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Optimal Sugar Compostitions And Conditions For Enhanced Biobutanol Bioproduction From Agriculture Residues

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OPTIMAL SUGAR COMPOSITIONS AND CONDITIONS FOR ENHANCED BIOBUTANOL BIOPRODUCTION FROM AGRICULTURE RESIDUES

By

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Abstract

Production of alternative non-fossil biofuels based on renewable resources has been the focus of research in the past few decades due to its environmental and economical advantages. The current study focuses on testing two Clostridia strains towards production of butanol. The work was performed in three parts: the first part includes introducing *C. acetobutylicum* ATCC 4259 for butanol production and identifying the proper working conditions for this strain. The following part includes extending investigation of production to examine *C. beijerinckii* BA101 and compare with results obtained from *C. acetobutylicum*. In the last part, an optimization study was conducted on a presently derived mathematical model in order to predict the best sugar composition in the feedstock for maximum production of butanol. Results showed that the agriculture residues are potential biomass resource for biofuel industry since both Clostridia strains were successfully able to utilize all types of agricultural sugars including hexose and pentose. However, using *C. beijerinckii* resulted in 53% higher butanol concentration than using introduced *C. acetobutylicum.* The yield was fairly comparable, while high acid accumulation found when using *C. acetobutylicum* made this strain inapplicable to anaerobic batch fermentation without effective system of pH control.

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Chapter 1
Introduction

Green house concerns, energy scarcity and the desire for energy independence are increasing the pace and intensity of biofuel research and commercialization. Since the late 1990s global biofuel research has steadily been on the rise, with new modified micro-organisms, pretreatments, process configurations and technologies improving conversion efficiencies and decreasing production cost. In first-generation biofuel, plant sugars and starch from food crops were fermented to biofuel by yeast. The advent of second-generation biofuels broadened the feedstock base to include non-food cellulosic biomass by incorporating chemical or enzymatic hydrolysis in various process configurations (Olive 2008). Third-generation biofuels employ enzyme-producing micro-organisms to both hydrolyze plant polymers and ferment the resulting sugars. The Canadian government supports the development of biofuel production through a $2 billion commitment in the 2007 Federal Budget. A long-standing federal and provincial excise tax exemption on renewable fuels has encouraged the renewable fuels to be competitive with gasoline. In United States, according to the Department of Energy (DOE) "Roadmap for Biomass Technology in the United States" biobased transportation fuels are projected to increase from the 0.5% of U.S consumption in 2001 to 4% in 2010, 10% in 2020, and further to 20-30% in 2030, or about 60 billion gallons of gasoline equivalent per year (SRI Consulting Business Intelligence 2008). This is in addition to 10–12 billion pounds of butanol annually required for industries rather than biofuel (Donaldson et al. 2007). Meeting the timeline was responsible to initiating the race in commercializing the fermentation production of alternative fuels such as ethanol and butanol.

Although there was more focus on fermentation of ethanol due to the vast applications in industry, more interest has recently intensified on butanol for the similar characteristics to gasoline which allow the direct use of butanol in any gasoline engine without modification and/or substitution (Qureshi et al. 2007a).

Butanol, a product of acetone-butanol-ethanol (ABE) fermentation, is an excellent feedstock chemical in the plastics industry, a food-grade extractant in the food and flavor industry and, more importantly, a superior fuel to ethanol (Formanek el al. 1997, Parekh et al. 1998 & 1999). Butanol contains 22% oxygen making it an excellent fuel extender and a cleaner burning fuel (Ezeji et al. 2005a & 2006, Ladisch 1991). Butanol's energy content is 30% more than ethanol and is closer to gasoline. Its low vapor pressure facilitates its application in existing gasoline supply channels, it is not sensitive to water, less volatile, less hazardous to handle, less flammable than ethanol (Zverlov et al. 2006, Hector et al. 2007). While the current automotive engine can not tolerate more than 15% ethanol, n-butanol can be used up to 100% in unmodified 4-cycle ignition engines or blended up to 30% (70% diesel) in a diesel compression engine or to 20% (80% kerosene) in a jet turbine engine (Schwarz et al. 2006, Antoni et al. 2007). Table 1.1 summarizes the properties of common fuels with respect to gasoline.

Table 1.1 Properties of common fuels (Qureshi et al. 2007a)

Fuel Type	**Energy Density** (MJ/L)	**Air-Fuel Ratio**	**Specific Energy** (MJ/kg air)	**Heat of Vaporization** (MJ/kg)	**Research Octane Number**	**Motor Octane Number**
Gasoline	32	14.6	2.9	0.36	91–99	81–89
Butanol Fuel	29.2	11.2	3.2	0.43	96 -105	78- 89
Ethanol Fuel	19.6	9.0	3.0	0.92	129	102
Methanol	16	6.5	3.1	1.20	136	104

Chapter 2
Literature Review

2.1 History of Butanol

Biological production of butanol has a long history as an industrially significant fermentation process. After Pasteur discovered bacterial butanol production from his landmark anaerobic cultivation in 1861, fermentative ABE production prospered during the early 20th century and became after ethanol the second largest industrial fermentation process in the world. In 1945, two thirds of industrially used butanol was produced by fermentation in U.S (Jones and Woods 1986). However, the ABE fermentation process had lost competitiveness by 1960s due to the increase of feedstock costs and advancement of the petrochemical industry except in Russia and in South Africa, where the substrate and labor costs were low. The ABE fermentation processes in South Africa and Russia continued to operate until the late1980s to early 1990s (Zverlov et al. 2006).

Competitiveness of ABE process depends on several major features, including the cost of substrate, the product yield, and the product recovery. In addition, the recovery and complete utilization of the by-products (such as CO_2 and H_2) could significantly affect the total value obtained from the process (Sang et al. 2008). The use of food crops, such as corn, as substrates raises conflicts with its nutritional purposes, especially in areas concerned with potential food shortages. The prices of related materials have been nearly doubled in concert with the growing demand in biobased industries. For example the price of corn grain was increased to nearly double from 2004-2007 (Olive 2008). Thus, biofuel production from nontraditional food biomass, such as agricultural residues, thought to be a potential solution for biofuel industry (Lynd 1989). It has recently been reported that Russian fermentation industry is concentrating on the conversion of

agricultural biomass into butanol (Sang et al. 2008). Furthermore, BP Biofuels in United Kingdom is working with DuPont in United States to develop and commercialize biobutanol process using lignocellulosic feedstocks. BP announced a huge grant of $500 million for the next ten years to establish an Energy Biosciences Institute at the University of California, with the overall goal of making the large-scale use of this technology viable (SRI Consulting Business Intelligence 2008).

2.2 Agricultural Biomass

Agricultural residues and wastes used for the production of biofuels include wheat straw, rice straw, wood (hardwood), byproducts left over from the corn milling process (corn fiber), dried distillers' grains with solubles (DDGS), annual and perennial crops, and waste paper (Tabka et al. 2006, Qureshi and Blaschek 2005, Ezeji and Blaschek 2008). Agriculture biomass are composed mainly of three biobased chemicals called cellulose (35-48 % dry wt), hemicellulose (22-48%) and lignin (15-27%) (Scurlock and Jonathan 2004, Sun 2002). Together, they are called lignocellulose, a composite material of rigid cellulose fibers embedded in a cross-linked matrix of lignin and hemicellulose that bind the fibers. Lignocellulose material is by necessity resistant to physical, chemical, and biological attack, but it is of interest to biorefining because the cellulose and hemicellulose can be broken down through a hydrolysis process to produce fermentable, simple sugars (Hopkins 1999, Saha 2003).

Cellulose is a very large linear polymer exists in native in the form of microfibrils. These microfibrils (Figure 2.1) are composed of many hundreds or thousands of glucose molecules (polysaccharide). Unlike starch, the glucose monomers of cellulose are linked

together resulting in tightly packed and highly crystalline structures that are resistant to hydrolysis (Carpita and McCann, 2000). Therefore, pretreatment of lignocellulosic biomass before hydrolysis is a vital step. Hemicellulose consists of short, highly branched, chains of sugars. It contains five-carbon sugars (usually xylose and arabinose) and six-carbon sugars (glucose, galactose and mannose) and uronic acid. Hemicellulose is amorphous and relatively easy to hydrolyze to its constituent sugars (Wisconsin biorefining 2004). Lignin is a polymer constructed of non-carbohydrate, alcohol units that are not fermented such as p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Klinke et al. 2004). While cellulose and hemicellulose contribute to the amount of fermentable sugars for butanol production, products of lignin degradation are recognized as a potential source of microbial inhibitors (Ezeji et al. 2007a, Fan et al. 1987).

Producing commercial products through fermentation of lignocellulose is a multi-step process: pre-treatment and hydrolysis of the lignocellulose to release fermentable simple sugars, fermentation of simple sugars by living microorganisms to produce hydrocarbons such as organic acids or alcohols, recovery from the fermentation broth of the desired fermentation products, and utilization of the byproducts. Although the process steps are described separately, the steps may be integrated to optimize production performance. (Qureshi et al. 2008a and 2008b).

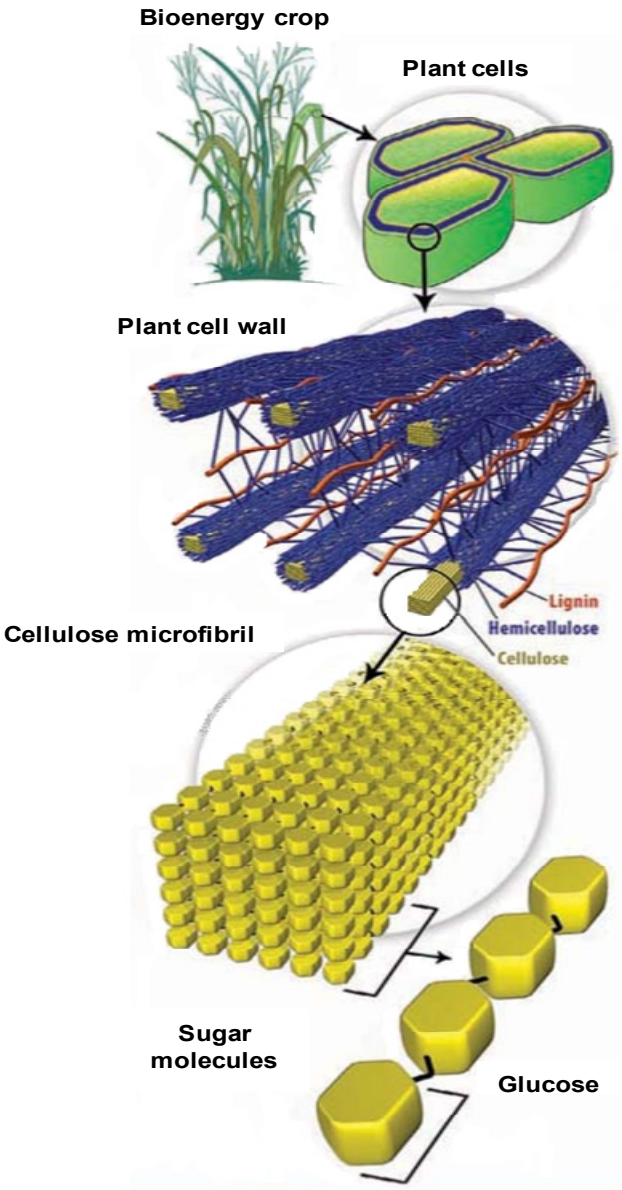


Figure 2.1 Agriculture crop composed of three major constituents: cellulose, hemicellulose and lignin (Wyman and Yang 2009)

2.3 Pretreatment and Hydrolysis

The purpose of pretreatment is to break the lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials (Sun 2002). Due to the recalcitrant nature of these lignocellulosic feedstocks, their pretreatment often requires a combination of physical, chemical, and heat treatments to disrupt the structure and convert it into a more hydrolysable form. However, the complete depolymerization of this renewable feedstock in a cost-effective manner with minimal formation of degradation products represents a significant challenge for microbiologists and chemical engineers. However, during pretreatment and hydrolysis, a complex mixture of microbial inhibitors is generated (Ezeji et al. 2007b). Various pretreatment methods such as the use of dilute acid (Purwadi et al. 2004), hot water controlled pH (Mosier et al. 2005), and ammonia fiber expansion (AFEX) are now available to solubilize and depolymerize biomass. Dilute acid pretreatment methods generate significant microbial inhibitors, while hot water and AFEX pretreatment methods generate only low concentrations of inhibitors (Bruce et al. 2008, Teymouri et al. 2005). Enzymatic hydrolysis is the most favorable hydrolysis techniques being used due to the low cost compared to acid or alkaline hydrolysis since enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45 to 50°C) and does not have a corrosion problem. During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ABE (Dien et al. 2008). Cellulases are usually a mixture of several enzymes which are highly specific. At least three major groups of cellulases are involved in enzymatic hydrolysis process: 1) Enzymes which attack regions of low crystallinity in the cellulose fiber, creating free-chain ends, 2) Enzymes which

degrades the molecule further by removing cellobiose units from the free-chain ends, 3) Enzymes which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl 1988). Table 2.1 summarizes the sugar composition of typical agriculture residues using sulfuric acid and enzymatic hydrolysis pretreatment.

Table 2.1 Sugar content of agriculture residues

	WS		**CF**		**DDGS**		**Molasses**	
Sugars	Actual g/l	wt/wt %	Actual g/l	wt/wt %	Actual g/l	wt/wt %	Actual g/l	wt/wt %
Glucose	28.9	**48.0**	37.2	**53.4**	23.6	**44.9**	14.0	**25.0**
Xylose	20.1	**33.4**	17.6	**25.3**	16.7	**31.7**	0.0	**0.0**
Arabinose	5	**8.3**	11.3	**16.2**	10.3	**19.6**	0.0	**0.0**
Galactose	3.5	**5.8**	3.6	**5.1**	1.2	**2.3**	0.0	**0.0**
Mannose	2.7	**4.5**	0.0	**0.0**	0.8	**1.5**	0.0	**0.0**
Sucrose	0	**0.0**	0.0	**0.0**	0	**0.0**	28.0	**50.0**
Fructose	0	**0.0**	0.0	**0.0**	0	**0.0**	14.0	**25.0**
Total sugar	60.2	**100%**	69.6	**100%**	52.6	**100%**	56.0	**100**
References	Qureshi et al. 2007a		Qureshi et al.2008a		Ezeji & Blaschek 2008		Shin et al. 1983	

WS: Wheat straw, CF: Corn Fiber, DDGS: Dry Distiller Grain and Solubles

As shown in Table 2.1, various sugar compositions are available in agriculture residues at different sugar content. This would affect the metabolism of clostridia organism and yield different fermentation products. Table 2.2 review the research work done utilizing the agriculture residues as carbon source for butanol fermentation processes. Control experiment using glucose was also listed for base of comparison. In order to better understand the effect of various sugar compositions on fermentation process, the metabolism of clostridia would be introduced in the following section.

