DEVELOPMENT OF NOVEL CLOSTRIDIAL FUSANTS FOR ENHANCED BIOBUTANOL PRODUCTION

by

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B.A.Sc. Chemical Engineering
University of Toronto, Toronto, Canada, 2010

A Thesis

presented to Ryerson University

in partial fulfilment of the requirements

for the degree of

Master of Applied Science
in the Program of
Chemical Engineering

Toronto, Ontario, Canada, 2012

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ABSTRACT

Biobutanol, a fuel with higher energy content than ethanol was produced in the present work through Simultaneous Saccharification and Fermentation (SSF) of wheat straw (WS) that traditionally produces acetone, butanol and ethanol solvents (ABE). Novel Clostridium strains were developed and tested for enhanced biobutanol production. Thermal stability was imparted to two mesophilic clostridial wild strains through protoplast fusion with that of a corresponding thermophilic clostridial strain. This novel development eliminated the need to add external enzymes during the SSF process, allowed for SSF to be conducted at an elevated temperature of 45^{o}C, enhanced the activity of internally produced enzymes and tolerated butanol toxicity to new limits. In order to determine the stability of the genetically modified strains, the parent fusants were passed on to five successive growth cycles. The fused strains of each growth cycle were inoculated with substrate and examined for solvent production and compared to the parent generation. Polymerase Chain Reaction Technique (PCR) followed by Agarose Gel Electrophoresis demonstrated reproducible genetic stability of the fused strains. Highest biobutanol production of 13.8 g/L (almost twice the concentration reported by other laboratory scale batch SSF research studies) was produced by one of the novel fusants. Generally, fused strain that achieved the highest biobutanol production exhibited relatively constant butanol concentration over the five growth cycles. Optimistic results obtained from batch SSF at lab scale demonstrate a clear potential of these novel strains to improve productivity and yield of biobutanol at a large-scale facility.

ACKNOWLEDGMENTS

I would like to take this opportunity to acknowledge the people who contributed to the successful completion of this project.

Firstly, I would like to show my sincere gratitude to my supervisor Dr. Yaser Dahman for giving me the opportunity to work on this project. Without his continuous encouragement, assistance and inspiration it would have been almost impossible to deliver my full potential to this project.

Furthermore, I would like to thank my lab colleagues Banafesheh Mohtasebi, Hamza Nakhoda, Sarkar Begum and Wahib AlAbdallah who have shown initiative in discussing aspects of my project and created a friendly and amiable atmosphere in the laboratory making experimental work more enjoyable. I would like to thank the "Ryerson University Analytical Centre (RUAC) for letting me use the HPLC for quantitative analysis. I would also like to thank the administrative, academic staff and my oral examination committee at the Department of Chemical Engineering at Ryerson University for doing their best in making graduate studies interesting, challenging and rewarding.

A special thanks is due to Miranda Maki who is a PhD student in the Department of Biology at Lakehead University for performing stability studies for the strains.

Finally I would like to acknowledge the unconditional love and support I received from my parents (Riaz and Atya) and my sister (Rakshan) during my years at Ryerson University.

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CHAPTER 1

INTRODUCTION

The endless reliance of the global economy and industry on decreasing fossil fuel reserves has generated amplified interest in non-fossil based energy primarily from fermentation of agricultural residues like wheat straw, bagasse and corn stover. The presence of the largest crude oil deposits in the most antagonistic regions has fostered research on biofuels like ethanol and butanol.

Alternative biomass based green energy from renewable resources has been the focus of research in the past few decades primarily due to its environmental and economical advantages. In Canada, biomass remains an important part of our country's energy picture, supplying about 4.7 percent of our primary energy demand, the second largest source of renewable energy after hydroelectricity. In 2006, Canada's bioenergy based generating capacity was 1652 MW with Ontario's contribution amounting to 19 percent (Government of Canada, 2009). Apart from its use in forest products, pulp and paper industries, burning of biomass to provide heat to generate steam and electricity, it is also used to generate liquid fuels. Currently existing in Canada, the ethanol fuel industry produces about 238 million liters of ethanol each year mainly from corn and wheat. However, butanol as a biofuel exhibits immense potential due to its relatively high carbon content in comparison with ethanol.

Butanol can be prepared from Acetone-Butanol-Ethanol (ABE) fermentation process using agricultural substrates. Production of butanol through fermentation has been around for a while and is one of the oldest fermentation processes that commercialized the production of biofuels. Although there were large commercial operations during WWI and WWII, its production declined with the discovery of oil and inexpensive operating costs associated with crude oil based petroleum industries (Qureshi and Ezeji, 2008). The problem primarily associated with the decline of the commercial

fermentation process was the cost of substrates for making butanol (Ezeji, 2004). The substrates being used until recently were derived from edible parts of biomass like seeds, starches and crops. The immense demand for food crops not only for consumption but also for fuel production created a supply shortage thereby boosting the cost of substrates required for biofuel production. (Ezeji, 2004). With world population on the rise, more food crops were grown for feeding rather than producing fuel. However, utilizing agricultural wastes left out after harvesting can diminish the economic concerns of higher substrate costs.

With the vast reserves of crude oil around the world, the reader might wonder the need to invest in biofuels. The answer lies in sustainability of our current energy resources. The never-ending demand for fuel has increased production from large oil fields that are declining at a rate of 4-5% annually. The most important reason to switch to biofuels is that global statistics forecast that production of oil and gas is approaching its maximum and the world is now finding one new barrel of oil for every four it consumes (Nigam and Singh, 2011).

