer (1M)).

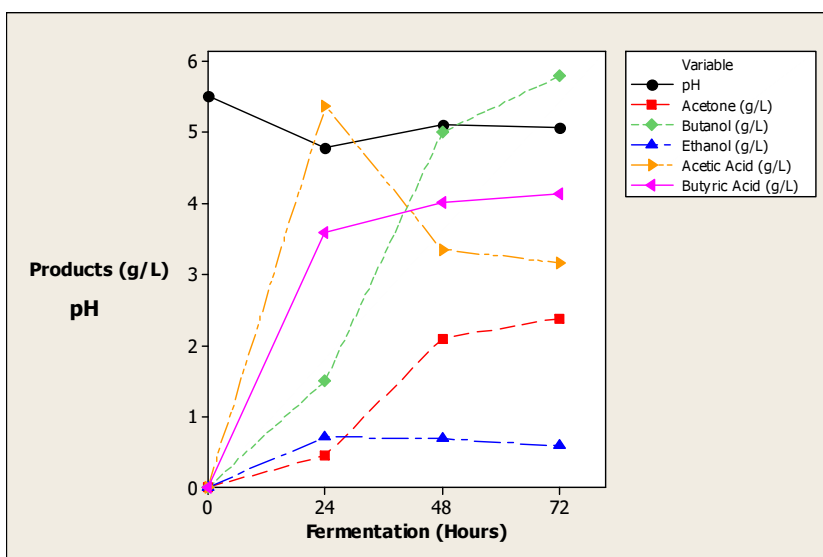


Figure 3.7b. pH, acid and solvent production in white grape pomace (12.5 % w/v); (11 % (v/v) inoculum, 1 % (w/v) Y.E. and pH 5.5 adjusted with potassium phosphate buffer (1M) over 72 hours of fermentation).

3.4 Search for an alternative growth factor to Yeast Extract

Yeast Extract played an important role in the fermentation in grape pomace. However, the fermentation of grape pomace would be non-profitable on an industrial scale due to the high cost of yeast extract. Thus, the following experiment was carried out to determine what component could be used to replace yeast extract as a growth factor for successful fermentation using a substrate of white grape pomace.

3.4.1 Nitrogen salts

Either ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ or ammonium phosphate $(\text{NH}_4)_2\text{PO}_4$ at a concentration of 2 g/L was used to replace Yeast Extract. The concentration used was based on the composition of semi-synthetic medium in the study done by Roos, McLaughlin, & Papoutsakis (1985).

Using 2 g/L ammonium sulphate or ammonium phosphate led to an acidogenic fermentation. Figure 3.8a shows that at 2 g/L, the concentration used previously by Roos et al. (1985) no gas production occurred which was indicative of growth. The pH measurements did not show the presence of pH breakpoint throughout the fermentation (Table 3.8). In addition, a lack of consumption of reducing sugars was observed with fermentation using ammonium sulphate or ammonium phosphate.

It was possible that using 2 g/L of ammonium salts, which were inorganic salts had a detrimental effect on *C. saccharobutylicum* due to mineral toxicity (Qureshi & Maddox, 1995). As a result, a lower concentration of the salts was used. Using a one-eighth of the original concentration of ammonium sulphate and ammonium phosphate at 0.25 g/L also led to an acidogenic fermentation. Figure 3.8b shows the lack of gas production in the fermentation media with ammonium sulphate or ammonium phosphate, which indicated absence of active growth. Results of pH measurements did not show the presence of a pH breakpoint (Table 3.8). The concentration of reducing sugars remained constant over 48 hours of fermentation (Table 3.8).

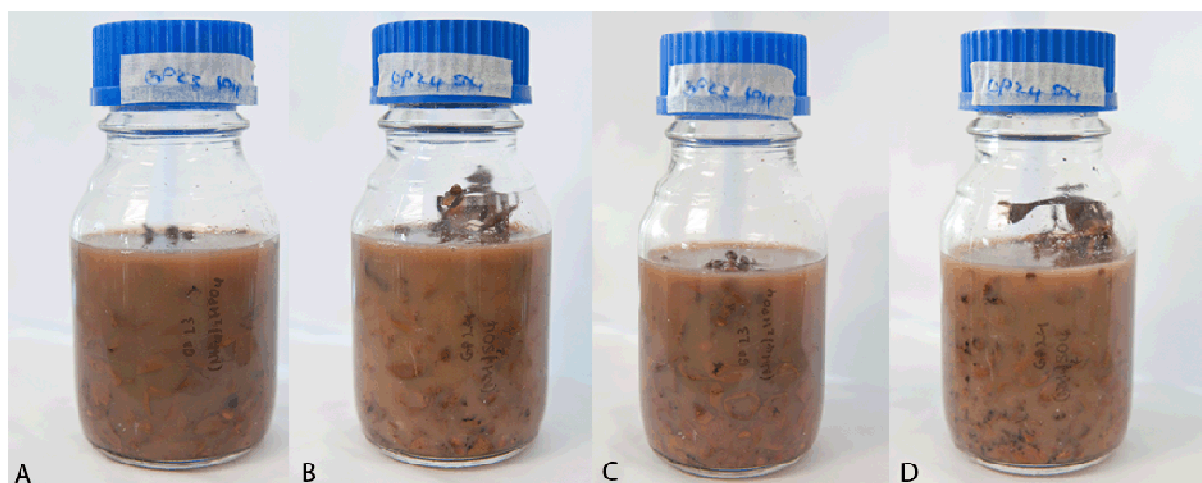


Figure 3.8a. Photographs of fermentation of grape pomace (12.5% w/v) using nitrogen compounds at 2 g/L; (11 % (v/v) inoculum, pH 5.5 adjusted with KH_2PO_4 (1M)). (A) and (B) Fermentation of $(\text{NH}_4)_2\text{PO}_4$ and $(\text{NH}_4)_2\text{SO}_4$, respectively after 24 hours of fermentation. (C) and (D) Fermentation of $(\text{NH}_4)_2\text{PO}_4$ and $(\text{NH}_4)_2\text{SO}_4$, respectively after 48 hours of fermentation.

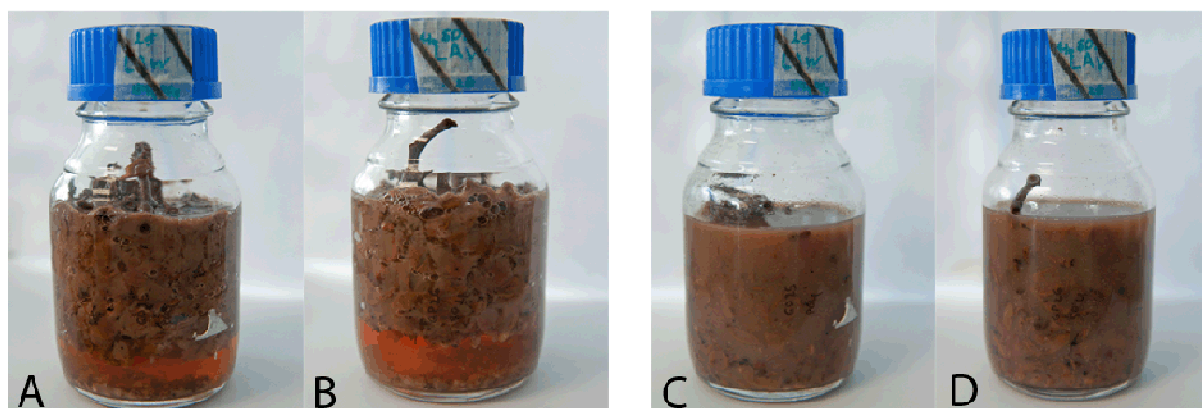


Figure 3.8b. Photographs of fermentation of grape pomace (12.5% w/v) using nitrogen compounds at 0.25 g/L; (11 % (v/v) inoculum, pH 5.5 adjusted with KH_2PO_4 (1M)). (A) and (B) Fermentation of $(\text{NH}_4)_2\text{PO}_4$ and $(\text{NH}_4)_2\text{SO}_4$, respectively after 24 hours of fermentation. (C) and (D) Fermentation of $(\text{NH}_4)_2\text{PO}_4$ and $(\text{NH}_4)_2\text{SO}_4$, respectively after 48 hours of fermentation.