Table 2.2 Properties comparison of most used agriculture residues on production of butanol by fermentation

Feed Stock Type[a]	**WS**	**WS + glucose**	**Corn Fibers**	**DDGS**	**Glucose (control)**	
ABE concentration, g/l	21.4	28.0	9.3	8.1	20.1	15
A:B:E ratio[b]	20.4:45.5:1	11.9:12: 1	3:6:1	13:26:1	11:21:1	33:61:1
ABE Productivity, g/l.hr	0.31	0.63	0.10	0.34	0.28	0.27
ABE Yield	0.41	0.42	0.39	0.48	0.41	0.25
Initial Sugar, g/ l	62.1	93.1	25.0	52.6	62.0	50
Sugar utilized, g/l	52.0	68.0	23.6	16.5	49.0	-
Mico-organism (Strain)	*C. beijerinckii* P260	*C. beijerinckii* P260	*C. beijerinckii* P260	*C. beijerinckii* P260	*C. beijerinckii* P260	*C. beijerinckii* NRRL B592
pH	5.0	6.5	6.8	4.8	6.5	4.7-5
Time, hr	72	72	72	72	72	1,600
Temperature,˚C	35	35	35	35	35	34
Pretreatment type	Acid + enzyme hydrolysis	Acid + enzyme hydrolysis	Acid + enzyme hydrolysis	Acid + enzyme hydrolysis	Not needed	Not needed
Process type	Batch	Batch	Batch	Batch	Batch	2 stages Continuous
ABE recovery technique	Gas stripping	Gas stripping	None	None	Gas stripping	Gas stripping
Reference:	Qureshi et al. 2008	Qureshi et al. 2007a	Qureshi et al. 2007a,b	Ezeji & Blaschek 2008	Qureshi et al. 2007a	Mutschlec-hner et al. 2000

[a] feedstock used is actual agricultural waste

2.4 Clostridia and Fermentation Metabolism of Butanol

Butanol (as well as acetone, ethanol, and isopropanol) are naturally formed by a number of clostridium strains. In addition, clostridia can produce chiral products which are

difficult to make by chemical synthesis (Rogers 1986) and degrade a number of toxic chemicals (Francis et al. 1994, Spain 1995). Clostridia are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes. Solventogenic clostridia can utilize a large variety of substrates from monosaccharides which include hexose and pentose, to polysaccharides (Jones and Woods 1986). Complex nitrogen sources such as yeast extract are generally required for good growth and solvent production, but otherwise the nutrient requirements for the growth of clostridia are rather simple (Monot et al. 1982). Among many solventogenic clostridia, *C. acetobutylicum*, *C. beijerinckii*, and *C. saccharobutylicum* are primary solvent producers (Durre 2005 & Sang et al. 2008). A typical feature of the clostridial solvent production is biphasic fermentation via Embden-Meyerhoff Pathway (Bowles and Ellefson 1985). The first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. This acidogenic phase usually occurs during the exponential growth phase (Andersch et al. 1983, Hartmanis and Gatenbeck 1984). The second phase is the solventogenic phase during which acids are reassimilated and used in the production of acetone, butanol and ethanol (or isopropanol instead of acetone in some *C. beijerinckii* strains) (Ezeji et al. 2003). Although the transition mechanism to switch acetogenic to solvogenic phases has been investigated, however not been determined yet (Qureshi 2008). Durre P (2005) explained the transition as a result of changes in fermentation conditions (such as pH). Whereas, recent research studies related the transition as a result of dramatic change in gene expression pattern (Durre 1987, Ezeji et al. 2007b). It is important to denote here that metabolic engineering studies showed the transcription factor (CoA) which is responsible for solvent production, is also

responsible on initiating the sporulation of *C. acetobutylicum* which makes solventogenesis closely coupled to sporulation (Bahl et al. 1995, Paredes et al. 2005). Figure 2.2 illustrate the primary fermentation metabolism of biomass by solventogenic clostridia. Simple organic end products are formed from the anaerobic dissimilation of glucose (or other carbon sources). Energy in the form of adenosine triphosphate (ATP) is generated through the dehydrogenation reactions that occur as glucose is broken down enzymatically. The simple organic end products formed from this incomplete biologic oxidation process also serve as final electron and hydrogen acceptors. On reduction, these organic end products are secreted into the medium as waste metabolites (usually alcohol or acid). The organic substrate compounds are incompletely oxidized by bacteria, yet yield sufficient energy for microbial growth (Medical Microbiology 1996). The presence of ferrodoxin (Pyruvate to Actyl-CoA reaction) is common among the solventogenic clostridia and the direction of electron flow around reduced ferrodoxin could have a crucial impact on the type and quantity of fermentation products produced. In addition, the ability of the solventogenic clostridia to grow under a low redox potential enables them to undertake a variety of stereospecific reductions, yielding chiral products that are difficult to synthesize chemically (Tomas el al. 2005). As the electron flow can be reversed, butanol yield should respond to factors that influence the direction of electron flow. This observation has caused researchers to test the effect of numerous reducing compounds, such as carbon monoxide gassing, addition of methyl viologen, and the addition of neutral red into the fermentation medium during the ABE fermentation. In the presence of these electron carriers, butanol and ethanol formation were stimulated at the expense of acetone synthesis (Mitchell 1998). In addition, a completely different product,

1,3-propanediol, was synthesized by *C. acetobutylicum* when glycerol, which is a more reduced substrate than glucose, was used as the carbon source (Gonzalez-Pajuelo 2006). Summary of desirable metabolic engineering alterations and the potential impact on bioprocessing is given in Table 2.2.

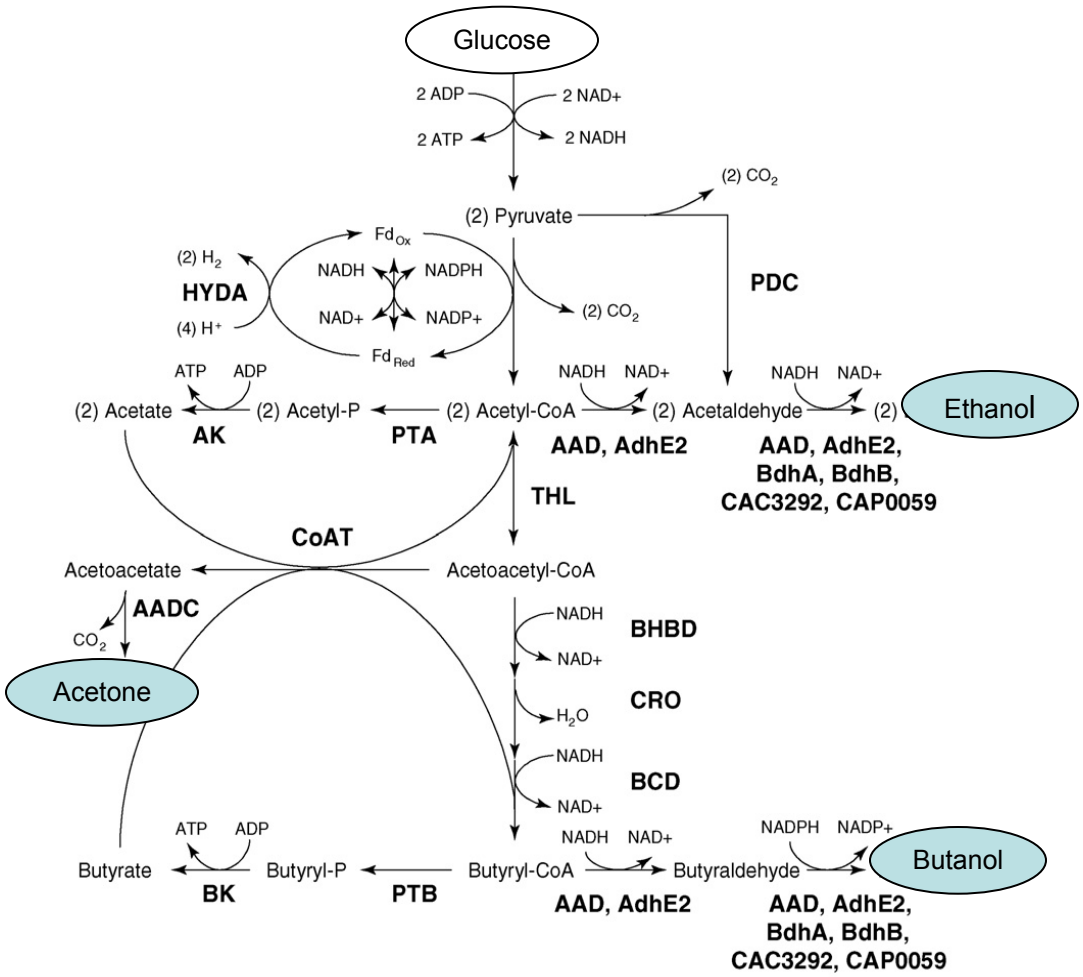


Figure 2.2 Primary metabolism of biomass by solventogenic clostridia involving different enzymes. These are: phosphotransacetylase (PTA), acetate kinase (AK), thiolase (THL), b-hydroxybutyryl dehydrogenase (BHBD), crotonase (CRO), butyryl-CoA dehydrogenase (BCD), CoA Transferase (CoAT), acetoacetate decarboxylase (AADC), butyrate kinase (BK), phophotransbutyrylase (PTB), alcohol/aldehyde dehydrogenase (AAD), hydrogenase (HYDA), pyruvate decarboxylase (PDC).(Papoutsakis 2008, Ezeji et al. 2007b, Hartmanis and Gatenbeck 1984).

Table 2.3 Summary of desirable metabolic engineering alterations of solventogenic clostridia and the potential impact on bioprocessing (Mitchell 1998, Papoutsakis 2008)

Strain or trait	Likely Impact
Tolerate Oxygen and possibly carrying out some aerobic metabolism	Simplify bioprocessing owing to less strict requirements for anaerobics; aerobic metabolism could enable higher cell densities without accumulating higher acid levels
Grow to much higher cell densities	Better volumetric productivity and faster completion of fermentation
Prolonged cell viability	Enhanced volumetric and cell specific productivity; prolonged productive fermentation; possibility of multi-cycle fed-batch fermentation
Direct utilization of cellulosic	Decreased substrate costs; possibly higher butanol tolerance owing to the impact of solute and insoluble macromolecular carbohydrates
Asporognous solvent-production strain	Improved specific cell productivity and volumetric productivity; ability to use continuous or semi-continuous bioprocessing
Solvent that do not degenerate (degeneration leads to reduced or no solvent production)	Ability to carry out multi-cycle fed-batch or continuous fermentations
Solvent tolerance	Higher solvents titers; improved cell-specific and volumetric productivity
Improved butanol selectivity (butanol becomes the sole or major solvent produced)	Better butanol yield per unit carbon substrate; simplified downstream processing; no undesirable side products
Inducing additional redox potential	Results in increased butanol and ethanol formation with reduced acetone formation
Purging with electron carrier gas	Improve butanol yield per unit carbon substrate: influence the direction of electron flow to stimulate butanol formation

The following equations represent the overall metabolism reactions of bio-base butanol fermentation (Ye and Zhihao 2009, Sun 2008):

$$\underset{\textbf{Cellulose}}{(C_6H_{10}O_5)_{12}} + 12\ H_2O \xrightarrow{\text{Hydrolysis}} \underset{\textbf{Glucose}}{12\ C_6H_{12}O_6} \tag{2.1}$$

$$\underset{\textbf{Glucose}}{12\ C_6H_{12}O_6} \xrightarrow{\text{Fermentation}} \underset{\textbf{n-Butanol}}{6\ C_4H_{10}O} + \underset{\textbf{Acetone}}{4\ C_3H_6O} + \underset{\textbf{Ethanol}}{4\ C_2H_6O} + 16\ H_2 + 28\ CO_2 + 2\ H_2O \tag{2.2}$$

The micro-organism used for butanol production will determine the maximum possible yields of butanol from available sugar. The mass-based theoretical yield of butanol from stoichiometry of glucose conversion shows 0.41 g butanol/g glucose based on the formation of one mole butanol from every mole of glucose consumed. But this yield is not practically achievable, since other than butanol is being produced in the biological process of fermentation, Equation 2.2. However, some micro-organism strains could come remarkably close. Mutant strains, normally generated by random mutagenesis (Sang et al. 2008) showed to produce higher yield and higher tolerance to butanol toxicity than wild strains (Soni & Jane 1997, Formanek et al. 1997).

Table 2.4 summarizes the fermentation micro-organisms and related maximum butanol obtained.

Table 2.4 Butanol fermentation micro-organisms and maximum butanol production.

Micro-organism	Solvents Yield	Sugar utilized g/l[a]	Sugar substrate/l	Process	Reference
C. acetobutylicum ATCC 4259	0.41[a] mol/mol	-	31g-Glucose Glycerol mix	Continuous (chemostat)	Andrade & Vasc.2003
	0.349 [a]	64.7	80g-starch	Continuous	Soni & Jane 1997
C. acetobutylicum ATCC 824	0.32[a] mol/mol	-	29g-Glucose Glycerol mix.	Continuous (chemostat)	Andrade & Vasc.2003
	0.354 [a]	52.0	60g-GXMA (5:4:2:1) mix.	Batch	Ezeji & Blaschek 2008
C. saccharobutylicum P262, (originally called *acetobutylicum*)	0.3 [a]	44.5	60g-GXMA (5:4:2:1) mix.	Batch	Ezeji & Blaschek 2008
C. acetobutylicum E-604 (mutant of *acetobutylicum* 4259)	0.36[a]	72.4	80g-starch	Continuous	Soni & Jane 1997
C. beijerinckii NCIMB 8052 (originally called *acetobutylicum*)	0.29[a]	16.0	22g-Glucose	Batch	Gottschal & Morris 1981
C. bejerinckii BA101 (mutant of *beijerinckii NCIMB 8052*)	0.39	45.4	60g-Glucose	Batch	Ezeji et al. 2004, Formanek et al. 1997
	0.47	500	100+426g-Glucose supply	Fed-Batch	Ezeji et al. 2004
	0.353 [a]	51.0	60g-GXMA (5:4:2:1) mix.	Batch	Ezeji & Blaschek 2008
C. bejerinckii P260	0.28	48.9	62g-Glucose	Batch	Qureshi et al 2007a
	0.42	67.1[a]	60g-WSH +35Glucose	Batch	Qureshi et al 2007a
	0.37[a]	54.8	60g-GXMA (5:4:2:1) mix.	Batch	Ezeji & Blaschek 2008
C. *butylicum* 592	0.355 [a]	55.5	60g-GXMA (5:4:2:1) mix.	Batch	Ezeji & Blaschek 2008
C. beijerinckii 55025	0.28	34.5	50g-Glucose	Batch	Liu et al. 2010

[a] calculated values
GXMA: is a blend of Glucose, Xylose, Mannose and Arabinose

2.5 Butanol Production Process Configurations

Biofuel fermentation is anaerobic process whereas the anaerobic mico-organism tends to preserve significant amount of the original substrate's energy. Anaerobic metabolism of sugars provides much less energy to the organism than aerobic metabolism (Antoni et al. 2007). This is a boon for biotechnology since an anaerobic organism would need to consume about ten times more substrate to obtain the same amount of energy as an aerobic organism, and by so doing produces far more fermentation products. Depending on the process configuration, fermentation may occur separately after hydrolysis, or simultaneously accomplished with hydrolysis (i.e. continuous scarification) (Saha et al. 2005, Qureshi et al 2008a). Batch fermentations are often preferred in the bioindustry due to simple operation and reduced risk of contamination. However, the productivity achievable in a batch reactor is low due to the lag phase, product inhibition as well as down time for cleaning, sterilizing, and filling. The preparation time and lag phase can be eliminated using continuous culture and the problem of product inhibition can be solved using an *in situ* product removal system (Ying 2003). It should be noted that single stage continuous fermentation is not feasible due to the complexity of butanol production in *clostridia*. To avoid substrate inhibition and to increase cell mass, fedbatch fermentation has been applied to the butanol production (Sang 2008). Fed-batch fermentation is an industrial technique where the reactor is started with a relatively low substrate concentration (to reduce substrate inhibition) and a low volume. As the substrate is consumed, it is replaced by adding a concentrated substrate solution at a low rate while keeping the substrate concentration in the reactor below the toxic level (Qureshi and Blaschek 2001, Qureshi and Maddox 1990, Qureshi and Blaschek 2000). Fed-batch

fermentation is advantageous in cases where an initial high substrate concentration is toxic to the culture. However, when applied to systems such as butanol, which is toxic, a product-removal technique should be applied in combination with the fed-batch fermentation. By feeding the reactor at a slow and controlled rate, substrate toxicity can be kept below inhibitory levels, while the product-removal technique can be applied simultaneously to remove butanol toxicity. Thus, application of these two engineering techniques solves two toxicity problems: one for substrate inhibition and another for butanol inhibition (Ezeji et al. 2004).

Immobilized cell bioreactors and cell recycle reactors have also been applied to butanol production, in order to increase productivity. *C. beijerinckii BA101* was immobilized onto clay brick particles and the fermentation was performed for ABE production (Qureshi et al. 2005, Qureshi and Maddox 1988). As expected, the yield and concentration increased with lower dilution rate. The process suffered from the fact that only a fraction of the biomass was in the solventogenic state and a significant amount of biomass was present as inactive biomass, spores (Qureshi et al. 2004). Therefore, it is suggested that sporulation should be blocked to achieve higher productivity. Nevertheless, immobilized cell continuous reactor is a strong candidate for an industrial fermentation process. Membrane cell recycle bioreactors are another option to improve productivity. A hollow-fiber ultrafilter was applied to separate and recycle cells in a continuous fermentation (Pierrot et al. 1986, Huang et al. 2004). However, fouling of the membrane with the fermentation broth was a major obstacle of this system. Lipnizki et al. (2000) suggested a way to overcome this problem by allowing only the fermentation broth to undergo filtration by using the immobilized cell system. To enhance the performance of

fermentation micro-organism, continuous product recovery is requested in the various process configurations.

2.6 Product Recovery

High product recovery cost is another problem in biological butanol production. Besides the traditional distillation process, several other processes including pervaporation, adsorption, liquid–liquid extraction, gas stripping, and reverse osmosis have been developed to improve recovery performance and reduce costs (Qureshi and Blaschek 1999, Qureshi et al. 1992). The traditional recovery process employing distillation suffers from a high operation cost due to the low concentration of butanol in the fermentation broth. To solve this problem and the solvent toxicity problem at the same time, *in situ* recovery systems have been employed. From an economic point of view, reverse osmosis is most preferable. However, it has disadvantages of membrane clogging or fouling. In contrast, liquid–liquid extraction has high capacity and selectivity, although it can be expensive to perform (Durre 1998, Groot at al. 1990). Thus, there are advantages and disadvantages of using each recovery system, which need to be thoroughly examined. Gas stripping is a simple but efficient way to recover butanol from the fermentation broth. The fermentation gas is bubbled through the fermentation broth then passed through a condenser for solvent recovery. The stripped gas is then recycled back to the fermentor and the process continues until all the sugar in the fermentor is utilized. Gas stripping enables the use of a concentrated sugar solution in the fermentor (Qureshi and Blaschek 2001) and a reduction in butanol inhibition and high sugar utilization (Maddox

et al. 1995). Table 2.5 shows a comparison between various process configurations on total solvent production using glucose as substrate.

Table 2.5 Comparison of ABE production at various process configurations

Fermentation Process	Micro-organism	Glucose utilized g/l	ABE conc. g/l	ABE Yield	Reference
Batch (control)	*C. beijerinckii* BA101	45	17.6	0.39	Ezeji et al. 2004
Continuous immobilized reactor	*C. beijerinckii* BA101	-	7.9	0.38	Qureshi et al. 2000
Continuous membrane cell recycle bioreactor	*C. acetobutylicum*	20 g/l of cell mass	13.0	0.35	Pierrot et al. 1986
Product recovery by gas stripping:					
Batch	*C. beijerinckii* BA101	161	75.9	0.47	Ezeji et al. 2003
Fed-batch	*C. beijerinckii* BA101	500	233	0.47	Ezeji et al. 2004
Continuous	*C. beijerinckii* BA101	1163	460	0.4	Ezeji et al. 2005b
Product recovery by pervaporation:					
Batch	*C. acetobutylicum*	78	32.8	0.42	Evans & Wang 1988
Fed-batch	*C. acetobutylicum* 824	384	155	0.43	Groot et al. 1984
Product recovery by liquid-liquid Extraction:					
Continuous membrane bioreactor	*C. acetobutylicum*	7 g/l of cell mass	14.5	0.3	Eckert & Schugerl 1987

In this study, anaerobic batch fermentations experiments were conducted using *C. acetobutylicum* as well as *C. beijerinckii* as micro-organism for fermentation of butanol. No *in situ* recovery was implemented but instead, low sugar concentration was used to prevent accumulation of butanol product to more than 16g/l which is the lethal value for Clostridium (Ezeji et al. 2007b). Next chapter would explain fermentation set up and experimental work in details.

Chapter 3
Experimental Work

This chapter describes the material and methods used in conducting fermentation experiments for butanol production as well as the sampling and analysis techniques. Software packages used in optimizing the sugar composition of feedstock and in developing the empirical model for butanol production are also described in this chapter.

3.1 Preparation of Micro-organisms and Culture Conditions

Two micro-organism strains were used in the present study. These are *Clostridium acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101. Strains were purchased from American Type Culture Collection (ATCC), and unless otherwise specified all chemicals were purchased from Sigma-Aldrich/Canada. Stock cultures were maintained as suspensions in double distilled water at 5˚C. Whenever needed, culture inoculum was prepared using 5 vol% stock culture in culture medium grown anaerobically inside incubator (VWR, Canada) for 12-18 hrs at 35˚C.

Two inoculum media were tested for best growth of *C. acetobutylicum* ATCC 4259: Rainforest media recommended by ATCC (composition details are given in Appendix A), and Andrade & Vasconcelos inoculum medium (Andrade & Vasconcelos 2003). *C. acetobutylicum* strain visually tested to be growing within 35 hrs in Rainforest medium, while it took more than 50 hrs to visualize the growth in Andrade & Vasconcelos inoculum medium. Thus, Rainforest medium was chosen throughout this study to culture *C. acetobutylicum* ATCC 4259.

For *C. beijerinckii* BA101, Cooked Meat Medium (CMM- Sigma Aldrich) was used as recommended from the literature (Qureshi et al. 2008b).

3.2 Fermentation Media

Two fermentation media were used throughout this study. These are the Andrade & Vasconcelos medium (Andrade & Vasconcelos 2003) used in continuous fermentation by *C. acetobutylicum* and the P2 medium used for batch fermentation using C. *beijerinckii* BA101 (Ezeji et al. 2007a, Qureshi and Blaschek 1999). The composition of the two fermentation media is given in Table 3.1.

Table 3.1 Composition of butanol fermentation media used in this study

Andrade & Vasconcelos medium	**P2 medium**
Carbon source: 15 or 30 g/l • Glucose • Other agriculture sugars Nitrogen source • Yeast extract 1 g/l • Nitrogen gas Vitamins • p-aminobenzoic acid 8 mg/l • Biotin 0.04 mg/l • $COCl_2.6H_2O$ 0.01 g/l Buffers[a] • KH_2PO_4 0.5-5.4 g/l • K_2HPO_4 0.5-8.45 g/l • H_2SO_4 9.7 M, 0.1 ml Minerals • $MgSO_4.7H_2O$ 0.2 g/l • $FeSO_4.7H_2O$ 0.01 g/l • NH_4Cl 1.5 g/l pH 6.6 ± 0.1	Carbon source: 15 or 30 g/l • Glucose • Other agriculture sugars Nitrogen source • Yeast extract 1 g/l • Nitrogen gas Vitamins • p-aminobenzoic acid 1 mg/l • Biotin 0.01 mg/l • Thiamin 1 mg/l Buffers • KH_2PO_4 0.5 g/l • K_2HPO_4 0.5 g/l • Ammonium acetate 2.2 g/l Minerals • $MgSO_4.7H_2O$ 0.2 g/l • $MnSO_4.7H_2O$ 0.01 g/l • $FeSO_4.7H_2O$ 0.01 g/l • NaCl 0.01 g/l pH 6.6 ± 0.1

[a] four different buffer contents were tested

3.3 Batch Fermentation Experiments

Fermentation experiments were done using 175ml Wheaton serum bottles containing 150 ml medium. The procedure for anaerobic batch fermentation of butanol is illustrated in Figure 3.1.

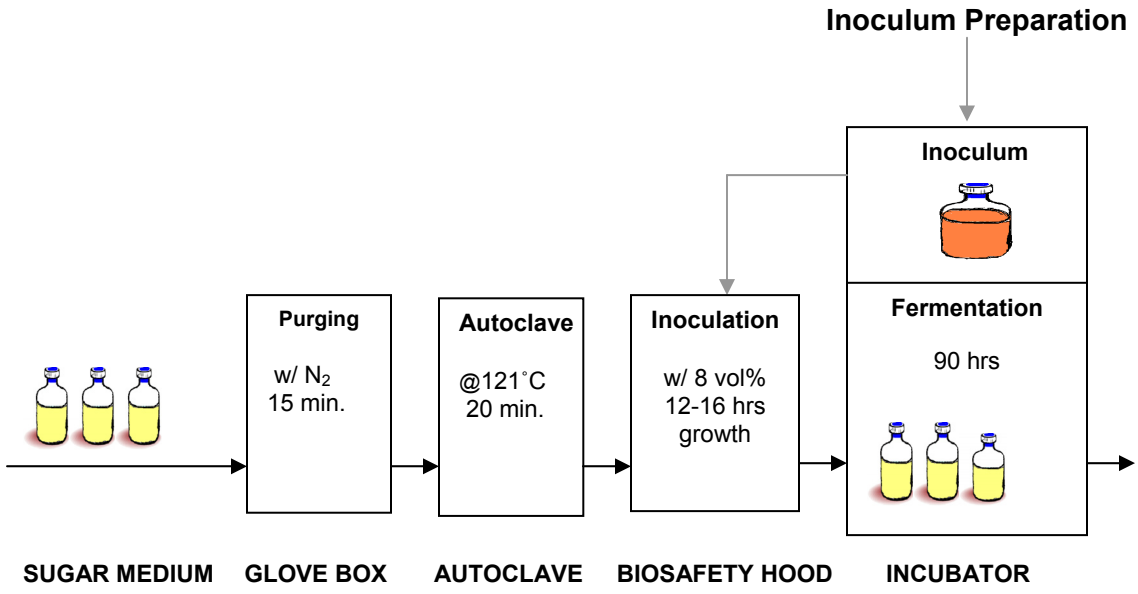


Figure 3.1 Procedure for anaerobic batch fermentation of butanol

As seen in this figure, pure sugars and stock solutions of vitamins, buffers, and minerals were first prepared into the serum bottles prior to purging with Nitrogen gas (99.9 % purity, Linde, Canada) at 14.7 psig for 15 minutes to insure anaerobic medium condition. Also 0.5g/l L-Cysteine HCL and 0.01g/L Rasazarin salt were added to the bottles using an anaerobic glove box (Terra Universal, Canada) to confirm an oxygen free medium. Following that, serum bottles containing the medium were crimped with rubber caps inside the anaerobic glove box prior to autoclaving at 121˚C for 20 minutes using a VWR (Canada) autoclave. The serum bottles were placed inside a biosafety hood (LABGUARD Class II-type A2, Canada) to bring down to room temperature. Before

inoculation, the serum bottles were placed inside the incubator (VWR-Canada) for an hour to reach fermentation temperature of 35˚C. Inoculation was made from one culture inoculum bottle at 8 vol% of 12-16 hrs age already prepared inoculum by inserting a sterilized syringe through the bottles rubber cap, inside the biosafety hood. Rubber cap was sterilized by an ethanol spray before and after each syringe insertion. Then, the inoculated serum bottles were placed in the incubator for 90 hrs until the end of fermentation time. No agitation was used as the agitation would negatively affect culture cell growth. The incubator temperature was set at 35 ˚C as an average temperature between the optimum cell growth of 37˚C (Fan et. al 1987, Shin et al. 1983), and optimum acids uptake of 30˚C (Soni and Jain 1997). This fermentation temperature was been used for other butanol production strains successfully (Ezeji et. al 2007a, Andrade and Vasconcelos 2003).

Various parameters were investigated through fermentation experiments. These include two initial sugar concentrations at two inocolum volumes, four different buffer contents, and various types of agriculture sugars (25g/l either of glucose, xylose, arabinose, galactose, and mannose added to base of 6 g/l glucose). The role of fermentation gases were also examined by running two experiments at which the fermentation gases were released every 24 hrs in one experiment, while kept until the end of fermentation in the second experiments. Testing the pH of fermentation broth (bacterial extracellular pH) was used as good prediction for the bacteria intracellular acidity level since the intracellular pH is highly related to the extracellular pH and recorded to be greater than the extracellular pH by only 0.22 units over pH range of this study (Bowles and Ellefson 1985).

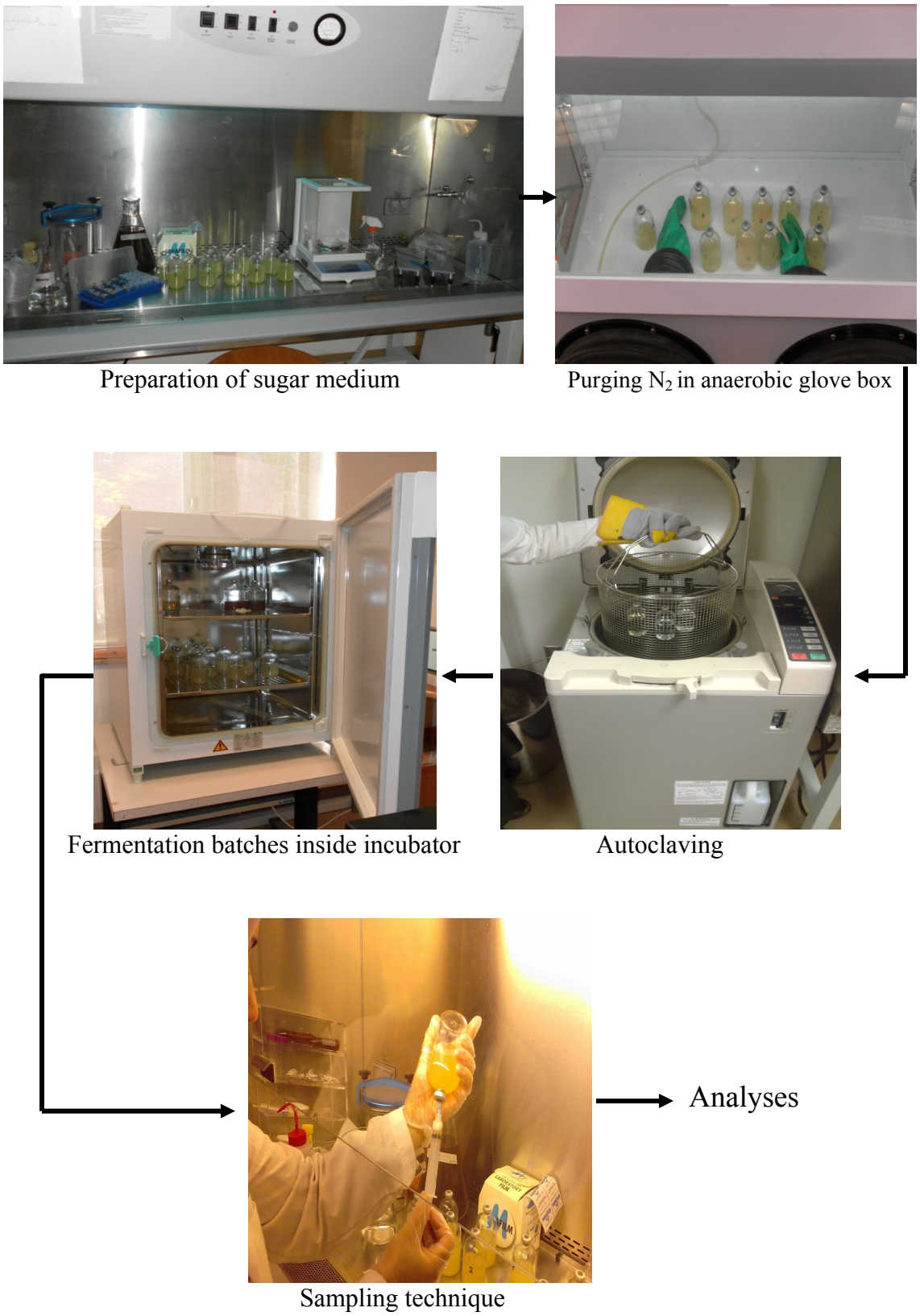


Figure 3.2 Experimental units for anaerobic batch fermentation of butanol

3.4 Sampling

For all fermentation experiments and culture inoculums, sampling was made inside the biosafety hood cleaned with ethanol and left in the UV light for 10 min. All other tools such as syringes and needles were washed with ethanol and left under the UV light for 10 minutes as well. The serum fermentation batches were taken out of incubator, placed inside biosafety hood and washed with ethanol prior to sampling. According to Ye and Zhihao (2009), 1.5 moles of hydrogen and 2.33 moles of carbone dioxide gases are expected to be produced for each mole of sugar consumed. Thus, a sampling method was designed to capture fermentation gases inside fermentation bottles until the end of fermentation. This was achieved by inserting a sterilized syringe-needle combination through the serum bottle's rubber stopper and sampling only the liquid phase of fermentation medium which contained the liquid products (solvents, acids, sugars) and the culture cells, while leaving the gases to continue their role in fermentation process. Four-milliliter samples were taken every 8 hrs and kept until analyzed in 2x 2-ml Eppendorf tubes at -80°C in ultra low freezer (Thermo, Canada).