Table 3.8: Summary of fermentations using inorganic nitrogen salts as growth factors.

Salt	Fermentation (Hours)	pH	Reducing Sugars (g/L)	Growth
(NH ₄) ₂ PO ₄ (2 g/L)	0	5.35	31.40	Negative
	24	5.34	34.27	
	48	5.31	37.45	
(NH ₄) ₂ SO ₄ (2 g/L)	0	5.18	44.64	Negative
	24	5.13	47.21	
	48	5.12	49.98	
(NH ₄) ₂ PO ₄ (0.25 g/L)	0	5.35	32.3	Negative
	24	5.35	33.26	
	48	5.39	34.28	
(NH ₄) ₂ SO ₄ , (0.25 g/L)	0	5.22	35.76	Negative
	24	5.26	35.79	
	48	5.22	38.79	

3.4.2 Use of mineral salts from a semi-synthetic medium adopted from Roos et al. (1985) in fermentation free of Y.E.

Since the use of nitrogen compounds did not lead to the fermentation of grape pomace, other salts which include from manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) were incorporated into grape pomace. This was designed based on the semi-synthetic medium used in the study performed by Roos et al. (1985) and the composition is shown in Table 3.9.

Table 3.9: Mineral salts in the semi-synthetic medium used by Roos et al. (1985).

Ingredients	Concentrations
Manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$)	0.01 g/L
Magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$)	0.4 g/L
Ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.01 g/L
Ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$)	2.0 g/L
potassium diphosphate (KH_2PO_4)	0.75 g/L
dipotassium hydrogen phosphate (K_2HPO_4)	0.75 g/L

Using the salts and salt concentration similar to that of the semi-synthetic medium above (Roos et al., 1985), a solventogenic fermentation from grape pomace was obtained. Unlike the previous experiments using nitrogen compounds only, gas production, indicative of growth, occurred after 24 and 48 hours of fermentation (Figure 3.9).

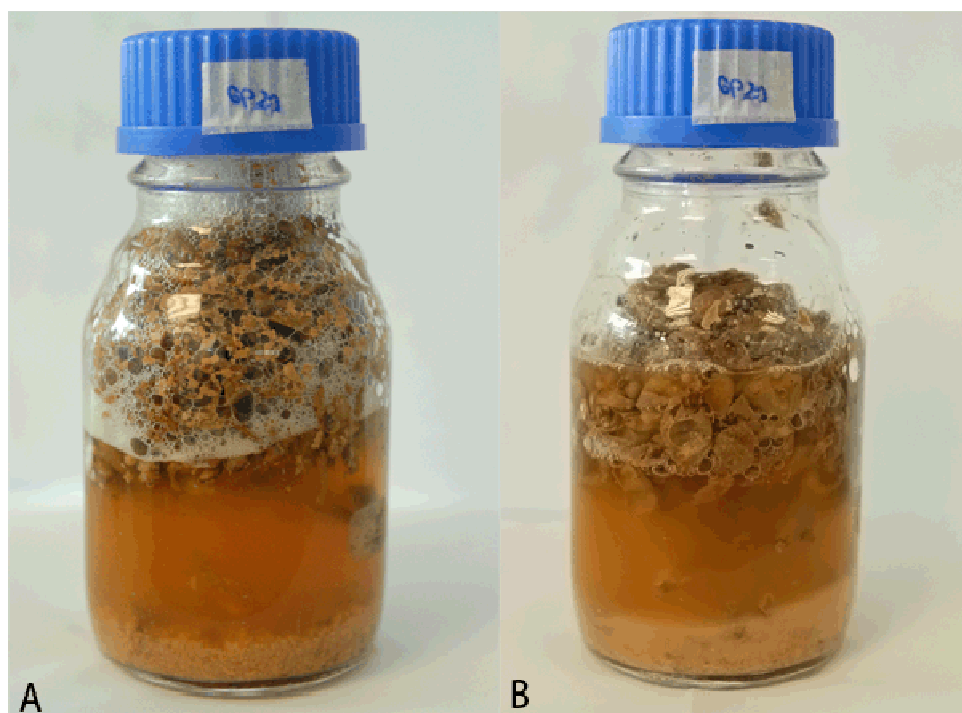


Figure 3.9. Photographs of white grape pomace with salts including manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$); (11 % (v/v) inoculum, with pH 5.5 adjusted with potassium phosphate buffer (1M)).

(A) Gas production after 24 hours of fermentation

(B) Gas production still present after 48 hours of fermentation

The results of the fermentation with salts were similar to the ones obtained from the fermentation medium with the parameters required for an ABE fermentation (Table 3.6 and 3.10). Figure 3.10a shows that 97.6 % of reducing sugars was consumed after 24 hours of fermentation. In comparison to the fermentation with the necessary parameters required for the production of solvents, the consumption was 97 % (Table 3.6). This high consumption of reducing sugars was reflected in the active growth of *C. saccharobutylicum* which produced a final biomass concentration of 0.85 g/L after 72 hours of incubation (Table 3.6).

A high concentration of acetic acid and of butyric acid were produced after 24 hours of fermentation and then decreased steadily after 48 hours (Figure 3.10b).

Acetone and butanol were produced after 48 hours of incubation and reached a high concentration of 2.83 g/L and 5.96 g/L after 72 hours, respectively (Table 3.10). This profile indicated the presence of a pH breakpoint after 48 hours.

Table 3.10 indicates that the fermentation produced a final concentration of 5.96 g/L of butanol, 2.83 g/L of acetone, and 0.51 g/L of ethanol, which were similar to the ones obtained from fermentation with the necessary parameters for an ABE fermentation (Table 3.6). The ratio of the solvents produced were, 6:3:1 (butanol-acetone-ethanol) which was similar to the Weizmann's fermentation (Jones & Woods, 1986) (Table 3.10).

In comparison to the fermentation with parameters required for an ABE fermentation with a pH breakpoint at 4.78 after 24 hours of fermentation, the fermentation using mineral salts as growth factors obtained a pH breakpoint at 4.64 after 48 hours (Table 3.10).

Table 3.10: Fermentation summary of white grape pomace with manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), 11 % (v/v) inoculum, with pH 5.5 adjusted with potassium phosphate buffer.

Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.49	29.33	0	0	0	0	0	0.59
24	5.06	28.84	0	0.53	1.55	6.13	3.39	0.65
48	4.67	15.77	0.87	2.99	0.93	5.7	2.98	0.80
72	4.75	0.71	2.83	5.96	0.51	3.03	2.49	0.81

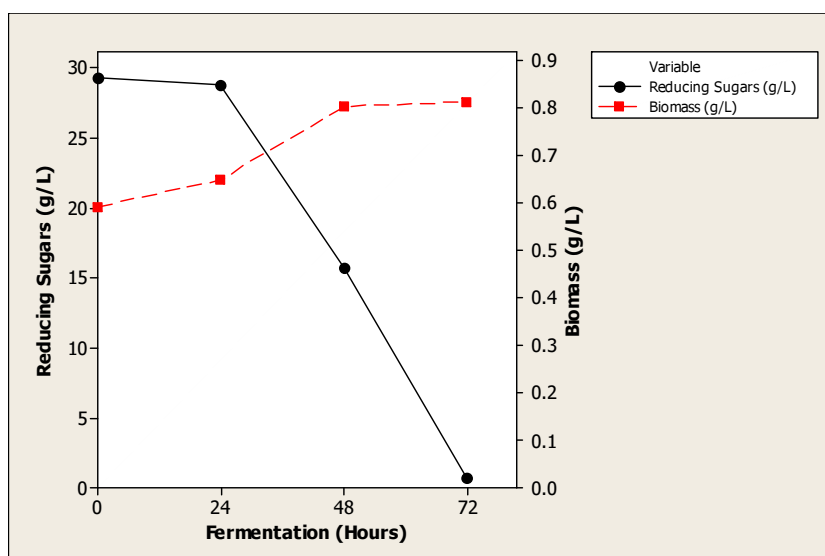


Figure 3.10a. Reducing sugars utilisation and biomass production in grape pomace unsupplemented Y.E., with salts ranging from manganese sulphate, magnesium sulphate, ferrous sulphate, potassium diphosphate, dipotassium hydrogen phosphate, and ammonium sulphate; (11 % (v/v) inoculum, and pH 5.5 adjusted with potassium phosphate buffer (1M)).

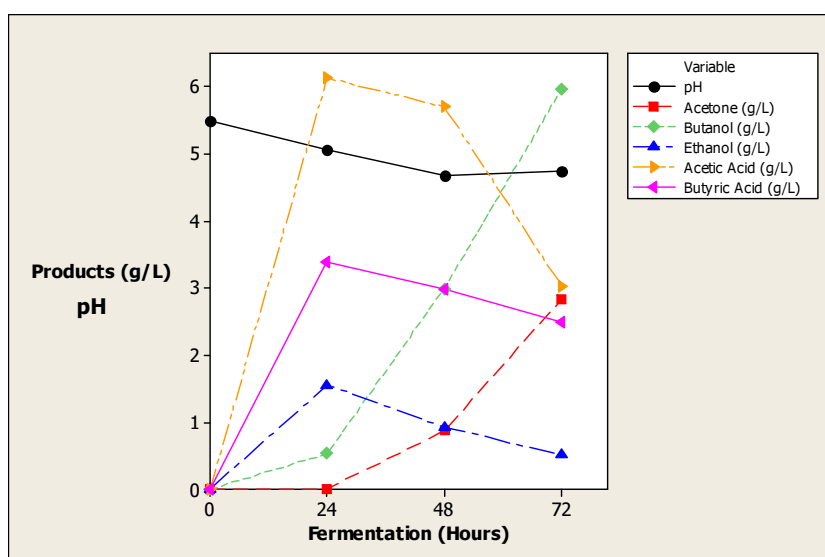


Figure 3.10b. Course of fermentation in grape pomace unsupplemented Y.E., with salts ranging from manganese sulphate, magnesium sulphate, ferrous sulphate, potassium diphosphate, dipotassium hydrogen phosphate, and ammonium sulphate; (11 % (v/v) inoculum, and pH 5.5 adjusted with potassium phosphate buffer (1M)).

Table 3.11 shows that the total solvent productivity and the solvent yield of the fermentation medium supplemented with salts were 0.19 g/L/h and 0.36 g/g, respectively. In comparison to fermentation supplemented with Yeast Extract and with parameters required for an ABE fermentation, which had a total ABE of 8.76 g/L (Table 3.7), the fermentation with mineral salts as growth factors produced a total solvents concentration of 9.08 g/L (Table 3.11).

The solvent yield and total solvent productivity from the grape pomace supplemented with Y.E. were 0.30 g/g and 0.21g/L/h respectively (Table 3.7). The solvent yield values were lower than that of the fermentation using mineral salts. The rate of utilisation of reducing sugars, 0.59 g/L/h (Table 3.11), was comparable with that of the fermentation medium with Y.E. which was 0.57 g/L/h (Table 3.7).

Table 3.11. Solvent yield, total ABE productivity and glucose utilisation based on white grape pomace unsupplemented with Y.E.; (manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), 11 % (v/v) inoculum, pH 5.5 adjusted with potassium phosphate buffer (1M)).

Total ABE produced after 72 hours of fermentation (g/L)	Solvent Yield (g/g) (a)	Total solvent productivity (g/L/h) (b)	Reducing sugars utilisation rate (g/L/h)	pH breakpoint
9.08	0.36	0.19	0.59	4.67

a. Grams of ABE solvents produced per gram of reducing sugars utilised.

b. Grams of ABE solvents produced per hour.

3.5 Improving the fermentation process

Optimisation of the fermentation process was attempted to obtain higher concentrations of solvent. During the fermentation, the grape pomace was not totally submerged due to gas production. As a result, it would prevent *C. saccharobutylicum* access to the reducing sugars that could be used to produce more butanol.

The attempt at optimising the fermentation using a magnetic stirrer led to an acidogenic fermentation. Figure 3.11b shows that the pH had barely changed after 24 hours of fermentation, but then decreased to pH 4.83 after 48 hours (Table 3.12). A pH breakpoint occurred after 48 hours of fermentation. The amount of sugar utilisation was equivalent to 73 % after 72 hours of fermentation (Figure 3.11a). The lack of gas production indicated that there was no vigorous fermentation. Acetic acid and butyric acid were the main end-products, and 5.14 g/L and 5.51 g/L, respectively were produced after 72 hours of fermentation (Table 3.12). Butyric acid was steadily produced whereas acetic acid peaked after 24 hours (Figure 3.11b). The conversion of acid to solvent started only after 48 hours of fermentation unlike the fermentation medium with the parameters required for an ABE fermentation (Section 3.3, Figure 3.7b) which started after 24 hour of fermentation. After 72 hours of fermentation, only 0.71 g/L of butanol and 0.88 g/L of ethanol were produced while acetone was not produced after 72 hours.

Table 3.12: Fermentation profile of white grape pomace (12.5 % w/v) optimised using a magnetic stirrer, 11 % (v/v) inoculum, 1 % (w/v) Y.E., pH 5.5 adjusted with potassium phosphate buffer (1M).

Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)
0	5.58	27.75	0	0	0	0	0
24	5.56	31.09	0	0	1.06	5.72	2.81
48	4.83	24.94	0	0	0.65	5.11	4.40
72	5.07	7.50	0	0.71	0.88	5.14	5.51

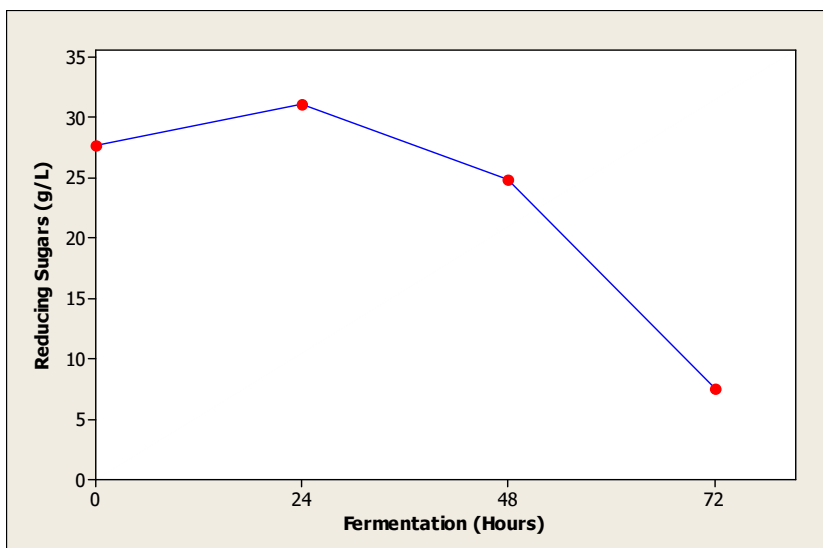


Figure 3.11a. Utilisation of reducing sugars of white grape pomace (12.5 % w/v) with a magnetic stirrer; (11 % (v/v) inoculum, 1 % (w/v) Y.E., and pH 5.5 adjusted with potassium phosphate buffer (1M)).