3.5 Analytical Techniques

3.5.1 Flow Cytometer

The number of culture cells/ml in all media was measured using a flow cytometer (Guava EasyCyte Mini, Guava Technologies, US). Cell count was done by the application of forward scattering light on stream of culture cells, whereas the refractions of light on culture cells facilitated towards cell count reading. Guava EasyCyte Mini flow cytometer has a blue laser beam (75 mW) of a single wavelength (532 nm) apply on stream of

diluted culture flowing at 5 µl/sec. Three detectors are available with a band pass filter ratio of 525/30 nm for Green, 583/26 nm for Yellow, and (690/50) for Red. These are aimed at the point where the flow stream passes through the light beam: one in line with the light beam (Forward Scatter, FSC) provides information on number and relative size of bacterial culture. The other two detectors are perpendicular to it (Side Scatter, SSC), and they provide information about the cellular granularity and complexity.

Figure 3.3 Flow Cytometer Guava EasyCyte Mini

Prior to running sample in flow cytometer, cleaning procedure was performed according to the manufacturer's guidelines using the Guava cleaning solution. Then 5 µl of the culture broth were diluted with 95 µl distilled water (i.e. 20 fold). The diluted sample was vortexed using VWR vortex, Canada for 30 sec to uniformly distribute the culture in testing sample and preventing bacterial cells to adhere together. Thereafter, sample was fed into the flow cytometer using automated feeding system, and the cell count readings was obtained in excel files after been analyzed by the Guava® ExpressPluss software module (built-in software).

3.5.2 High Performance Liquid Chromatograph (HPLC)

Quantitative analysis for butanol, acids and sugar concentrations was done using a High Performance Liquid Chromatograph (HPLC-Perkin Elmer), equipped with a gel column Aminex HPX-87H (300x 708 mm, Bio-Rad, Hercules, USA) and a refractive index detector (2414, Waters). Prior to testing, the samples and mobile phase were double filtered through 0.2 μm PTFE- filter (Whatman, USA). Sample was not centrifuged before filtering as the cell culture concentration was not high, and to eliminate solvent loss due to evaporation. One milliliter samples were injected using auto-sampling equipment (Series 200, Perkin Elmer). With a 5 mM H_2SO_4 mobile phase maintained at 0.6ml/min, the column temperature and pressure were maintained at 60°C and 1150 psi, respectively. Data were processed by the computer software (Turbochrom Navigator).

It was important to fill the HPLC testing vials to a minimum head space to reduce the loss of solvents in vapor phase. The reliability of HPLC column, and testing parameters was confirmed by running solvents, acids, and sugars standards in triplicates.

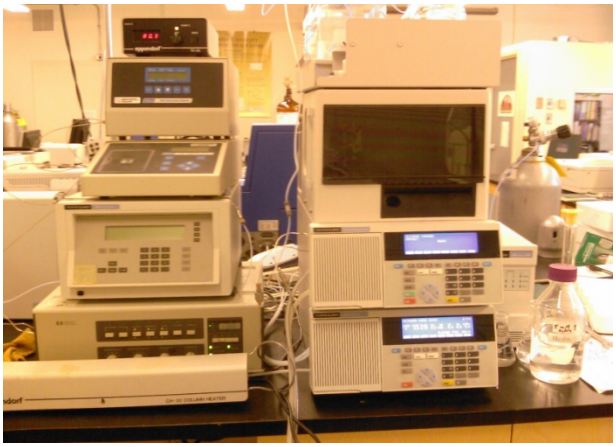


Figure 3.4 High Performance Liquid Chromatograph (HPLC)

3.5.3 Gas Chromatograph (GC)

The ratio of ABE (Butanol: Acetone: Ethanol) products at the end of fermentation was determined by a Gas Chromatograph (GC-Perkin Elmer XL Series). Two samples of fermentation media (100 µl each) were double filtered through 0.2 µm PTFE-filter (Whatman, USA) and diluted with distilled water prior to be injected in a Supelco Carboxen PLOT 1010 capillary column (30.53 m) followed by a thermal conductivity detector, with helium gas as carrier at 10 ml/minute and as a reference gas at 15 ml/minute. A head space auto sampler (Perkin Elmer) was used with heating to 60˚C to achieve proper peak separation.

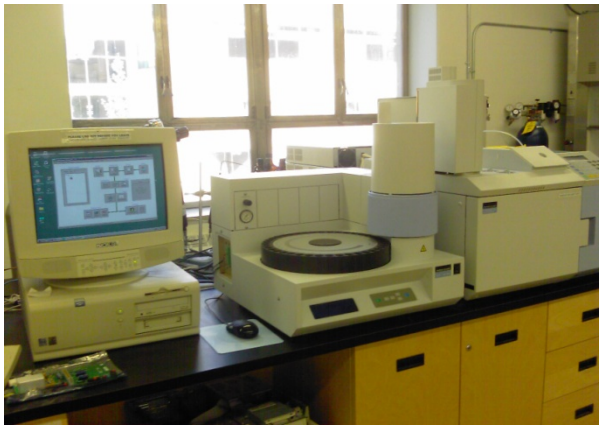


Figure 3.5 Gas Chromatograph (GC)

3.5.4 Inverted Microscope (IM)

Purity and the possibility of culture sporulation were examined using an Inverted Microscope (IM- ZEISS, Thornwood, NY). This was done by adding SYTO-9 fluorescent green DNA stain dye (Invitrogen, CA, USA) to 1ml culture in a 2-ml Eppendorf tube at a concentration of 0.025 mg/l. After leaving the stained culture for 10 minutes at room temperature without mixing, a 50-µl sample was taken and slide-mounted for microscopic examination.

3.6 Optimization of Sugar Composition

The objective of this part was to find the optimum sugar composition in feedstock resulted in maximum production of butanol. Knowing optimum composition, one will be able to utilize various types of agriculture wastes by adjusting the feedstock sugar composition rather than using mix culture (i.e. strains) at different optimum conditions, or applying multi stage process using different stains at their optimum conditions.

3.6.1 Factorial Design

Screening the sugar content in WS, CF and DDGS hydrolysates shows five types of sugars are available at various concentrations as illustrated in Table 3.2. By omitting galactose being the least favorable sugar towards butanol fermentation (Ezeji et. al 2007a), we applied full factorial design for four factors (n= 4 sugar types) at lower and upper concentration levels (k=2) given in Table 3.3.

Table 3.2 Sugar composition in control experiment, and in WS, CF and DDGS hydrolysates

	Control		**WS**		**CF**		**DDGS**	
Sugars	wt/wt %	Actual g/l	wt/wt %	Actual g/l	wt/wt %	Actual g/l	wt/wt %	Actual g/l
Glucose	**100**	30.0	**48.0**	14.4	**53.4**	16.0	**44.9**	13.5
Xylose	**0.0**	0.0	**33.4**	10.0	**25.3**	7.6	**31.7**	9.5
Arabinose	**0.0**	0.0	**8.3**	2.5	**16.2**	4.9	**19.6**	5.9
Mannose	**0.0**	0.0	**4.5**	1.4	**0.0**	0.0	**1.5**	0.5
Galactose	0.0	0.0	5.8	1.7	5.1	1.5	2.3	0.6
Total sugar	100%	30.0	100%	30.0	100%	30.0	100%	30.0
References	Qureshi et al. 2007a		Qureshi et al. 2007a		Qureshi et al.2008a		Ezeji & Blaschek 2008	

Table 3.3 Factors and levels for factorial design experiments

Factor (Sugar type)	**Lower level g/l (-1)**	**Upper level g/l (+1)**
Glucose	13.5	30.0
Xylose	0.0	10.0
Arabinose	0.0	5.9
Mannose	0.0	1.4

Using *C. beijerinckii* BA101, we ran k^n= 16 experiments in one replicate, plus 2 experiments at the center point for error analysis. All 18 experiments were run at once and at the optimum fermentation conditions presented later in Table 4.4. Factorial experiments design is shown in Table 3.4.

Table 3.4 Two levels four factors factorial design

Exp. No.	**Glucose**	**Xylose**	**Arabinose**	**Mannose**
1	-1	-1	-1	-1
2	+1	-1	-1	-1
3	-1	+1	-1	-1
4	+1	+1	-1	-1
5	-1	-1	+1	-1
6	+1	-1	+1	-1
7	-1	+1	+1	-1
8	+1	+1	+1	-1
9	-1	-1	-1	+1
10	+1	-1	-1	+1
11	-1	+1	-1	+1
12	+1	+1	-1	+1
13	-1	-1	+1	+1
14	+1	-1	+1	+1
15	-1	+1	+1	+1
16	+1	+1	+1	+1
17	0	0	0	0
18	0	0	0	0

3.6.2 MINITAB Software Package

MINITAB 15 statistical software was chosen to perform regression analysis. The multiple regression analysis helps in identifying the effect of four sugar types as independent variables (factors) on the dependent butanol concentration (as model respond). Confidence level at 95% probability was used as the minimum acceptable level for engineering analysis, thus α =1- 0.95= 0.05. The regression coefficients, β, were calculated by following equation (Montgomery and Runger, 2002):

$$\beta = \left(X'.X\right)^{-1} X'.y \qquad 3.1$$

$$X = \begin{bmatrix} X_{G1} & X_{X1} & X_{A1} & X_{M1} & X_{G1}X_{X1} & X_{G1}X_{A1} & X_{G1}X_{M1} & X_{X1}X_{A1} & X_{X1}X_{M1} & X_{A1}X_{M1} \\ X_{G2} & X_{X2} & X_{A2} & X_{M1} & X_{G2}X_{X2} & X_{G2}X_{A2} & X_{G2}X_{M2} & X_{X2}X_{A2} & X_{X2}X_{M2} & X_{A2}X_{M2} \\ X_{G3} & X_{X3} & X_{A3} & X_{M3} & X_{G3}X_{X3} & X_{G3}X_{A3} & X_{G3}X_{M3} & .. & .. & .. \\ X_{G4} & X_{X4} & X_{A4} & X_{M4} & .. & .. & .. & .. & .. & .. \\ X_{G5} & X_{X5} & X_{A5} & .. & .. & .. & .. & .. & .. & .. \\ X_{G6} & X_{X6} & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G7} & X_{X7} & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G8} & .. & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G9} & .. & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G10} & .. & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G11} & .. & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G12} & .. & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G13} & .. & .. & .. & .. & .. & .. & .. & .. & .. \\ X_{G14} & .. & .. & .. & .. & .. & .. & .. & .. & X_{AJ-2}X_{MJ-2} \\ X_{G15} & .. & .. & .. & .. & .. & .. & .. & X_{XJ-1}X_{MJ-1} & X_{AJ-1}X_{MJ-1} \\ X_{G16} & .. & .. & .. & .. & .. & .. & X_{XJ}X_{AJ} & X_{XJ}X_{MJ} & X_{AJ}X_{MJ} \end{bmatrix}$$

$$y' = \begin{bmatrix} y_1 & y_2 & y_3 & y_4 & y_5 & .. & .. & .. & y_{15} & y_{16} \end{bmatrix} \quad \text{Experimental design result}$$

$$\beta' = \begin{bmatrix} \beta_1 & \beta_2 & \beta_3 & \beta_4 & \beta_5 & .. & .. & .. & \beta_{15} & \beta_{16} \end{bmatrix} \quad \text{Regression coefficients}$$

To evaluate the effect of the factors on regression model and the analysis of ANOVA table (Montgomery and Runger 2002):

Let i=*G, X, A, M, GX, GA, GM, XA, XM, AM*

Then:

$$Contrast_i = \sum y_{i+} - \sum y_{i-}$$

y_{i+} and y_{i-} are the butanol concentrations for experimental design runs for each factor at the upper and lower levels, respectively

$$Effect_i = \frac{Contrast_i}{4n}$$

Sum of squares: $SS_i = \frac{(Contrast_i)^2}{n2^k}$

$$SS_R = \sum SS_i$$

$$SS_T = \sum y_i^2 - \frac{\left(\sum y_i\right)^2}{n2^k}$$

$$SS_E = SS_T - SS_R$$

Mean square: $MS_i = \frac{SS_i}{DF}$, DF=degrees of freedom

$$MS_R = \sum MS_i$$

F-test: $F = \frac{MS_R}{MS_E}$

Coefficient of multiple determinations: $R^2 = \frac{SS_R}{SS_T} = 1 - \frac{SS_E}{SS_T}$

3.6.3 Excel Optimization Solver Software

Excel optimization solver equipped with Widow XP 2003 has been used to find the optimum sugar composition in butanol regression model. Newton's method search (i.e. Newton's Raphson) was used as equivalent to quadratic model (Edgar et al. 2001). The objective function was the butanol regression model constructed by MINITAB package,

whereas constrains were represented by four sugar concentration limits, plus one total sugar content (30 g/l).

Mathematical formula for Newton's search is given in Equation 3.2.

$$X_i^{k+1} = X_i^k - \frac{f'(X^k)}{f''(X^k)} \qquad 3.2$$

When the function derivatives (*f'* and *f''*) for the butanol model are expressed by central finite difference approximation, equation 3.2 yields:

$$X_i^{k+1} = X^k - \frac{[y(X_i + h) - y(X_i - h)]/2h}{[y(X_i + h) - 2y(X_i) + y(X_i - h)]/h^2} \qquad 3.3$$

Where

X: sugar concentration, g/l

i= (G, X, A, M) denotes sugar type

k= no. of iterations

y=butanol concentration, predicted form butanol regression model constructed by Minitab software

f' , f'': are first and second derivatives of butanol regression model

h: step size, automatically selected by software to match the difference formula and computer precision in which the calculations were to be executed.

Chapter 4
Results and Discussion

4.1 Fermentation Conditions of *C. acetobutylicum*

Due to the lack of information about anaerobic batch fermentation of *C. acetobutylicum* ATCC 4259, investigating proper working conditions was challenging. The focus of this work was to characterize and define the optimum fermentation conditions of butanol production in anaerobic batch fermentation. Two fermentation media were tested for butanol production based on available information from the literatures (Ezeji et al 2007, Andrade & Vasconcelos 2003). These are P2 medium and Andrade & Vasconcelos medium. In a preliminary study to choose the medium of culturing, P2 medium produced almost no butanol compared to Andrade & Vasconcelos medium that produced 1.3 g/l butanol. Therefore, Andrade & Vasconcelos was used throughout this study as solvent production media with further adjustment in total buffer content.

4.1.1 Effect of Sugar Concentration and Inoculum Size

Table 4.1 summarizes results obtained for four experiments at two levels of initial sugar concentrations and two inoculum sizes. Examining products concentration at both 15g/l and 30g/l initial glucose levels reveals that either sugar concentration could be successfully used in batch experiments. Since there was no reduction in the products concentration or yield at 30g/l versus 15 g/l, one can assume that no limitation in initial sugar uptake was present up to 30 g/l. Although a 30 g/l initial glucose concentration did not result in higher butanol production, it will be used for future studies since it will allow variations in other factors (inoculum size and age, buffer content) to be evaluated.