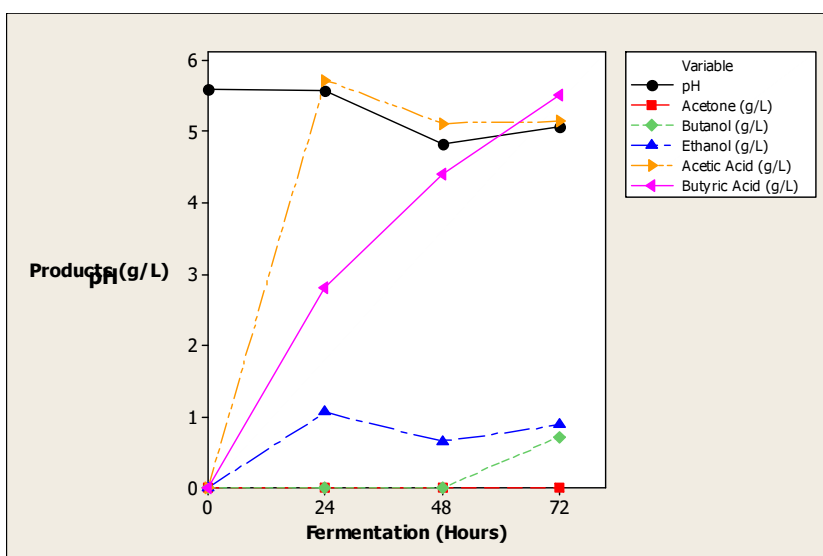


Figure 3.11b. Fermentation profile of white grape pomace (12.5 % w/v) with a magnetic stirrer; (11 % (v/v) inoculum, 1 % (w/v) Y.E., and pH 5.5 adjusted with potassium phosphate buffer (1M)).

Chapter 4

Discussion

4.1 Effect of grape pomace composition on growth and fermentation

The presence of the compounds including minerals, vitamins and nitrogen in the form of proteins and the high concentration of reducing sugars that remains in white grape pomace should permit the bacteria to perform an ABE fermentation.

Glucose, sucrose, and fructose are sugar found in grape juice. These sugars are also found in grape pomace. Chardonnay variety is known to have a high sugar content (Ribéreau-Gayon, Dubourdieu, & Donèch, 2006). The reducing sugar present in grape pomace measured by Bates and Regulski (1982) ranged from 8 to 48.8 % depending on the grape variety.

The sugar concentration in grape pomace suspension used in this study which could be fermented by *C. saccharobutylicum* ranged between 28.8 g/L and 44.6 g/L. According to Jones and Woods (1986), a batch system with less than 7 g/L of sugar would not lead to an solventogenic fermentation. Hence, the sugar concentration present in grape pomace should provide a high concentration of carbon source that would permit an ABE fermentation unlike fermentations with limited carbon source which are known to lead to an acidogenic fermentation (Jones & Woods, 1986).

Spanghero et al. (2009) determined the phosphate concentration of white grape pomace at 3.1 g/kg and 3.0 g/kg in seeds and pulp, respectively (Table 4.1). Since Bahl et al. (1982) stated that production of butanol is possible in a batch culture with *C. saccharobutylicum* under phosphate limitation, phosphate is not a nutrient limiting growth factor. The nitrogen content of grape pomace ranged between 1.74 to 1.88 % (w/w) according to Bates and Regulski (1982).

Although nitrogen is present in grape pomace, in the present study, the nitrogen level was increased by adding yeast extract or nitrogen. Long, Jones, and

Woods (1984) determined that a batch chemostat with ammonium limited cultures did not lead to a solventogenic fermentation.

Table 4.1: Potassium and phosphate content in an Italian white grape pomace analysed by Spanghero et al. (2009).

White grape pomace	
Seeds (g/kg DM)	
P	3.1
K	8.0
Pulp (g/kg DM)	
P	3.0
K	23.1

4.1.1 Effect of initial pH of grape pomace

The adjustment of pH was necessary because the pH of the fermentation medium which contains an acidic grape pomace (pH 4.40) needs to be in the optimum range of pH 5.0 to 6.5 for the ABE fermentation to occur (Jones & Woods, 1986). It is noteworthy that the pH of grape pomace is acidic and ranging between pH 3.48 to 4.12 depending on the grape variety (Hang & Woodams, 2008). According to Jones and Woods (1986), the pH has a great influence on *C. saccharobutylicum* because it determines the outcome of the ABE fermentation. The pH of the fermentation medium should be between pH 5.0 to pH 6.5 for the optimum production of solvents. In addition, the optimum pH of *C. saccharobutylicum* to produce high concentration of solvents in a batch fermentation should be higher than 4.5 and a decrease below pH 4.5 is inhibitory to both growth and the metabolism of the cells and consequently the production of solvent does not occur.

Sodium hydroxide was initially selected instead of a buffer because of its low cost and its use would be economic at an industrial scale if its application were

successful. Sodium hydroxide was used to adjust the pH of grape pomace to 5.46 which decreased significantly to pH 4.58 after autoclaving. It is well known that the higher the temperature of heat treatment of growth media, the greater the decrease in pH is. Since the temperature during autoclaving reaches 121°C for 15 minutes, the pH of the grape pomace decreases to a more acidic level. As a result, sodium hydroxide, being an alkaline solution with no buffering capacity was unable to maintain the pH after autoclaving. The inability of maintaining the pH of the fermentation medium using sodium hydroxide led to an acidic pH of 4.58 which was not within the optimum pH for growth and production of solvents. The optimal pH range for solvent production should be between 5.0 and 6.5 (Jones & Woods, 1986). Previous studies have shown that a pH lower than 5.0 would result in low solvents concentration (Bahl et al., 1982). The pH of 4.58 of the fermentation medium should permit growth based on the studies which demonstrated that *C. saccharobutylicum* with pH below 4.50 inhibited the both growth and metabolism (Jones & Woods, 1986). However, it is possible that toxic compounds present in grape pomace prevented the growth of *C. saccharobutylicum*. Copper which is found in white grape pomace has antimicrobial effect on *Clostridium* species such as on *C. difficile* and *C. tyrobutyricum* (Spanghero et al., 2009; Weaver et al., 2008; Wheeldon et al., 2008). Although zinc is reported to have a lower antimicrobial effect on reducing sulphur bacteria than copper according to Sevinç et al. (2009), it is known to be present in grape pomace based on the analysis done by Spanghero et al. (2009). Hence, the presence of zinc in grape pomace might affect *C. saccharobutylicum*.

Sodium carbonate buffer was adopted from Voget et al. (1985). In their study they adjusted the pH of apple pomace was adjusted using sodium carbonate buffer before inoculating with *C. saccharobutylicum*. In the present study, sodium carbonate buffer was able to maintain the pH value about 5.0 (i.e. pH 5.22), however this had not resulted in an ABE fermentation due to the lack of gas production.

The pH of the fermentation medium adjusted to pH 5.22 with sodium carbonate should allow the growth and the production of solvents by *C. saccharobutylicum* since the adjusted pH was within the optimum pH range of 5.0 to 6.5 (Jones & Woods, 1986). This indicates that the presence of toxic compounds such as

copper prevented the growth of *C. saccharobutylicum* and consequently the production of solvents.

The absence of gas production in the fermentation of grape pomace adjusted with sodium carbonate buffer (1M) indicated that using yeast extract with a concentration of 0.5 % (w/v) did not help the growth of the bacteria cells. This concentration was adopted from previous studies for the culture development of *Clostridium* species. Rogers and Palosaari (1987) used yeast extract and several amino acids for the development of *C. saccharobutylicum* cells in their study while Qureshi and Maddox (2005) performed an ABE fermentation using *C. saccharobutylicum* with lactose as the main source of carbohydrates with 0.5 % (w/v) yeast extract. Roos et al. (1985) also applied that same yeast extract concentration when they studied the effect of pH on nitrogen supply on solvent production with *C. saccharobutylicum*.

4.1.2 Effect of copper and zinc on growth and fermentation

The environment can considerably affect the growth of *C. saccharobutylicum* and subsequently the outcome of the fermentation. In a batch fermentation, the bacteria can be under constant changing environment with varying concentration of toxic compounds (Häggström, 1985). The presence of copper and zinc in grape pomace might affect the growth of *C. saccharobutylicum*. (Table 4.2 show the copper and zinc concentration in grape pomace based on the study done by Spanghero et al. (2009). The copper concentration of white grape pomace is 49 mg/kg and 102 mg/kg in seeds and pulp, respectively. On the other hand, the zinc concentration of white grape pomace is 13 mg/kg and 12 mg/kg in seeds and pulp, respectively.

The presence of both elements in the grape pomace might have affected the growth of *C. saccharobutylicum*. Kuo and Genthner (1996) and Said and Lewis (1991) stated that heavy metals can interfere with microbial processes. Copper is known to affect the growth of vegetative cells *C. tyrobutyricum* and *C. difficile* (Mato Rodriguez & Alatossava, 2010; Wheeldon et al., 2008). According to Sevinç et al. (2009), copper has more antimicrobial effects than zinc on

sulphate reducing bacteria. Nies (1999) reported that zinc, nickel and more importantly copper are toxic at high concentration. As a result, the presence of copper and zinc in grape pomace might be the main inhibitors of *C. saccharobutylicum*.

Table 4.2: Copper and zinc content in an Italian white grape pomace analysed by Spanghero et al. (2009).

White grape pomace	
Seeds (mg/kg DM)	
Zn	13
Cu	49
Pulp (mg/kg DM)	
Zn	12
Cu	102

Yeast Extract can prevent the toxic effect of copper and zinc due to the presence of elemental iron. Iron is known to be present in Yeast Extract based on the analysis of by the mineral content of yeast extract by Grant and Pramer (1962).

The addition of iron is one method used to reduce the toxicity of copper. Kumar, Tewari, and Sharma (2008) prevented the oxidative damage of copper in maize by adding iron in surplus amount. Although their study is based on plant physiology, they claimed that iron has the ability to scavenge for peroxide radicals created by copper, the oxidative active agent.

The cementation reaction between elemental iron and copper ions which is known as reductive precipitation might explain how iron can detoxify grape pomace. Richardson (1997) stated that the removal of copper from an aqueous solution is possible by cementation whereby an elemental ion react with copper ions (Figure 4.1). Cementation is defined as “the removal of a metal ion from

solution by reduction of metal with a more electropositive material” (Richardson, 1997).

Zinc can also react with iron based on the oxidation-reduction reaction (Figure 4.2). Shuman (1977) and Kuo (1986) suggested that reactions occur between iron and zinc. With the inhibition of copper and zinc by iron provided by yeast extract being possible, the absence of growth in grape pomace supplemented with 0.5 % yeast extract indicated that the concentration of yeast extract was not sufficient to provide the iron level which could prevent the inhibitory effects of copper and zinc on *C. saccharobutylicum*.

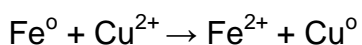


Figure 4.1: Cementation reaction between iron and copper (Richardson, 1997).

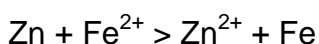


Figure 4.2: Reactions between iron and zinc (Chang, 2003).

Potassium phosphate buffer (1M) was then used after sodium hydroxide and sodium carbonate. The application of potassium phosphate buffer (1M) to adjust the pH to 6.10 before autoclaving resulted in a substrate with a pH of 5.5 after autoclaving. Using this buffer for grape pomace inoculated with bacteria at 11 % (v/v) and supplemented with 1 % (w/v) Y.E. allowed *C. saccharobutylicum* to grow in grape pomace and produce solvents which include butanol, acetone and ethanol.

Previous studies that involved *C. saccharobutylicum* with an acidic substrate have used pH buffers because the acidic pH of the substrate does not support the growth of *C. saccharobutylicum*. The effect of buffer is considered as a means to improve both growth of *C. saccharobutylicum* and the production of butanol (Bryant & Blaschek, 1988). According to Bryant and Blaschek (1988), when a highly buffered medium is used, there is not only an increase of cell

density during the stationary phase but also a high use of carbohydrate and production of butanol. More importantly, they determined that using phosphate buffer had a buffering effect which promotes the growth of *C. saccharobutylicum*. Hence, the application of this buffer not only helped the maintenance of the pH of the fermentation medium but it also helped the growth of *C. saccharobutylicum*.

In addition, the application of 1 % (w/v) yeast extract provided sufficient amount of iron to inactivate copper and zinc found in grape pomace and consequently allowed the growth and the production of solvents by *C. saccharobutylicum*.

4.1.3 Effects of phenolic compounds on growth and fermentation

Grape pomace is known to be a rich source of polyphenolic compounds including phenolic acids, flavonoids, anthocyanins and proanthocyanidins (Brenes et al., 2008; Lu & Yeap Foo, 1999). These chemical compounds have anti-oxidant activity and perform as scavenger for free radicals and are capable to bind to proteins (Bravo, 1998; Guerrero et al., 2008; Lu & Yeap Foo, 1999). The flavonoids which act as powerful antioxidants can stop oxidation reactions (Brenes et al., 2008). Phenolic compounds could affect the growth and fermentation of *C. saccharobutylicum* since *Clostridium beijerinckii* P260 and *Clostridium beijerinckii* BA101 seems to be affected by these inhibitors based on the results of Qureshi et al. (2010a) and Ezeji, Qureshi, and Blaschek (2007), respectively. Reddy, Pierson, and Lechowich (1982) also determined that phenols can inhibit the growth of *Clostridium botulinum*.

4.2 Effect of Yeast Extract and inoculum on the fermentation of white grape pomace

4.2.1 Effect of inoculum on the fermentation of grape pomace

Using 5.5 % (v/v) inoculum led to the formation of acids and a final biomass production of 0.69 g/L. This inoculum should be sufficient to perform an ABE fermentation since it is generally used in studies involving *C. saccharobutylicum* with batch fermentation. Qureshi, Saha, and Cotta (2007) and Qureshi et al. (2008) have used a batch fermentation system with 5 % (v/v) inoculum in substrate involving wheat straw hydrolysate and hydrolysed corn fibre, respectively.

In addition, the initial concentration of reducing sugar was 33.25 g/L for the fermentation inoculated with 5.5% (w/v) and this amount should be sufficient for the bacteria to perform an ABE fermentation. Studies involving *C. saccharobutylicum* had initial sugars concentration between 10 to 65 g/L. The concentration of sugars in the semi-synthetic medium used for the development of stock culture for *C. saccharobutylicum* by Roos et al. (1985) was 10 g/L. Jones and Woods (1986) stated the production of solvent does not occur when the concentration of sugar in a batch fermentation system is lesser than 7 g/L. As a result, the concentration of 33.25 g/L of sugars present in grape pomace with 5.5 % (v/v) inoculum and pH adjusted to 5.5 should be sufficient for the growth of *C. saccharobutylicum*.

The results involving 5.5 % (v/v) inoculum indicated that this concentration did not have a sufficient number of bacterial cells that would permit the ABE fermentation. According to Bahl, Andersch, and Gottschalk (1982), this parameter is so important that the inoculum can determine the outcome of the ABE fermentation. Most of the previous studies involving *C. saccharobutylicum* used 10 % (v/v) inoculum were based on continuous fermentation system, which differ from the present study using a batch fermentation system. For instance, Afschar, Biebl, Schaller, and Schügerl (1985) and Bahl, Andersch and Gottschalk (1982) have used 10 % (v/v) inoculum for their studies using *C. saccharobutylicum* in a continuous fermentation. The 5.5 % inoculum was used

initially to determine whether or not it was sufficient for fermentation of grape pomace. In addition this study involves a batch fermentation which differs from the continuous system of these studies.

Using 11 % (v/v) inoculum led to the production of solvents with ratio of 3:6:1 which was identical to the Weizmann's ABE fermentation (Jones & Woods, 1986). Increasing the concentration of inoculum from 5.5 % to 11 % (v/v) produced a higher number of highly motile cells characteristic of active growth and vigorous ABE fermentation. Masion, Amine, and Marczak (1987) determined that having a good cell development or a high biomass leads to the formation of the highest solvent production although the concentration of solvent produced does not reflect the quantity of biomass.

While the concentration of inoculum played an important role, it was not the only parameters that need to be adjusted correctly to permit a solventogenic fermentation of grape pomace. The concentration of yeast extract also affected the outcome of the ABE fermentation.

The phenolic compounds in grape pomace may also affect *C. saccharobutylicum* and using an inoculum of 0.5 % could not be sufficient to promote the growth of cells in grape pomace. On the other hand, using a higher concentration such as 1 % inoculum doubled the number of cells which could overcome to inhibitory effects of polyphenolic compounds by surviving and initiate growth and solvent production.