Furthermore, 60g/l were found to be successful with a *C. beijerinckii* mutant (Qureshi et al. 2008b). However, introducing larger size of inoculum showed considerable effect on product formation. When more than three times as much cells were inoculated, the yield (g of fermentative product/ g of sugar utilized) was only doubled, proving that increasing inoculum size results in increasing butanol production but not proportional relation since the cells at high concentration would suffer from nutrition limitation. Examining the pH after 120 hr shows that all fermentation medium were highly acidic (3.60, 3.90, 3.62, 3.84) compared to 6.62- 6.60 at inoculation time. That may explain the low consumption of glucose possibly due to the presence of partially dead cells or as a result sporulation at extreme conditions where pH is less than 4.5 (Paredes et al. 2005).

Table 4.1 Effects of initial sugar and inoculum size on fermentation products (T=35˚C, initial pH of 6.6, t=120hrs)

Glucose Initial sugar concentration g/l		**Culture inoculum [a] size**	**Product concentration g/l**		**Product Yield**		**pH Final**
Initial	**Consumption**	**vol%**	**Butanol**	**Acids**	**Butanol**	**Acids**	
15	4.88	5	0.77	1.12	0.16	0.23	3.60
15	11.29	18	3.12	4.59	0.28	0.41	3.90
30	4.71	5	0.71	1.03	0.15	0.22	3.62
30	11.82	18	3.18	4.71	0.27	0.40	3.84

[a] culture inoculum age is less than 24 hrs

As a result of this experimental set, we decide on using 30g/l glucose as initial sugar throughout the study and an inoculum volume of 8 vol% as the mid range of 5-10 vol% used for Clostridia strains in other work studies (Shin et al. 1985, Andrade & Vasconcelos 2003, Qureshi et al. 2007).

4.1.2 Inoculum Age

Through experimental replicates (data not shown), we found that using inculum culture age of less than 24 hrs, or of 20 hrs as indicated in other laboratories (Fan et al. 1987, Shin et al. 1983), the concentration of butanol was quite variable (0-3 g/l). That raises the question of how sensitive a fermentation process is with respect to the culture inoculum age. No information on this matter could be found in the literature.

Figure 4.1 shows different bacterial cell growth curves constructed for three different inocula (A, B, and C) that were kept at different temperatures of 35˚C, 5˚C, and -80˚C, respectively. As shown in Figure 4.1, different timing of vegetative phases can be distinguished for the different inoculums examined. The 35˚C inoculum was in a vegetative phase from 11hrs to 19 hrs. However, the phase for 5˚C inoculum was from sometime above 11hrs to 21hrs, and the phase of the -80˚C frozen inoculum was from 19 hrs to 26 hrs. Knowing the fact that culture at only vegetative phase is able to produce solvents in fermentation process, this would lead to the conclusion of using 15-17 hrs age inoculum when culture was maintained at 35˚C, while using 16-18 hrs age inoculum when culture was maintained cold at 5˚C, and using 21-22 hrs age inoculum when culture was maintained frozen at -80˚C. The three samples reached identical cell count at the end of 30 hrs incubation. Culture medium was slightly acidic with pH of 5.5-5.3 compared to 6.5 initially. To insure strain activity status, we examined culture at the vegetative phase for inoculum maintained at 35˚C using phase contrast microscopy as shown in Figure 4.2. The figure shows the culture is spores free and bacterial strain to have a rod shape as been described in literatures for *C. acetobutylicum* type. The bacterial strain was found to be held through a scaffold as shown in Figure 4.2. Being spores free culture indicate that

the culture is in active status and no further activation treatment was needed prior to inoculation into fermentation experiments.

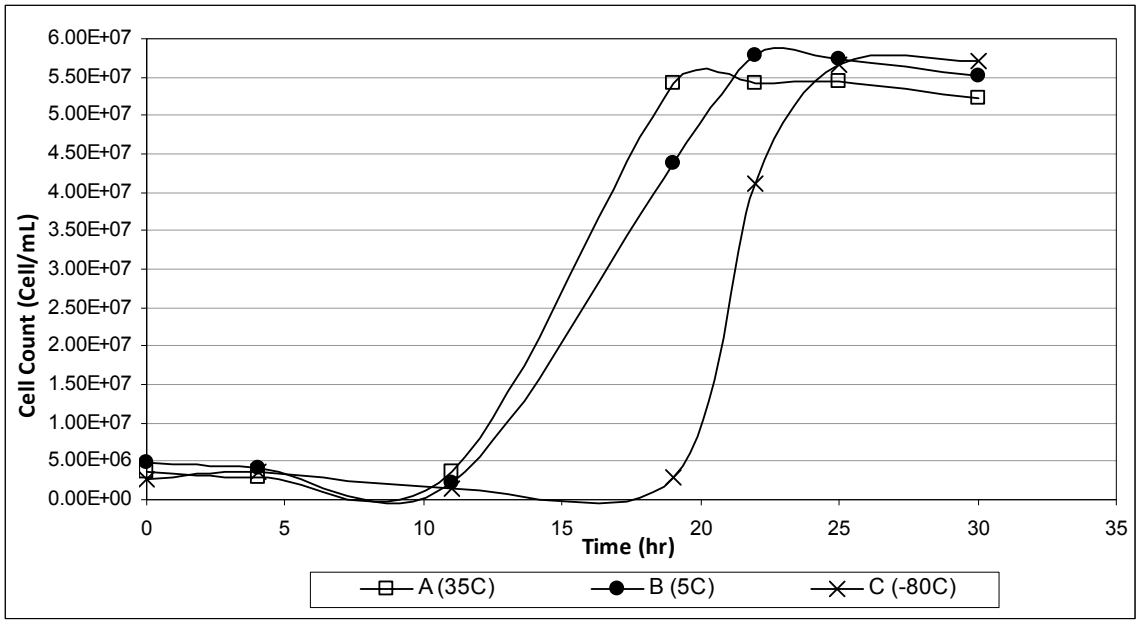


Figure 4.1 Growth of *C. acetobutylicum* ATCC 4259 at 35˚C in Rainforest medium inoculated from cultures stored at three temperatures.
Vegetative phase: **12-14 hr** @ 35˚C, **14-16 hr** @ 5˚C**, 21-23 hr** @ -80˚C

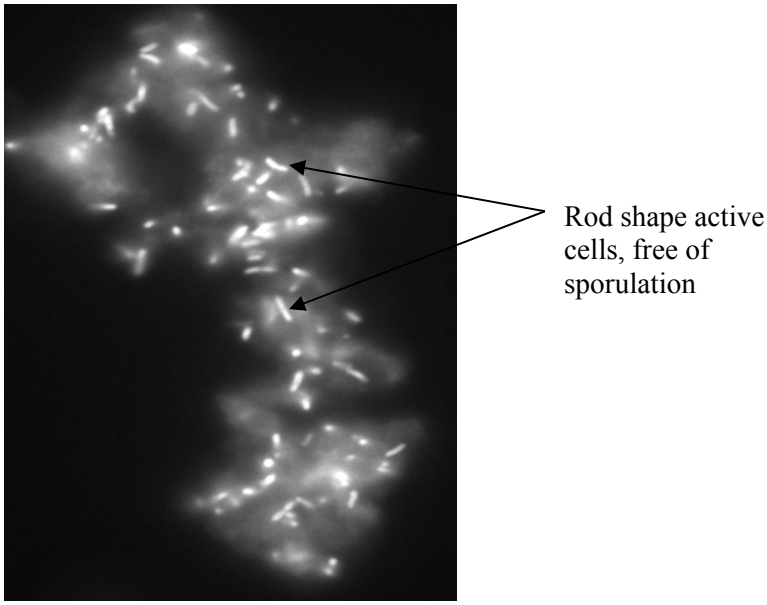


Figure 4.2 IM image of *C. acetobutylicum* ATCC 4259 at vegetative phase

4.1.3 Role of Fermentation Gases

In order to confirm the role of fermentation gases on the type and amount of final products, one fermentation procedure allowed gases to be released every 24 hrs while the gases were kept inside the flasks until the end of incubation in another one. Summary of observations is shown in Table 4.2.

Table 4.2 Effects of fermentation gases on fermentation products
(T=35˚C, initial pH of 6.6, 8 vol% inoculum)

Fermentation gases released every 24hrs	**Initial glucose g/l**	**Conc. g/l @24hrs**		**Conc. g/l @48hrs**		**Conc. g/l @90hrs**	
		Butanol	Acids	Butanol	Acids	Butanol	Acids
YES	30	-	4.4	-	6.2	0.3	7.6
NO	30	-	4.5	1.3	5.0	2.9	4.3

Examining the amount of butanol and acids concentrations (butyric acid & acetic acid) accumulated when releasing fermentation gases, we found the gases to play major role in fermentation reactions and the mechanism to switch from acidogenic to solventgenic cycles. By releasing fermentation gases, there was increasing amount of acids accumulated in reaction broth. This strongly supports the theory that intermediate acid products react with fermentation gases (CO_2 and H_2) to produce solvents (Wood 1961) as per following reactions:

$$\underset{\textbf{Glucose}}{C_6H_{12}O_6} \longrightarrow \text{butyrate} + \text{acetate} + CO_2 + H_2 \qquad (4.1)$$

$$\underset{\textbf{Glucose}}{C_6H_{12}O_6} + \text{butyrate} + \text{acetate} + CO_2 + H_2 \longrightarrow \underset{\textbf{n-Butanol}}{C_4H_{10}O} \qquad (4.2)$$

The CO_2 and H_2 gases play as electron donor to catalyze the butyric acid and acetic acid to butanol (Woods 1995). This observation on fermentation gases is strongly supported by studies on butanol fermentation in batch verses continuous processes. Batch fermentation showed successful production of butanol, whereas no butanol were produced in continuous fermentation unless external supply of CO_2 and H_2 been blend with nitrogen purging gas (Brosseau et al. 1986, Ezeji et al. 2005b). Moreover, enrichment of 10% H_2 found to produce fivefold higher solvents productivity, while enrichment of 10% CO_2 results in increasing the ratio of butanol/acetone solvent from 2.9 to 3.5 (Mollah and Stucky 1992). It is sufficient here to bring the attention that increasing the partial pressure of fermentation gases (especially H_2) would positively affect the production of butanol since higher pressure would enhance the low solubility of hydrogen gas (compared to carbon dioxide) in fermentation broth. This was taken in consideration when designing the fermentation experiment of this study by reducing headspace in fermentation batch to 25ml out of 175ml total fermentation batch. The maximum production of gases was shown to be occurring around 26-30 hrs of fermentation, detected as a large pressure when sampling with a syringe (results not shown). By confirming the role of fermentation gases it was important to set proper sampling procedures to prevent loss of gases while sampling.

4.1.4 Total Buffer Content

Examining the final pH of fermentation broth showed large reduction compared to the initial pH (i.e., pH of 3.6-3.9 was obtained compared to initial pH of 6.6). By knowing

that optimum media pH is 6.5, and the Clostridia's lethal pH value is just below 4.5, it was necessary to apply some kind of pH control through fermentation experiments to prevent pH drop to less than 4.5. This can be implemented either by running the fermentation experiments in chemostat fermenter, or by increasing the total buffer capacity of fermentation media (Bryant & Baschek 1988, Lee et. al. 2008). We implemented the total buffer capacity as presented in Table 4.3

Table 4.3 Effect of total buffer capacity on solvent production
(T=35˚C, 8 vol% inoculum)

K_2HPO_4 g/l	KH_2PO_4 g/l	Glucose g/l		pH @ 0hr	pH @ 90hr	Butanol	
		Initial	Utilized			Conc. g/l	Yield
0.5	0.5	15	4.1	6.6	3.8	0.80	0.20
1.0	1.0	15	5.7	6.6	4.0	1.25	0.22
4.24	2.7	15	9.5	6.6	4.5	2.70	0.28
8.48	5.4	15	8.7	6.6	4.9	2.30	0.26

As shown in Table 4.3, the reduction in pH of fermentation medium from 4.5 to 4.0 results in reducing butanol production by half. And more reduction occurs at pH lower than 4.0. In general, extreme extracellular acidity affects the cell membrane and limits nutrition's diffusion into the cell, restrict cell growth, and can lead to complete termination of activity by sporulation. The extreme intercellular acidity would results in serious genetic modifications which may no longer produce solvents, or even may results in bacteria cell death (Gottschal and Morris 1981).

Using a total buffer content of 6.94 g/l (4.24 g/l K_2HPO_4+ 2.7 g/l KH_2PO_4) resulted in the highest butanol yield. Using 13.88 g/l results in relatively lower butanol yield may be due to some toxicity effect of buffer chemicals on bacterial cells.

4.2 Utilizing Various Sugars in Feedstock

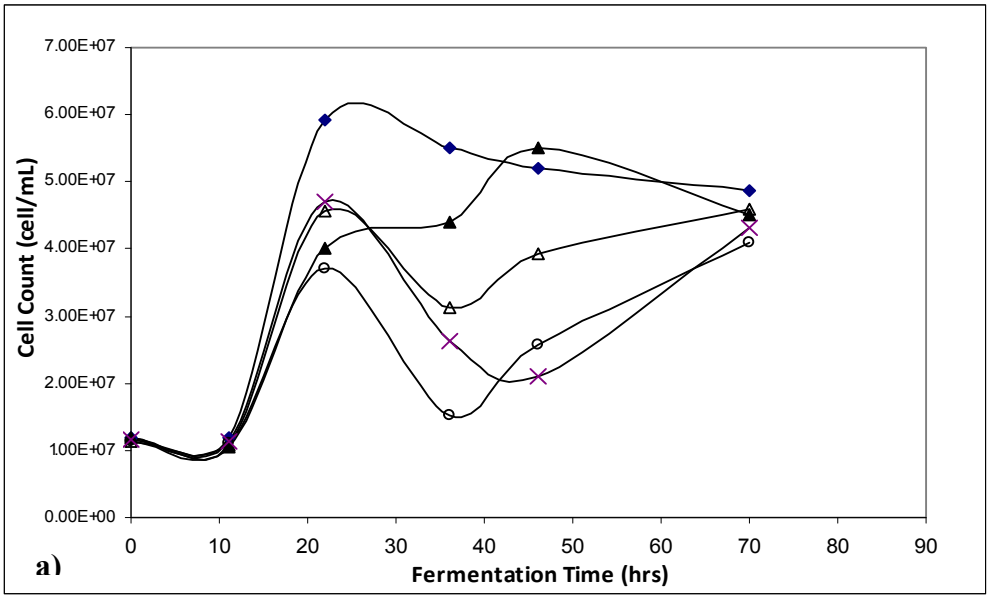
To evaluate the capability of *C. acetobutylicum* strain in fermenting different types of agricultural sugars biomass (i.e. WS, CF, DDGS), each type of sugar was separately evaluated.

4.2.1 Growth Curve on Various Sugars

Figure 4.3 a represents the growth curves of *C. acetobutylicum* ATCC 4259 in five sugar types. Same lag phase of 11hrs was found in all five fermentation media due to the presence of same amount of glucose. Around 22 hrs fermentation, reduction in cell count is been observed. This can be explained as a result of glucose consumption which requires the bacterial strain to enter a transition zone before start utilizing the other sugars. The reduction in cell count depends on digestibility of sugar by the strain. Glucose was the most digestible sugar followed by xylose, arabinose, mannose, and then galactose. By the end of fermentation experiment, the cell count for all five sugar types were comparable (average = $45x10^6$ cell/ml). This experiment indicates that agricultural residue is a potential biomass for fermentation of butanol since *C. acetobutylicum* was able to grow in all five types of sugars and reaches about the same cell concentrate but at different fermentation time.

4.2.2 Change of Fermentation pH with Various Sugars

As shown in Figure 4.3.b, testing pH over fermentation time showed sharp reduction in pH to less than 5 in 28-30 hrs, to reach a minimum pH= 4.3-4.5 at fermentation time of 42 hrs.