4.2.2 Effects of yeast extract on the fermentation of grape pomace

Yeast Extract affected the outcome of the grape pomace fermentation inoculated with *C. saccharobutylicum*. This study showed that the supplementation of 1 % (w/v) yeast extract in a fermentation of grape pomace led to the production of solvents. On the other hand, a fermentation that is unsupplemented or which contained 0.5 % yeast extract resulted in the production of acids and with the absence of growth.

The fermentation unsupplemented with yeast extract led to an acidogenic fermentation possibly due to the inhibitory effects of copper found in grape pomace and the lack of iron from Yeast Extract. On the other hand, the concentration of 0.5 % yeast extract did not provide sufficient amount of iron to prevent the inhibitory action of copper in grape pomace.

On the other hand, the supplementation of 1 % (w/v) yeast extract in grape pomace fermentation resulted in the production of solvents possibly because a sufficient amount of iron was provided from yeast extract. The high concentration of iron content present in 1 % Y.E. possibly reacted with copper found in grape pomace via cementation reaction or the scavenging properties of iron for reactive oxygen species (Kumar et al., 2008; Richardson, 1997). Iron is also known to react with zinc with a redox reaction. Consequently, the inhibitory effect of both copper and zinc being repressed would enable the growth and the production of solvents by *C. saccharobutylicum*. The results suggest that the concentration of Y.E. necessary for the ABE fermentation with *C. saccharobutylicum* in grape pomace was 1 % Y.E.

4.3 Fermentation of white grape pomace

This study has determined that the parameters required for a successful ABE fermentation using white grape pomace include 11 % (v/v) inoculum, supplementation with 1 % (w/v) Y.E., and the adjustment of pH 5.5 with phosphate potassium buffer (1M). Using 11 % (v/v) of inoculum allowed a high initial concentration of cells to grow in the fermentation medium while potassium phosphate buffer (1M) maintained the initial pH of the substrate at around 5.5. The presence of Yeast Extract at 1 % (w/v) provided the necessary concentration of iron to prevent the inhibitory effects of copper which is found in grape pomace.

The ratio of acetone, butanol, and ethanol obtained in this study was identical to Weizmann's ABE fermentation (Jones & Woods, 1986). In this study, the total solvent produced was 8.76 g/L which is half of the typical total solvent produced in a batch fermentation system (20-25 g/L) involving traditional substrate such as molasses, cornstarch or glucose with reducing sugars concentration of 60 g/L within 36-72 hours according to Qureshi and Ezeji (2008).

According to Kim, Bellows, Datta, and Zeikus (1984) and Häggström (1985), the stoichiometry of ABE fermentation with *C. saccharobutylicum* is that one mole of glucose yields 1 mol (0.41 g/g) of butanol. The theoretical yield of butanol based on the sugar concentration of 30.56 g/L in grape pomace should produce 12.53 g/L of butanol while in this study, 5.80 g/L of butanol was produced from grape pomace. This indicates that the fermentation process was not optimised. The presence of copper and zinc or other inhibitors might have interfered with the fermentation of *C. saccharobutylicum* even though iron was supplied using a concentration of 1 % yeast extract or that concentration is insufficient to prevent the inhibitory effects of copper and zinc. The presence of phenolic compounds in grape pomace which might have inhibited the growth of *C. saccharobutylicum*.

In addition, the productivity of the fermentation was 0.21 g/L/h, which is lower than a typical batch fermentation system since Qureshi and Ezeji (2008) indicated that productivity in batch fermentation rarely exceeds 0.5-0.6 g/L/h. The solvent yield in this study was 0.30 g/g and this value was typical of a batch

fermentation system according to Qureshi and Ezeji (2008) who stated in their review that a batch fermentation generally gives a low solvent yield of 0.30 g/g.

A comparison of the bioprocess and kinetic parameters between barley straw Qureshi et al. (2010a) and grape pomace used in this study shows that the fermentation was not optimised (Table 4.3). There seems to be inhibitors that could still be hindering the fermentation.

The bioprocess and kinetic parameters from grape pomace were similar to that of Qureshi et al. (2010a) when the barley straw hydrolysate contained inhibitors (Table 4.3). After the removal of inhibitor compounds, the fermentation of barley straw hydrolysate had higher bioprocess and kinetic parameters than their control experiment (glucose substrate) (Table 4.3). This comparison suggests that the grape pomace fermented with 1 % (w/v), pH 5.5 adjusted with potassium phosphate buffer (1M) still contained toxic compounds. The phenolic compounds of grape pomace are known to terminate oxidation reactions and bind to proteins (Bravo, 1998). These inhibitors are known to inhibit *C. botulinum* and their presence possibly affected the fermentation of *C. beijerinckii* might also hindered *C. saccharobutylicum* (Ezeji et al., 2007; Qureshi et al., 2010a; Reddy et al., 1982). The concentration of iron provided by 1 % Y.E. might not have been sufficient to prevent the antimicrobial activity of copper or zinc. Adding a higher concentration of ferrous salts might improve the fermentation process.

Table 4.3: Comparison of ABE fermentation using different substrates including agricultural residues, barley straw hydrolysate, treated barley straw hydrolysate, glucose substrate and grape pomace.

Substrates	White grape pomace	Barley straw hydrolysate (untreated) Qureshi et al. (2010a)	Barley straw hydrolysate after removal of inhibitors Qureshi et al. (2010a)	Glucose fermentation by Qureshi et al. (2010a)
Total ABE produced (g/L)	8.76	7.09	26.64	21.06
ABE productivity (g/L/h)	0.21	0.10	0.39	0.31
ABE solvent yield (g/g)	0.30	0.33	0.43	0.41

It could be possible that the fermentation with grape pomace still contained inhibitory compounds although copper and zinc were inhibited by iron present in 1 % (w/v) yeast extract and that the removal of those inhibitors could improve the fermentation of grape pomace.

The polyphenolic compounds found in grape pomace are potential inhibitors. Qureshi et al. (2010a) named the phenolic compounds as one of the inhibitors created by the sulphuric acid treatment of their substrate. According to Bravo (1998), polyphenolic compounds are reported to exert adverse effects due to their ability of binding and precipitating molecules such as proteins or carbohydrates. In addition, polyphenolic compounds which are known to have antioxidant properties can terminate oxidation reductions (Brenes et al., 2008). *C. saccharobutylicum* has several oxidation-reduction reactions involved during the acidogenic and solventogenic phases and the phenolic compounds can interfere with these reactions.

A comparison between the apple pomace fermentation involving *Clostridium* species for the production of butanol and the grape pomace fermentation also indicates that the fermentation supplemented with 1 % Y.E. was not optimal. Voget, Mignone, and Ertola (1985) used apple pomace with *Clostridium acetobutylicum* NRRL B 596, *Clostridium butylicum* NRRL B 592, and

Clostridium butylicum NRRL B 593 for the production of butanol. The butanol yield (based on substrate) was 0.25, 0.26, and 0.22 for B 592, B 593, and AB 596 strains, respectively. In this study, the butanol yield of the fermentation of grape pomace with the necessary parameters was 0.20 which is lower in comparison to the yield of the apple pomace fermentation by *C. acetobutylicum* AB 596. *C. acetobutylicum* AB 596 which is a similar strain to *C. saccharobutylicum* would have similar yield (Jones & Woods, 1986). Since the supplementation of 1 % Y.E. in grape pomace might not have been sufficient to inactivate the inhibitory components, the butanol yield was lower. On the other hand, the grape pomace supplemented with the mineral salts including manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) produced a butanol yield of 0.23 which is close to the butanol yield of *C. acetobutylicum* AB 596 using apple pomace as substrate. These mineral salts improved the grape pomace fermentation due to the use of analytical grade ferrous salts which are readily available.

Hang et al. (1986) were able to produce a high yield of ethanol (82 %) by yeasts in grape pomace involving a solid-state fermentation. The present study had a lower yield of ethanol (2 %) which might be due to the use of submerged fermentation which might accentuate the antimicrobial effects of inhibitors which become readily available to the bacteria cells in comparison to the solid-state fermentation. In addition, the high ethanol yield produced by Hang et al. (1986) is typical of wine yeasts since ethanol is the main end-product of the anaerobic glycolytic pathway of yeasts (Lee, 2006) while the solventogenic fermentation by *C. saccharobutylicum* yields ethanol with the lowest ratio in comparison to butanol (1:6) (Jones & Woods, 1986).