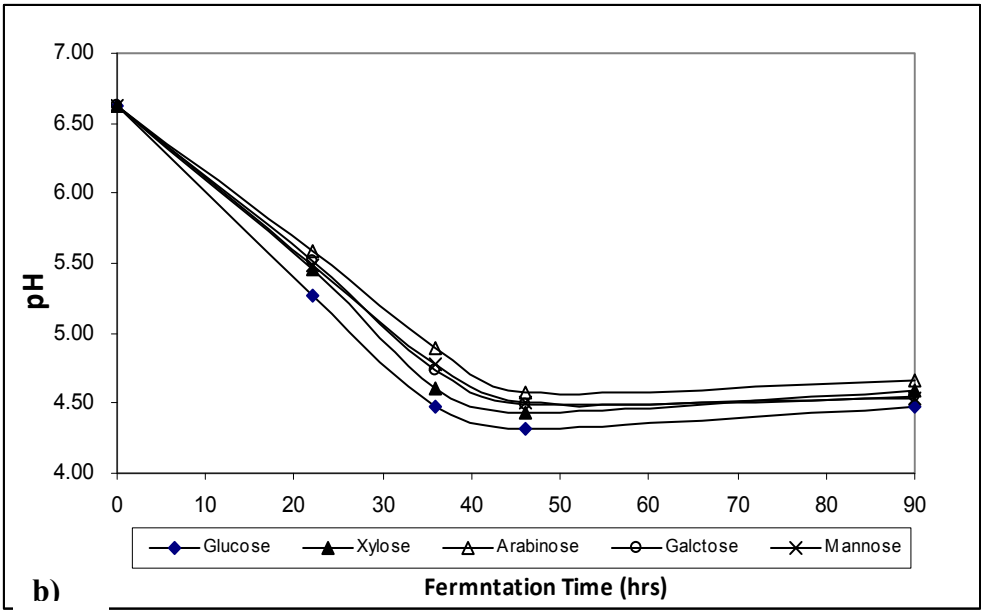


Figure 4.3 Growth of *C. acetobutylicum* ATCC 4259 with 24g of either glucose, xylose, arabinose, galactose or mannose added to 6 g glucose per liter of medium, a) growth curve, b) pH change (T=35˚C, initial pH of 6.6, inoculum 8 vol%)

All five sugar substrates showed comparable pH reading throughout fermentation experiments, which indicate same amount of acids formation mainly at the vegetative phase. There was no pronounced effect of sugar types on the acidity of fermentation broth since the large portion of these sugars were to be used for the formation of solvent products which would be reflected as variation in butanol product yield. Increasing total buffer content to 6.94g/l was shown to have limited effect on pH by only shifting up the pH from 3.6 to 4.5 at end of fermentation.

4.2.3 Yields Based on Various Sugars

Figure 4.4 represents the yield of butanol in different agricultural sugars. Variable yields of butanol were found using different sugars substrates. Glucose results in highest yield, followed by xylose, arabinose, mannose then glucose. These results were in agreement with Qureshi's observation when testing *C. beijerinckii* strain on different sugar substrates (Qureshi et al. 2007). Breakdown of sugar to pyruvate and utilization of pyruvate (Figure 2.2) requires the induction of enzyme pathways in different ratios. These are: ferredoxin (Feox), phosphotransferase (PTS), acetate kinase (AK) pathways, and thiolase (THL). Reported observations by Mitchell (1996) showed that mono- and di-saccharide uptake occurs mainly by the phosphotransferase (PTS) system at various rates, which explains the difference in sugar utilization, with galactose being the only sugar to be transported by a non-PTS mechanism which explain the additional reduction of butanol yield compared to the other sugars.

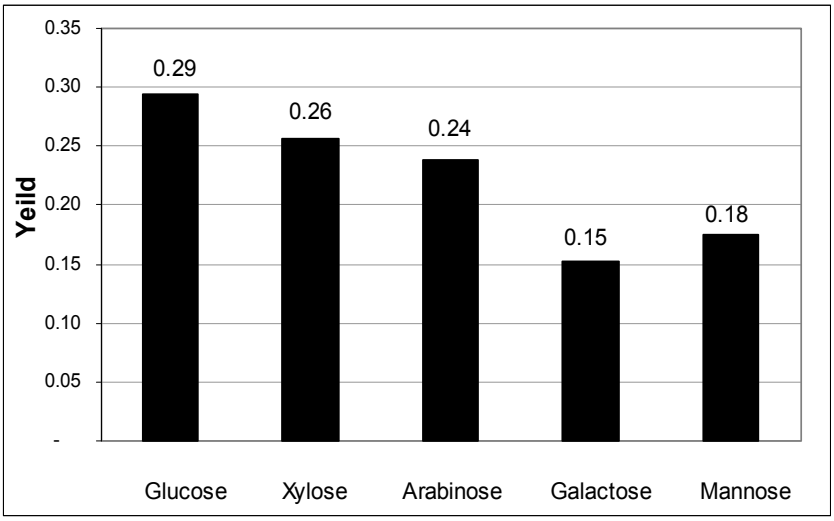


Figure 4.4 Butanol yields in different sugars using *C. acetobutylicum* ATCC 4259

It is important here to highlight the capability of solventogenic clostridia to efficiently utilize pentose sugars including xylose and arabinose in which these sugars are hardly utilized by ethanologenic micro-organisms (Andrade and Vasconcelos 2003), while they consist about (42-50%) of the total agriculture sugar. This makes the agriculture residues a promising feedstock to butanol fermentation industry, in specific.

As a result of this set of experiments, we were able to set optimum fermentation parameters for production of butanol using *C. acetobutylicum* ATCC 4259 as given in Table 4.4. These optimum parameters will be used as the base of comparison between the two Clostridium strains in the next section.

Table 4.4 Optimum fermentation parameters for *C. acetobutylicum* ATCC 4259

Parameter	**Value**
Fermentation Temp., ˚C	35
Fermentation time, hr	90
Initial sugar, g/l	30
Culture inoculum age, hrs	12 -14
Inoculum, vol %	8
Acidity of fermentation broth, pH	6.6 - 3.8
Total buffer content, g/l	1- >7.4
Type of sugar	Glucose
Gases of fermentation to be kept through experiment	

4.3 Comparison of *C. acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101

This part presents a comparison between the two *Clostridium* strains to find the best bacterial strain for the production of butanol. Two experiments were conducted in glucose base at the optimum fermentation parameters set in Table 4.4, and the results were as follows.

4.3.1 Growth Curve

Figure 4.5-a represents the growth curve for the two clostridium strains in two fermentation experiments. Both strains had a lag phase of around 11hrs. After 11hrs, *C. acetobutylicum* ATCC4259 started its vegetative phase to reach a maximum cell count of $60x10^6$ cell/ml at 24 hrs, compared to $12x10^6$ cell/ml at start of fermentation. This was followed by a slight decrease in cell count to $48x10^6$ cell/ml prior to initiating the stationary phase.

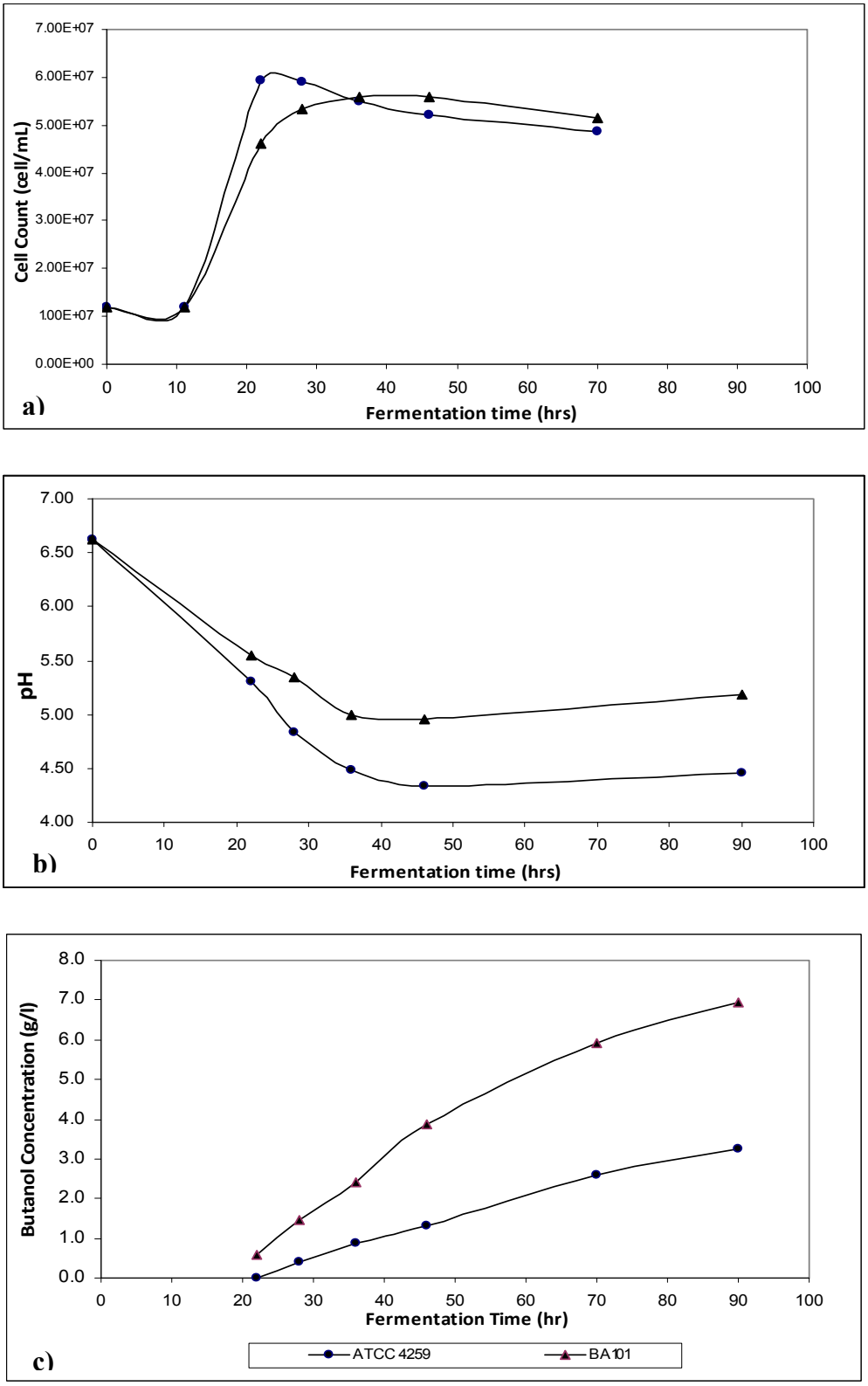


Figure 4.5 Comparison of *C. acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101 in fermentation medium a) growth curve b) pH variations c) butanol product concentration

For *C. beijerinckii* BA101, the vegetative phase reach a maximum of $55x10^6$ cell/ml prior to initiate the stationary phase at same cell concentration as the *C. acetobutylicum* one. The final cell count for both strains was found to be comparable at close to $50x10^6$. This can be explained as the maximum density of Clostridium bacterial in nutrition medium. Variations in pH, however, were quite pronounced.

4.3.2 Change of Fermentation pH

Figure 4.5-b represents the change in pH over the two fermentation experiments. The figure shows that pH started at 6.6 in both media, then decreases in almost linear fashion to pH of 4.3 in *C. acetobutylicum* ATCC4259 fermentation medium, and pH of 5.0 in *C. beijerinckii* BA101 medium. The pH of *C. acetobutylicum* found much less than the other due to extensive formation of acids (acetic + butyric acids) mainly during the vegetative phase (Table 4.2). This acids formation is found to be a characteristic of *C. acetobutylicum* strain (Handbook on Clostridia 2005). This extreme acidity may explain the limitation in sugar uptake as well as in butyric acid uptake for the strain towards production of solvents. More investigations were needed to determine whether sporulation would be initiated at pH below 4.5 which restricted the sugar uptake and limited butanol production, or whether *C. acetobutylicum* strain suffers physiological changes preventing it from producing butanol (Gottschal and Morris 1981).

In *C. beijerinckii* BA101 fermentation medium, the pH value did not go below 5.0 until the end of experiment for 90 hrs. This would possibly explain the capability of *C. beijerinckii* stain to produce higher concentration of butanol over the *C. acetobutylicum*

strain. At end of fermentation, a slight increase in the pH of *C. beijerinckii* experiment is shown as a result solvents accumulation.

4.3.3 Butanol Product Concentration

Figure 4.5-c shows a minimum butanol concentration detected was 0.4 g/l at 24 hrs in *C. acetobutylicum* ATCC4259 fermentation media. This concentration increased to 3.24 g/l by the end of 90hrs of fermentation. The low butanol production is thought to be a result of poor sugar uptake as well as poor acids uptake of *C. acetobutylicum* ATCC4259 strain. These strain characteristics can be further improved by generating mutant version of *C. acetobutylicum* ATCC 4259 with higher capability of sugar uptake. In the case of *C. beijerinckii* BA101, the production concentration increased from 0.6 g/l at 22 hrs fermentation to 6.95 g/l at the end of 90 hrs fermentation. This value is more than double the concentration produced from the C. *acetobutylicum* but with double consumption of sugar. In other word the yields are equals.

4.3.4 Product Selectivity and ABE Ratio

Butanol selectivity (g/g) is defined as the concentration of butanol produced to the total ABE concentration produced in fermentation batch. This indicates how efficient the micro-organism in producing the desired product is. Since solvents are produced in mixture of acetone, butanol and ethanol, the ABE ratio would be considered. In order to illustrate the difference in concentrations of the three solvents produced, the concentrations and yields of products at the end of 90 hrs are first calculated as shown in Table 4.5. In this table, *C. acetobutylicum* ATCC 4259 showed to produce lower yields of

butanol and total solvents (0.29 & 0.40) compared to *C. beijerinkcii* one which results in butanol and total solvents yields of (0.30 & 0.44) respectively. In other word, the *C. beijerinckii* produced more solvents but with lower butanol selectivity than *C. acetobutylicum*.

Table 4.5 Concentrations and yields for solvents and acids products of two Clostridia strains.

Micro-organism (strain)	Sugar utilized, g/l	**BUTANOL**		**ACETONE**		**ETHANOL**		**ACIDS**		**ABE**			**Butanol Selectivity**
		Conc. g/l	Yield g/g	Conc. g/l	Yield g/g	Conc. g/l	Yield g/g	Conc. g/l	Yield g/g	Conc. g/l	Yield g/g	Ratio	
***C. acetobutylicum* ATCC4259**	11	3.24	0.29	0.91	0.08	0.22	0.02	4.75	0.43	4.37	0.40	4:15:1	0.74
***C .beijerinckii* BA101**	24	6.95	0.30	3.10	0.13	0.60	0.03	2.60	0.11	10.65	0.44	5:12:1	0.66

The A:B:E ratio for *C. acetobutylicum* was calculated as 4:15:1 compared to 5:12:1 for *C . beijerinckii* which results in selectivity values of 0.74 and 0.66 respectively. Acids yield (acetic acid & butyric acid) was found to be four times higher in *C. acetobutylicum* in spite of using 6.94 g/l total buffer content. Concentrations and selectivity results of 90 hrs fermentation were also summarized in Table 4.5, whereas the yields for the two Clostridia were presented in Figure 4.6. Summary of this set of experiments for *C. acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101 strains comparison is given in Table 4.6.

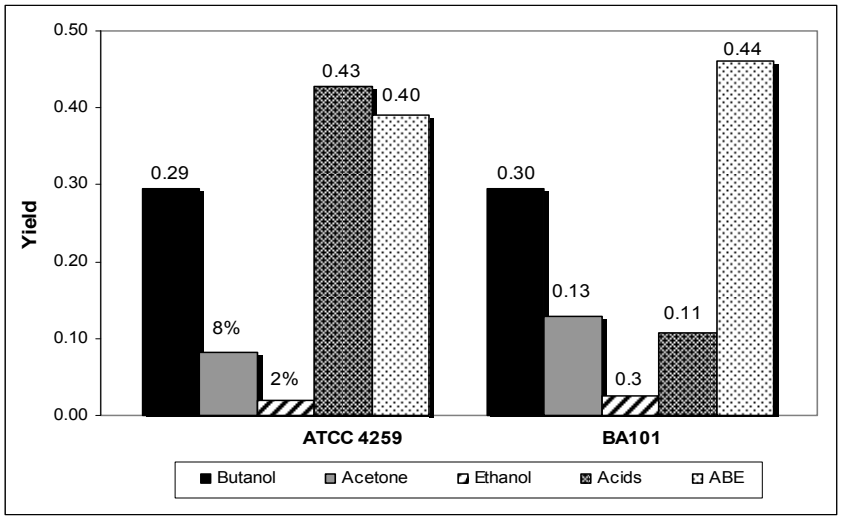


Figure 4.6 Solvents and acids yields using C. *acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101 strains

Table 4.6 Summary of fermentation results for *C. acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101 strains.

Property	***C. acetobutylicum* ATCC 4259**	***C. beijerinckii* BA101**
Culture cell count, cell/ml	48,735,022	51,415,578
Acidity of fermentation medium, pH	6.6- 4.2	6.6- 5.0
Sugar utilized, g/l	11.0	24.0
Butanol concentration, g/l	3.24	6.95
Butanol Yield, g/g	0.29	0.30
ABE concentration, g/l	4.37	10.65
ABE Yield, g/g	0.40	0.44
Acid concentration, g/l	4.75	2.60

At the current stage in biofuel researchs, the total solvent production is of more interest especially that butanol yield is fairly close in both strains (0.30 and 0.29). Not to forget the cost of acids byproducts separation that may result in serious corrosion risk of pipelines and storage reservoirs. Therefore it been decided to use the *C. beijerinckii* BA101 in next section for determining the optimum sugar composition in feedstock for

maximum production of butanol, until resolving the poor sugar uptake issue associated with *C. acetobutylicum* ATCC 4259 by developing mutant version of this strain.

Evaluating Yields Compared To Latest Research Work: Table 4.7 represents a results comparison of this study with the latest research works in results in uncontrolled pH batch fermentation using glucose sugar as substrate.

Table 4.7 Comparison of current results with other research work in uncontrolled pH batch fermentation using glucose substrate

Micro-organism	Butanol Yield	Solvent Yield	Acid Yield	ABE ratio	Selectivity	Reference
C. acetobutylicum ATCC4259 *C. beijerinckii* BA101	0.29 0.30	0.40 0.44	0.43 0.11	4:15:1 5:12:1	0.75 0.66	Current study
C. beijerinckii 55025	0.178	0.28	0.015	1.8: 4.7:1	0.62	Liu et al. 2010
C. beijerinckii P260	0.26	0.28	0.14	11:21:1	0.64	Qureshi et al. 2007
C. beijerinckii BA101	0.26	0.39	0.02	10.4:24:1	0.67	Ezeji et al. 2004

This study results in butanol and solvents yields of 69- 60% higher than the *C. beijerinckii* 55025 study by Liu with higher selectivity of butanol product. However, Liu's strain showed to produce the minimal fermentation acids. Our results were also 17-7 % higher in butanol and solvents yields than *C. beijerinckii* P260 by Qureshi, and in high selectivity as well. However, 2004 study using same C. *beijerinckii* strain by Ezeji gives almost same butanol selectivity but fewer yields when using as much as double the initial sugar used in our experiments.

4.4 Optimization of Sugar Composition in Feedstock

The objective of this part was to find the optimum sugar composition in feedstock which results in maximum production of butanol. By knowing optimum composition, we will be

able to utilize various types of agriculture waste by adjusting the feedstock sugar composition rather than using blend of strains at different optimum conditions, or applying multi stage process using different stains at their optimum conditions. Applying 4-factors 2-levels full factorial design, section, we ran 18 fermentation experiments at once using *C. beijerinckii* BA101 at the optimum fermentation parameters given earlier in Table 4.4. Sugar composition and butanol product for factorial experiments were given in Table 4.8.

Table 4.8 Sugar composition and butanol product in factorial design experiments.

Exp. No.	Glucose g/l	Xylose g/l	Arabinose g/l	Mannose g/l	Butanol g/l
1	13.50	0.00	0.00	0.00	2.00
2	30.00	0.00	0.00	0.00	5.80
3	13.50	10.00	0.00	0.00	3.84
4	30.00	10.00	0.00	0.00	6.80
5	13.50	0.00	5.80	0.00	3.35
6	30.00	0.00	5.80	0.00	6.60
7	13.50	10.00	5.80	0.00	5.00
8	30.00	10.00	5.80	0.00	8.50
9	13.50	0.00	0.00	1.40	2.15
10	30.00	0.00	0.00	1.40	4.90
11	13.50	10.00	0.00	1.40	3.90
12	30.00	10.00	0.00	1.40	6.95
13	13.50	0.00	5.80	1.40	3.30
14	30.00	0.00	5.80	1.40	6.55
15	13.50	10.00	5.80	1.40	5.10
16	30.00	10.00	5.80	1.40	8.20
17	21.75	5.00	2.80	0.70	5.10
18	21.75	5.00	2.80	0.70	4.70

Analyzing the butanol product readings by MINITAB package, taking in consideration the effect of individual sugars plus the 2-way interactions of glucose (using confidence level of 0.95), then the output for the analysis of variance (ANOVA) as table 4.9, and the effect of each factor and its coefficients in the regression model as table 4.10.

Table 4.9 Analysis of variance (ANOVA)

Source	**DF**	**SS**	**MS**	**F**
Main Effects (*i=G,X,A,M*)	4	93.0158	23.2539	914.77
2-Way Interactions (*i=GX,GA*)	2	0.2004	0.1002	3.94
Residual Error (*E*)	10	0.2542	0.0254	-
Lack of Fit	-	0.1530	0.0170	0.17
Pure Error	1	0.1012	0.1012	-
Total	17	95.3724	-	-

Table 4.10 Factors effects and coefficients in regression model

Factor	**Effect**	**Effect** (Standardized)	**Coefficient** (β_i)
Constant	-	-	-0.371080
Glucose	3.896	48.028	0.220982
Xylose	2.266	27.934	0.211127
Arabinose	1.714	21.129	0.191602
Mannose	-0.019	-0.234	-0.0133929
Glucose*Xylose	0.034	0.419	0.000409091
Glucose*Arabinose	0.221	2.724	0.00454545
R^2 = 99.73%			

Applying regression coefficients into linear polynomial formula results in following fitted regression model for butanol production:

$$\boldsymbol{y = -0.3710 + 0.2209\, X_G + 0.2111\, X_X + 0.1916\, X_A - 0.1339\text{x}10^{-1}\, X_M + 0.4090\text{x}10^{-3}\, X_G X_X + 0.455\text{x}10^{-2}\, X_G X_A} \quad (4\text{-}3)$$

Where

y: concentration of butanol product in g/l

X_G, X_X, X_A, X_M:concentration of glucose, xylose, arabinose and mannose, g/l, respectively

The effect of individual sugars and sugar interactions in butanol production model are well presented Figure 4.7 and Figure 4.8. Figure 4.7 shows positive individual effect of glucose, xylose and arabinose while negligible effect was found for mannose on the mathematical model. Figure 4.8 shows positive interaction effect between glucose-xylose, glucose-arabinose, and xylose-arabinose at both lower and upper concentration limits, while there is no interaction of mannose with other types of sugar. This can be related to the relatively hard metabolism of mannose compared to other sugars, and due to its low concentration (< 1.4 g/l) in agriculture residues.

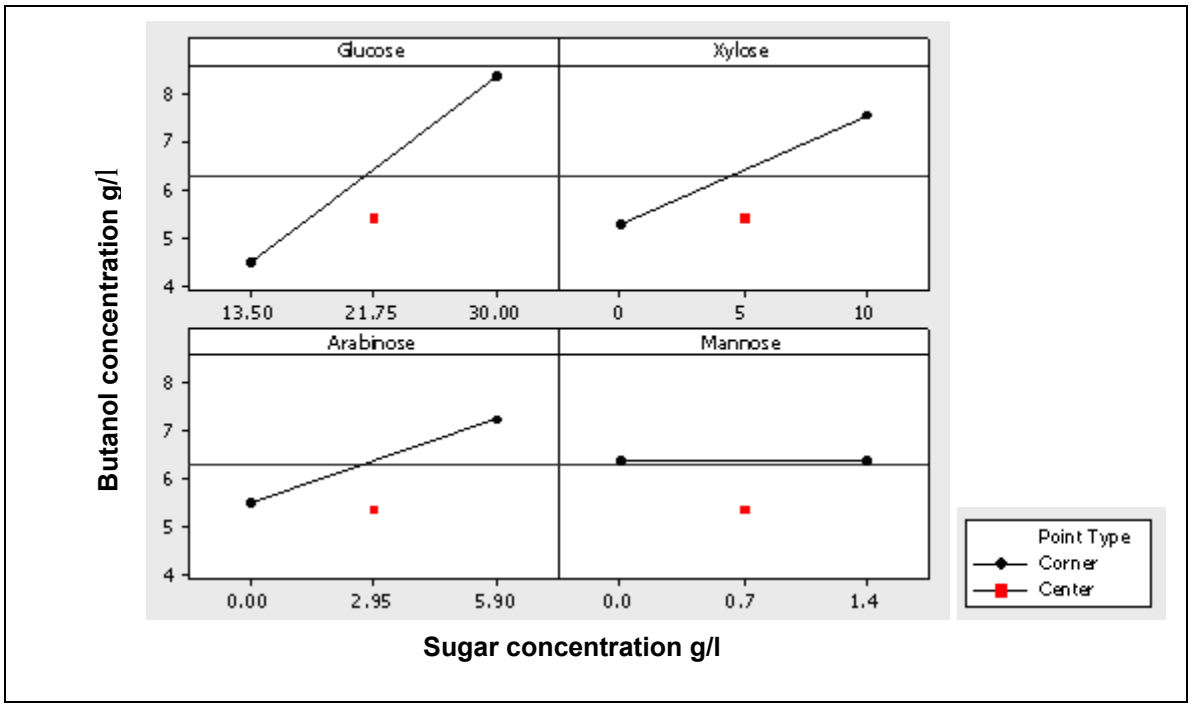


Fig 4.7 Plot of individual sugar effect on butanol production model (MINITAB package)

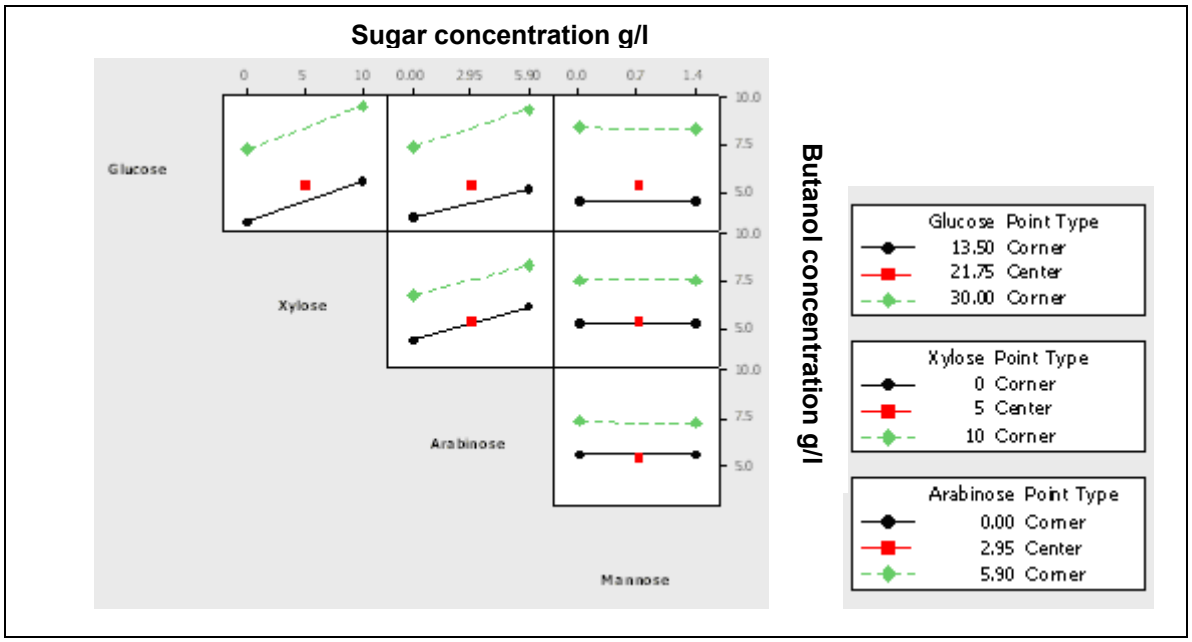


Fig 4.8 Plot of 2-way sugar interaction effects on butanol production model (MINITAB package)

Furthermore, Fig 4.9 shows that only X_G, X_X, X_A and $X_G X_A$ are the significant terms in the model. Thus the mathematical model formula can be simplified to:

$$y = -0.371 + 0.221\, X_G + 0.211\, X_X + 0.192\, X_A + 0.455\text{x}10^{-2}\, X_G X_A \quad (4.4)$$

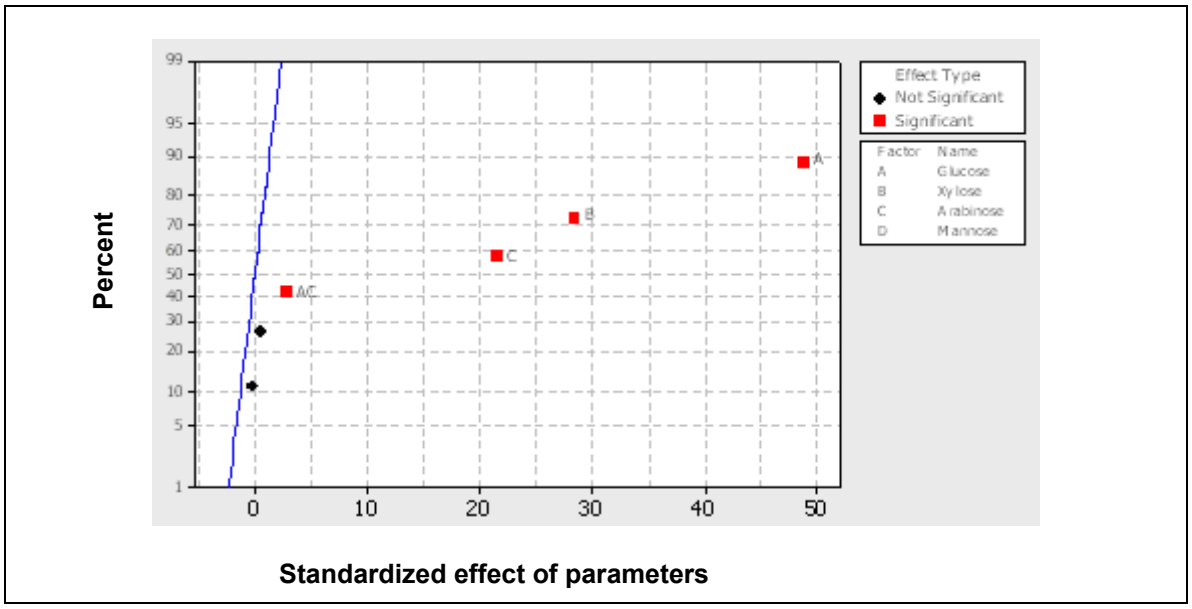


Figure 4.9 Plot of significant terms in butanol production model, i.e. normal plot of standardized effects (MINITAB package)

Equation 4.4 shows that the control experiment (100% glucose) would always results in highest butanol production followed by CF, then WS and finally DDGS. But the reality is that agriculture residues contain no more than 53.3% glucose plus other types of sugars. To find the optimum sugar composition in agriculture residues, the Excel optimization solver has been applied on equation 4.3 with following sugar constraints:

X_G: 13.5- 16.0, X_X: 7.6-10.0, X_A: 2.5-5.9, X_M : 0.0-1.4, $X_G + X_X + X_A + X_M = 30$

Optimization solver results are summarized in Table 4.11

Table 4.11 Optimum sugar composition and maximum butanol production (Excel optimization solver)

Property	**Optimum**	**WS**+adjustment	**CF**+adjustment	**DDGS**+adjustment
Sugar composition, wt/wt %				
Glucose	53.33	48.00 + **5.33**	53.4	44.9 + **8.43**
Xylose	27.00	33.40	25.3 + **1.70**	31.5
Arabinose	10.67	8.30 + **2.37**	16.2	19.6
Mannose	0.00	4.50	0.0	1.5
Maximum butanol production at 30g/l initial sugar	6.49 g/l			
Maximum butanol production At 60g/l initial sugar	12.97 g/l			

By knowing the optimum sugar composition to be 53.33% glucose, 27.00% xylose, 10.67% arabinose, with no need to mannose, we can use various agriculture biomasses as feedstock to fermentation process at same operation conditions in Table 4.4. I.e. we can use CF with an additional supplement 1.7 % xylose, WS with an additional supplement of 5.33 % glucose plus 2.37 % arabinose, or DDGS with an additional supplement of 8.43 % glucose. This is to mimic the optimum sugar composition required for maximum butanol production of 12.97 g/l. The variation in sugar content of agricultural restudies was not been discussed here because it is completely dependent on the efficiency pretreatment and hydrolysis of agriculture waste. However, corn fiber was found to contain higher sugar content (69.6 g/l) compared to wheat straw (60.2 g/l) and dry distilled grain solubles (52.2 g/l) which would results in higher butanol production.