4.4 Fermentation with salts and unsupplemented with Y.E.

Yeast Extract is expensive and its cost ranges between US \$ 180 to \$280 per kilo. Using this essential component for the fermentation of grape pomace at an industrial scale would render the production of biobutanol non-economic. Unlike Y.E., grape pomace has no cost associated with it since it is a waste product of winemaking (Bates & Regulski, 1982).

4.4.1 Nitrogen salts

The addition of inorganic salts in the substrate is commonly performed when ABE was produced at an industrial scale (Jones & Woods, 1986). Generally inorganic nitrogen such as ammonium salts were used in previous studies to understand the effect of nitrogen on the solvent production by *C. saccharobutylicum* (Roos et al., 1985). Nitrogen compounds in the form of ammonium sulphate and ammonium phosphate were used in the present study at concentrations based on those used in a semi-synthetic medium of Roos et al. (1985). The lack of gas production which is indicative of growth was not observed in the fermentation of grape pomace supplemented with nitrogen salts (Section 3.4.1). A lower concentration of 0.25 g/L for ammonium sulphate and ammonium phosphate was used because 2 g/L might be detrimental to *C. saccharobutylicum*. Qureshi and Maddox (1995) reported that mineral toxicity can occur in a batch fermentation which is a closed fermentation system and it was possible that 2 g/L of ammonium phosphate had inhibitory effects on *C. saccharobutylicum*.

The fermentation of grape pomace with 0.25 g/L of nitrogen salts did not yield any growth as indicated by the lack of gas production which accompanies growth.

The fermentation run solely with nitrogen salts did not lead to a solventogenic fermentation possibly because the medium was not supplemented with iron which would have interfered with the copper present in grape pomace.

According to the mineral analysis done by Spanghero et al. (2009), iron is known to be present in white grape pomace at a concentration of 58 mg/kg and 111 mg/kg in seeds and pulp respectively. The results of the fermentation with nitrogen salts at concentrations of 0.25 and 2 g/L and that in a medium unsupplemented with Y.E. suggest that the iron content of grape pomace did not react with copper. On the other hand, the iron supplied from Y.E. as demonstrated in Section 3.3 probably enabled the production of solvents. The iron content in grape pomace might not be readily available that could react with copper.

4.4.2 Use of mineral salts of semi-synthetic medium adopted from Roos et al. (1985) in fermentation medium free of Y.E.

The addition into grape pomace of manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium diphosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) at concentrations based on the semi-synthetic medium (Roos et al., 1985) enabled the growth of bacteria cells and led to the production of solvents with ratio similar to the Weizmann's fermentation of 3:6:1 (acetone, butanol, ethanol) (Jones & Woods, 1986).

A comparison of the mineral composition of yeast extract analysed by Grant and Pramer (1962) and the mineral salts adopted from Roos et al. (1985) revealed that ferrous salts were also found in yeast extract (Table 4.4). As a result ferrous sulphate might react with copper via precipitation reaction and prevented the inhibitory effect of copper on *C. saccharobutylicum*. A higher solvent yield and glucose utilisation rate obtained when the mineral salts were applied could be due to the use of magnesium, manganese, phosphate salts. The results indicated that the use of mineral salts as substitute for Y.E. enabled an improved production of total solvents in comparison to the fermentation with Y.E.

Table 4.4. Mineral salts used in the fermentation of grape pomace adopted from Roos et al. (1985) and elemental iron in Y.E. (Grant & Pramer, 1962).

	Mineral salts used by Roos et al. (1985)	Minor elements in Y.E. analysed by Grant & Pramer (1962)
Minor elements / Salts	Potassium diphosphate	None
	Dipotassium phosphate	None
	Manganese sulphate	Manganese
	Magnesium sulphate	Magnesium
	Ammonium sulphate	None
	Ferrous sulphate	Iron

Table 4.5: Comparison of kinetic parameters between fermentation of grape pomace supplemented with 1 % (w/v) Y.E. and fermentation of grape pomace supplemented with manganese sulphate, magnesium sulphate, potassium phosphate, dipotassium phosphate, ferrous sulphate, and ammonium sulphate.

	Grape pomace fermentation with 1 % (w/v) Y.E.	Grape pomace fermentation with mineral salts adopted from Roos et al. (1985)
Total ABE (g/L)	8.76	9.08
Solvent Yield (g/g)	0.30	0.36
Total solvent productivity (g/L/h)	0.21	0.19
Reducing sugars utilisation rate (g/L/h)	0.57	0.59

The fermentation of grape pomace containing mineral salts and unsupplemented with Y.E. had a higher concentration of ABE produced, solvent yield and, glucose utilisation rate than that of grape pomace with 1 % (w/v) Y.E (Table 4.5).

The use of analytical grade of the mineral salts meant that the mineral salts were readily available in the grape pomace. Ferrous sulphate could have also improved the production of solvents. Bard and Gunsalus (1950) reported that iron is essential for the metabolic processes of *Clostridium* species. The supply of sulphate minerals could greatly induce the production of solvents by *C. saccharobutylicum* as demonstrated by Bahl and Gottschalk (1984).

Although the fermentation of white grape pomace would not be considered economically viable since Woods (1995) stated that a total solvent production between 22 and 28 g/L is the range that is considered to be economically competitive, the zero cost of grape pomace would still be an potential incentive for the production of butanol. The use of mineral salts which optimised the fermentation of grape pomace in comparison to the supplementation of yeast extract could make the production of biobutanol from grape pomace considerably cheaper and more appealing because these minerals are commercially produced as fertilisers in the agriculture industry.

4.5 Optimisation of ABE fermentation with grape pomace.

During the fermentation, some of the grape pomace solids and bacterial cells came out of the suspension and adhered the top wall of the bottle due to gas formation by *C. saccharobutylicum*. This, therefore, reduced the number of cells in the substrate and consequently decreased the available substrate and reducing sugars which the bacteria could have utilised for growth and solvent production.

Due to time and financial constraints, an attempt to optimise ABE fermentation using grape pomace involved a simple set-up using a magnetic stirrer to keep the grape pomace in suspension and prevent wall growth. This resulted mainly in an acidogenic fermentation. The technique worked well with the wall growth being prevented and grape pomace consistently submerged. However, stirring introduced oxygen into the system which was detrimental to the anaerobic bacteria in this fermentation.

The neck of the bottle still had a headspace or inside the anaerobic jar that might contain oxygen which is being introduced back to the fermentation medium when the magnetic stirrer was activated. Since *C. saccharobutylicum* is an anaerobic bacteria and is consequently sensitive to oxygen (Jones & Woods, 1986), a significant number of bacteria would not grow significantly leading to a low number of bacteria cells which was not sufficient enough to perform an ABE fermentation.

The use of a batch bioreactor with controlled agitation could prevent the phenomenon of substrate adhesion to bioreactor wall. In addition, a pH control and supply of anaerobic gas also optimise the fermentation.

Further research into this fermentation could also determine the success in using treated grape pomace to remove toxic compounds.

Chapter 5

Conclusions

Problems associated with the dependence on fossil fuel are not solely based on running out of oil but mainly on the economic and social issues (Sperling & DeLuchi, 1989). As awareness regarding global warming increases, the negative impact of using fossil fuels on the environment, especially greenhouse gas emission, and the dependence on petroleum-based fuels, the search for alternative fuels is attracting worldwide attention (Balat, 2009). The prospect of converting renewable resources such as organic wastes into useful chemicals is promising (Grube et al., 2002). Currently, researchers have studied the use of agricultural residues such as barley straw as potential substrate for the production of butanol using *Clostridium* spp. The present study could be a precursor for providing one of many solutions to reduce the dependence on fossil fuels and their associated environmental and economic issues by utilising the main waste in wine processing called grape pomace.