4.4.1 Validation of Butanol Production Model

To evaluate butanol production model (Equation 4.3), we fitted data from previous research paper at the same sugar constraints and the data output is presented in Table 4.12. This table shows the model to result in error of (12.1-9.6) % when fits with other

research work. In addition, the model results in an error of 10 % when applying data from previous fermentation experiment (section 5.3). This leads to the conclusion that the model is a good representation for effect of sugars on butanol production in batch fermentation using *C. beijerinckii* BA101.

Table 4.12 Error percentages of Butanol Production Model

Reference	**Initial Sugar Concentration**	**Butanol Production g/l**		**Model Error %**
		Observed	**Calculated[a]**	
Queshi et al. 2007	Glucose: 55 g/l	13.40	11.78	12.1
	Sugar mixture: 55 g/l X_G:X_X:X_A:X_M;(5:4:2:1)	12.80	11.57	9.6
Figure 4.5	Glucose: 30 g/l	6.95	6.26	10.0

[a] using Equation 4.3

4.4.1 Estimating Error in Fermentation Experiments

To minimize the error in butanol fermentation experiments and the testing analysis, batches subjected to comparison were all prepared and ran at once. Analytical testing was performed at same time headed and followed by running calibration standards for all sugars, solvents, and acids. By following this technique we were able to reduce the experimental error from more than 25% to a maximum of 15% which represents good confidence level for such kind of biological systems. Figure 4.10 represents the maximum experimental error in butanol production results for fermentation experiment in Table 4.1 at 30 g/l initial sugar concentration and 5 vol% inoculum.

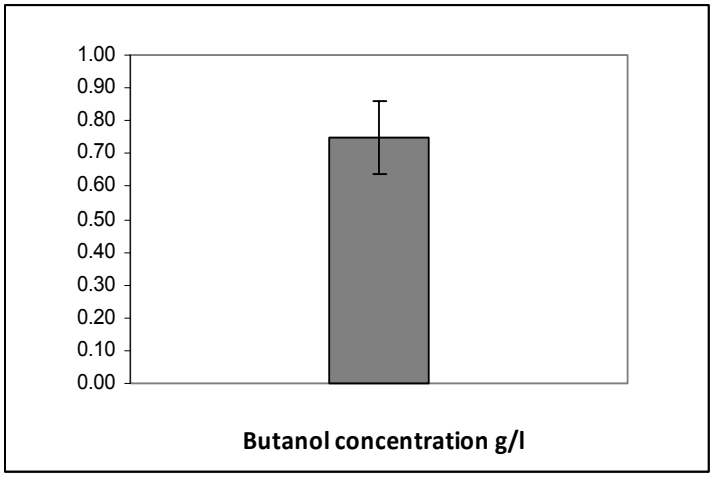


Figure 4.10 Maximum error in butanol fermentation experiments

This error can be further reduced if fermentation set up was modified to set a serialized anaerobic glove box as incubator in which sampling can be handled at constant temperature, with minimal chance of oxygen contamination.

Chapter 5
Conclusion and Future Work

5.1 Conclusion

This study has been successful in addressing all three core project research questions: "Are agricultural residues a potential feedstock for the biobutanol industry?", "What are the suitable bacterial strain and fermentation conditions for the anaerobic batch fermentation of butanol?" and "Are we able to optimize the sugar composition of agricultural feedstock so we can use various types of waste at the same optimal fermentation conditions?"

Screening of agricultural residues showed potential feedstock to biofuel industry. Using low cost or sometimes negative cost residues would feasibly reduce the cost of biofuel formation by biological processes. Results of investigating the production conditions of *Clostridium acetobutylicum* ATCC 4259 in anaerobic batch fermentation of butanol showed the fermentation gases to play major role in fermentation reactions. They work as intermediates to react with acids product and form the butanol. The increase of culture inoculum volume into fermentation batch was shown to positively affect butanol yield. The inoculum culture was found to be only productive when inoculated during its vegetative phase. The beginning and end of vegetative phase was greatly dependant on the temperature at where the stock culture been maintained prior to inoculation. The type of fermentable sugar was shown to affect butanol production as well: glucose yielded the highest butanol, followed by xylose, arabinose, mannose then galactose.

Testing two Clostridium strains for the fermentation of butanol showed the *C. beijerinckii* BA101 to be a preferred strain over *C. acetobutylicum* ATCC 4259. This was due to the high capability of the mutant C. *beijeirinckii* to uptake the sugar towards the production

of butanol. On the other hand, *C. acetobutylicum* suffered poor sugar uptake which resulted in limited production of butanol while high concentration of acid products were accumulated in the fermentation broth. The amount of produced acids was enough to lower the pH to low value which makes the strain inapplicable to batch fermentation without effective system for pH control. Even with an improved buffer content in the medium, the pH decreased to 4.5 at the end of fermentation, a value known to prevent the growth of the bacterium.

Optimization of feedstock composition showed an optimum sugar composition of 53.33% glucose, 27.00% xylose, 10.67% arabinose, and 0.0% mannose. This suggest to adjust the composition of wheat straw feedstock by adding supplement of 5.3% glucose plus 2.37% arabinose, corn fiber feedstock by adding supplement of 1.7% xylose, and the dry distilled grain solubles by adding supplement of 8.43% glucose.

5.2 Future Work

As a future work, would be important to use actual agriculture residues as feedstock to fermentation experiments instead of a mixture of pure sugars, and compare the results with current study results. This comparison should also take in consideration the effects of inhibitors as a result of pretreatments and hydrolysis which needs to be applied to feedstock biomass prior to fermentation. This should be followed by fermentation experiments in fed-batch bioreactor with continuous recovery of solvent products to minimize the toxicity effect of butanol product on bacterial culture and allow the use on higher sugar content close to 75g/l to mimic the actual sugar content of agricultural waste. If *C. acetobutylicum* ATCC 4259 strain were to be utilized in anaerobic batch fermentation, it would be required to develop a mutant version with high capability to uptake various agriculture sugars towards butanol production since the overall competitiveness of the bioprocess highly depends on the strain performance, successful metabolic. Protoplast diffusion using 2-deoxyglucose is currently being undertaken in our lab to develop novel mutant of *C. beijerinckii* with *C. thermocellum* which is expected to produce higher concentration of butanol, in addition to high capability of hydrolyzing the biomass feedstock as a result of loading the enzyme-producing organism. It would be also beneficial to examine the effect of heat shock treatment on the activity of bacterial culture prior to inoculation into fermentation batch. Activating the heat shock protein is expected to improve the tolerance to butanol as demonstrated by Ezeji et al. 2007 and Gape et al. 2000.

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Appendix A
Composition of Rainforest Clostridia Medium

Table A-1, Typical formula for Rainforest clostridia medium

Composition	g/l
Glucose	5.0
Soluble starch	1.0
Yeast extract	3.0
Lab-Lemco powder	10.0
Peptone	10.0
L-Cysteine HCL	0.5
Sodium chloride	5.0
Sodium acetate	3.0
Agar	0.5

Preparation: an amount of 38 g of Rainforest medium powder (Sigma-Aldrich, Canada) was suspend in one liter distilled water (pH=6.8 ± 0.2 at 25˚C). Mixture was brought to boil for complete dissolving of solids. Then mixture was sterilized by autoclave at 121C for 15 minutes.

Appendix B
Results Tables

Table B-1 (for Figure 4.1) Growth of *C. acetobutylicum* ATCC 4259 in Rainforest culture medium for inocula stored at different temperatures

Culture Cell Count grown at 35˚C, cell/ml			
Exp, hr	**A @ 35˚C**	**B @ 5˚C**	**C @ -60˚C**
0	3,584,372	4,817,638	2,558,046
4	2,911,541	4,199,298	3,645,199
11	3,577,090	2,243,991	1,400,634
19	54,219,671	43,867,279	456,472
22	54,275,051	57,776,299	41,198,055
25	54,439,459	57,222,855	56,557,559
30	52,147,127	55,184,815	57,017,279

Table B-2 (for Figure 4.4) Butanol yields in different sugars using *C. acetobutylicum* ATCC 4259

Property	**Glucose**	**Xylose**	**Arabinose**	**Galactose**	**Mannose**
Butanol, g/l	3.24	2.84	2.58	1.22	1.49
Sugar utilized, g/l	11	11.1	10.8	8	8.5
cell/ml	48,735,022	44,980,138	45,951,000	40,909,000	43,151,706
Y , g/g	0.29	0.26	0.24	0.15	0.18

Table B-3 (for Figure 4.3) *C. acetobutylicum* ATCC 4259 in different sugar substrates prepared by adding 24g/l of either of glucose, xylose, arabinose, gactose and mannose to a base of 6g/l glucose. (T=35˚C, initial pH of 6.6, inoculum 8vol%)

a) Growth curve

Culture Cell Count, cell/mL					
Exp., hr	**Glucose**	**Xylose**	**Arabinose**	**Galactose**	**Mannose**
0	12,006,868	11,770,852	11,477,226	11,459,896	11,750,152
11	11,816,865	10,568,374	10,735,866	11,026,342	11,241,792
22	59,338,810	40,249,998	45,636,082	37,001,946	47,086,766
36	55,011,052	43,949,282	31,313,000	15,152,000	26,331,122
46	52,023,579	54,979,514	39,255,000	25,602,434	21,108,516
70	48,735,022	44,980,138	45,951,000	40,909,000	43,151,706

b) pH changes

pH					
Exp., hr	**Glucose**	**Xylose**	**Arabinose**	**Galactose**	**Mannose**
0	6.62	6.62	6.62	6.62	6.62
22	5.27	5.45	5.58	5.51	5.49
36	4.48	4.61	4.89	4.73	4.78
46	4.32	4.43	4.58	4.49	4.51
90	4.48	4.59	4.67	4.55	4.53

Table B-4 (for Figure 4.5) Comparison of *C. acetobutylicum* ATCC 4259 and *C. beijerinckii* BA101 in fermentation medium

a) Growth curve

Culture Cell Count, cell/ml		
Exp. time, hr	***C. acetobylicum* ATCC 4259**	***C. bejeirinckii* BA101**
0	12,006,868	12,000,624
11	11,816,865	12,012,658
22	59,326,823	45,996,586
28	59,003,211	53,485,858
36	55,011,052	55,866,818
46	52,023,579	55,851,834
70	48,735,022	51,415,578

b) pH changes

pH		
Exp time, hr	***C. acetobylicum* ATCC 4259**	***C. bejeirinckii* BA101**
0	6.62	6.62
22	5.30	5.55
28	4.84	5.35
36	4.48	5.00
46	4.34	4.96
90	4.46	5.19

c) butanol product concentration

Butanol Concentration, g/L		
Exp time, hr	***C. acetobylicum* ATCC 4259**	***C. bejeirinckii* BA101**
22	-	0.60
28	0.40	1.46
36	0.89	2.42
46	1.33	3.87
70	2.59	5.90
90	3.24	6.95

Appendix C
Sugars, ABE, and Acids Standards

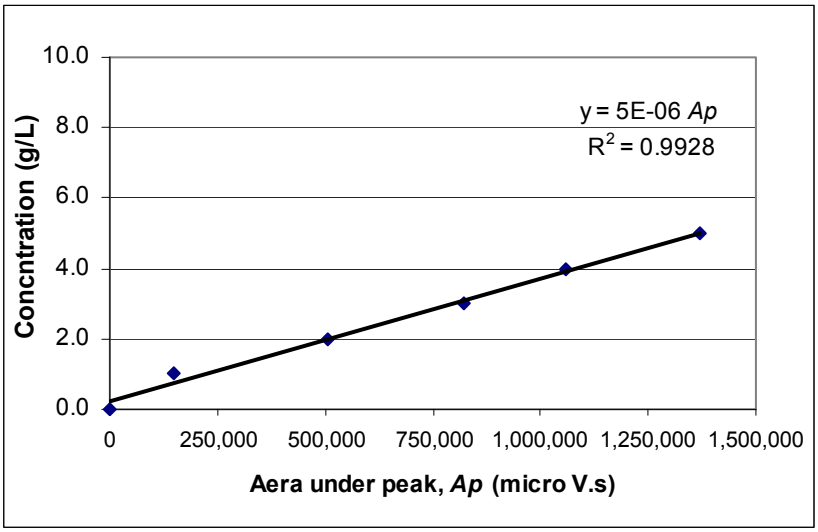

Figure C-1 HPLC standard curve for Butanol

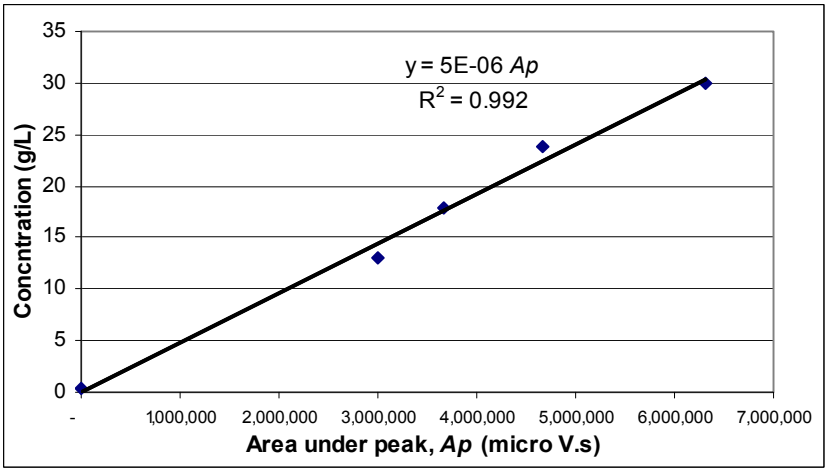

Figure C-2 HPLC standard curve for Glucose

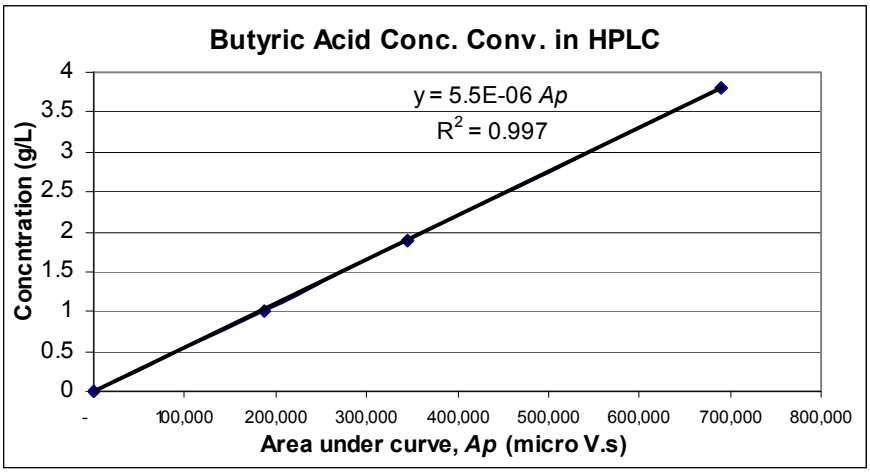

Figure C-3 HPLC standard curve for Butyric Acid