Grape pomace is a great candidate substrate for ABE fermentation because it does not compete with food resources in contrast to corn which is used for the production of ethanol. The New Zealand wine export accounts for 285 thousand tonnes of grapes produced in the country (New Zealand Wine, 2010). The large production of white wine provides an opportunity to convert 64 thousand tonnes of white grape pomace into a biofuel, butanol using an anaerobic bacterium, *Clostridium saccharobutylicum*. This microorganism has been used with different types of substrate such as noncellulosic to agricultural wastes for the production of solvents. The production of butanol at an industrial scale is performed by *Clostridium saccharobutylicum* using the ABE fermentation. This fermentation is currently receiving renewed attention because of its potential as an alternative and environmentally-friendly process for the production of solvents, particularly with the fluctuations in oil prices, increasing concerns of fossil fuels depletion and environmental problems (Grube et al., 2002; Zheng et al., 2009).

Since most studies concentrated on the extraction of health promoting compounds from the grape pomace, there have been few studies concentrating on the conversion of grape pomace as a biomass to a biofuel such as butanol using an industrial strain *C. saccharobutylicum*. As a result, most of the efforts in this study were to determine the parameters such as the concentration of inoculum to be used, the yeast extract concentration and the pH for the production of solvents.

This study has demonstrated that the parameters required for solventogenic fermentation of grape pomace (12.5 % w/v) with *Clostridium saccharobutylicum* are an inoculum concentration of 11 % (v/v), supplementation with 1 % (w/v) Yeast Extract at % (w/v) and an initial pH of 5.5 obtained by using potassium phosphate buffer (1M). The use of buffers is necessary to maintain the pH at 5.5 which is within the optimum pH range for *C. saccharobutylicum* to produce solvents. The use of 5.5 % (v/v) inoculum would be sufficient to perform an ABE fermentation but an inoculum of 11 % (v/v) provides a higher concentration of vigorous fermentative cells.

The absence of growth, acidogenic fermentation and solventogenic fermentation in grape pomace may be explained by its phenolic content. These phenomena could also be attributed to the presence of zinc and copper which might be inhibitory to *C. saccharobutylicum*. Zinc and copper are known to be present in white grape pomace based on the study by Spanghero et al. (2009). Copper toxicity is known to affect the vegetative cell growth of *C. tyrobutyricum* and *C. difficile* (Mato Rodriguez & Alatossava, 2010; Weaver et al., 2008). Since studies have shown that copper is present in grape pomace and it has antimicrobial effects on *Clostridium* species, it is possible that copper inhibited the growth of *C. saccharobutylicum*.

The presence of Y.E. appears to be essential for growth and solventogenic fermentation in grape pomace. This is attributed to the suppressive effect of Y.E. components, such as iron, on the antimicrobial activity of copper. Results suggest that a low concentration of Y.E. (0.5%) does not provide sufficient amount of iron to prevent the toxic effect of copper and zinc on the bacteria.

The use of nitrogen salts as sole mineral salt supplement to replace yeast extract does not lead to an ABE fermentation probably due to the lack of iron.

This suggests that the iron content of grape pomace is not sufficient to disable copper or zinc in the substrate.

This study demonstrates that the production of solvents is possible when mineral salts including manganese sulphate, magnesium sulphate, potassium phosphate, dipotassium phosphate, ferrous sulphate, and ammonium sulphate were used in a fermentation medium free of yeast extract. In addition, the use of the mineral salts mentioned previously is a better alternative than Yeast Extract resulting in higher kinetic parameters. The fermentation with mineral salts produced a total ABE of 9.08 g/L, a solvent yield of 0.36 g/g, and a total solvent productivity of 0.19 g/L/h. These bioprocess and kinetic parameters are better in comparison to those obtained from fermentation of grape pomace with yeast extract.

The attempt in optimising the fermentation process by keeping the substrate submerged with *C. saccharobutylicum* using a magnetic stirrer led to an acidogenic fermentation because oxygen was reintroduced into the batch system when stirring was applied. The use of a batch bioreactor with controlled stirring and pH would probably give a higher solvent production and productivity.

YE provides growth factors such as vitamins, amino acids and peptides while mineral salts do not. The minerals do not replace YE but are relevant in ABE fermentation of grape pomace. The addition of mineral salts comprising of manganese sulphate, magnesium sulphate, potassium phosphate, dipotassium phosphate, ferrous sulphate, and ammonium sulphate as an alternative to the costly yeast extract could improve the prospect of producing butanol from grape pomace.

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Appendices

APPENDIX A

Accuracy of DNS

Known amount of reducing sugar	Measurements using DNS	Accuracy
10.015	9.77	93.40%
	9.51	
	8.80	

APPENDIX B

Alkaline solution and Buffer

Sodium hydroxide		
Fermentation (Hours)	pH	Reducing Sugars (g/L)
0	4.58	29.21
24	4.52	30.35
48	4.51	29.32
72	4.52	28.93

Sodium carbonate		
Fermentation (Hours)	pH	Reducing Sugars (g/L)
0	5.23	28.16
24	5.24	32.30
48	5.16	31.60
72	5.18	31.79

APPENDIX C

Effect of Yeast Extract on the fermentation and inoculation concentration of white grape pomace

5.5 % (v/v) inoculum								
Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.51	33.25	0	0	0	0	0	0.45
24	5.48	31.60	1.02	0.13	0.07	1.96	2.46	0.52
48	5.27	34.02	0.98	0.13	0.00	1.95	2.38	0.69

11 % (v/v) inoculum								
Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.56	34.00	0	0	0	0	0	0.54
24	4.70	19.45	1.39	1.21	0.00	2.74	3.60	0.75
48	5.49	3.76	3.75	5.84	0.05	1.64	2.79	0.83

Unsupplemented with yeast extract								
Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.76	30.20	0	0	0	0	0	0.69
24	5.64	29.62	0	0	0.64	4.66	2.37	0.68
48	5.65	28.34	0	0	0.51	4.68	2.50	0.69
72	5.45	27.71	0	0	0.64	5.28	2.77	0.71

APPENDIX D

Fermentation of white grape pomace with 11 % (v/v) inoculum, 1 % (w/v) Y.E., adjusted with pH 5.5. with potassium phosphate buffer (1M)

Grape pomace fermentation								
Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.52	30.56	0	0	0	0	0	0.69
24	4.78	19.93	0.44	1.50	0.71	5.37	3.58	0.73
48	5.11	6.19	2.10	5.00	0.68	3.35	4.02	0.81
72	5.07	0.88	2.38	5.80	0.58	3.17	4.13	0.85

APPENDIX E

Nitrogen salts

(NH₄)₂PO₄ (2 g/L)

Fermentation (Hours)	pH	Reducing Sugars (g/L)
0	5.35	31.40
24	5.34	34.27
48	5.31	37.45

(NH₄)₂SO₄ (2 g/L)

Fermentation (Hours)	pH	Reducing Sugars (g/L)
0	5.18	44.64
24	5.13	47.21
48	5.12	49.98

**(NH₄)₂PO₄
(0.25 g/L)**

Fermentation (Hours)	pH	Reducing Sugars (g/L)
0	5.35	32.30
24	5.35	33.26
48	5.39	34.28

**NH₄)₂SO₄,
(0.25 g/L)**

Fermentation (Hours)	pH	Reducing Sugars (g/L)
0	5.22	35.76
24	5.26	35.79
48	5.22	38.79

APPENDIX F

Fermentation with salts ranging from manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), magnesium sulphate ($\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$), ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) in addition to ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$)

Fermentation with salts								
Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Biomass (g/L)
0	5.49	29.33	0	0	0	0	0	0.59
24	5.06	28.84	0	0.53	1.55	6.13	3.39	0.65
48	4.67	15.77	0.87	2.99	0.93	5.70	2.98	0.80
72	4.75	0.71	2.83	5.96	0.51	3.03	2.49	0.81

APPENDIX G

Improving the fermentation process

Optimisation of the fermentation							
Fermentation (Hours)	pH	Reducing Sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)
0	5.58	27.75	0	0	0	0	0
24	5.56	31.09	0	0	1.06	5.72	2.81
48	4.83	24.94	0	0	0.65	5.11	4.40
72	5.07	7.5	0	0.71	0.88	5.14	5.51