A recent classification of liquid biofuels includes first, second and third generation biofuels. The primary distinction between them is the feedstock used. The classification is pictured in Figure 1.1. The first-generation of secondary biofuels are generally produced from sugars, grains or seeds and require a simple process to convert it into biofuel. The viability of this generation is questionable due to its conflict with food supply. The second generation are produced from biological or thermochemical processing of agricultural biomass. This generation has a significant advantage over the previous generation as it utilizes the non-edible residues of food crops like wheat and corn. However, the production of second generation biofuels requires not only sophisticated processing technologies but also more investment per unit of production accompanied by large-scale facilities (Nigam and Singh, 2011). The biomass substrates undergo different technological treatments including thermochemical and biochemical to convert it to biofuels. The last generation of biomass sources such as microbes and microalgae are considered to be viable alternative energy resource.

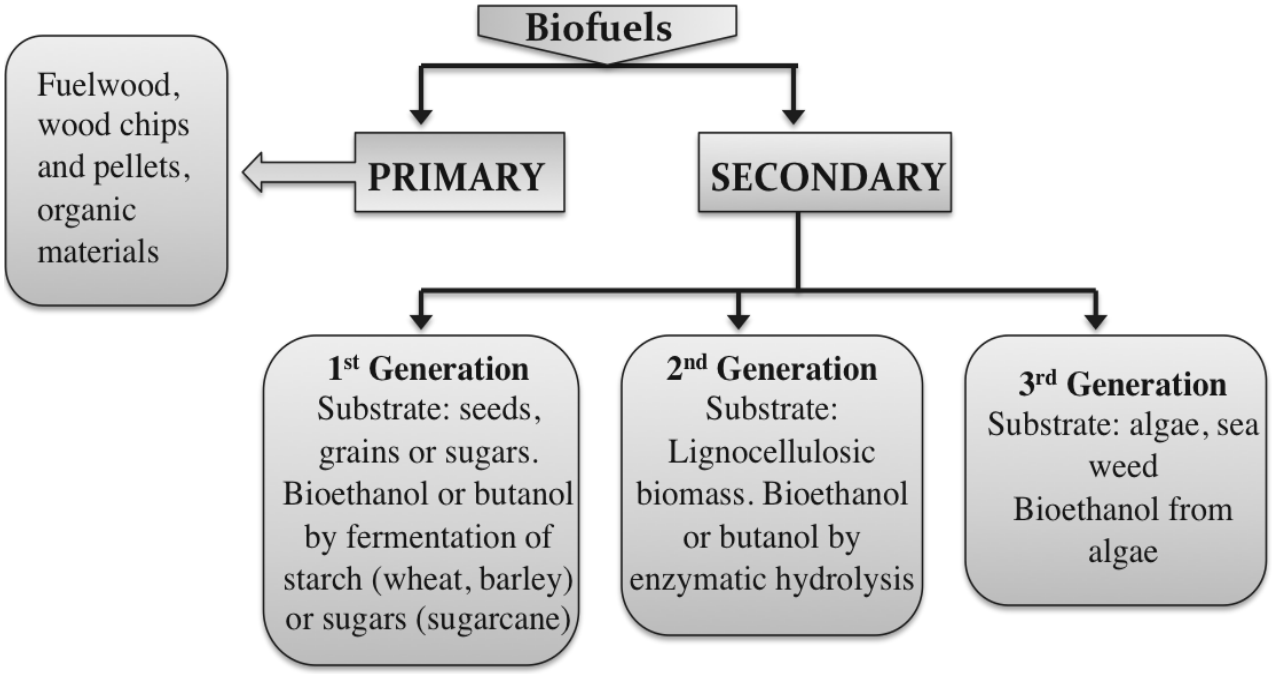

Figure 1. 1 Characterization of biofuels based on substrate source

With so many biomass resources available for production of biofuels, the reader might wonder about the distinctiveness of wheat straw as a substrate for our current study. Previous attempts at producing butanol from Wheat Straw Hydrolysate (WSH) have been very successful. In these carefully constructed experiments, not only was the fermentation more rapid than the ones in which glucose (as a substrate) was exclusively used, but also there was an absence of inhibition due to salts or other inhibitory products (Qureshi and Ezeji, 2008).

Now that we have established the uniqueness of wheat straw and the advantages of using WSH as a substrate, we turn to a more imperative question. Why butanol and not ethanol? Butanol as a biofuel has some attractive properties that other biofuels like ethanol do not possess. It is worthwhile to note that butanol's energy content is 30% more than ethanol and is closer to gasoline. Biobutanol, in its pure form can be blended in any concentration with gasoline unlike ethanol which can only be blended up to 85% (Dürre, 2007). Introduction of biobutanol into the existing car engines as a combustible fuel

requires minimal modification to the engine technology. The products lower vapour pressure makes it safer to handle. Butanol is not hygroscopic which allows blending with gasoline at a refinery way ahead of storage and distribution. This is in stark contrast to ethanol, which requires blending to occur shortly before distribution (Dürre, 2007). Therefore, if we cogitate and recapitulate the benefits associated with using butanol, we can comprehend that it is a more practical solution to address current and imminent energy crisis. In Table 1.1, some properties of gasoline like calorific value, air-fuel ratio and research octane number are compared to biofuels like ethanol and butanol.

Table 1. 1 Properties of gasoline in comparison with liquid biofuels

Fuel	Calorific value (MJ/L) (Dürre, 2007)	Air-fuel Ratio	Research Octane number (Dürre, 2007)
Gasoline	32.5	14.6	91-99
Ethanol	21.2	3	129
Butanol	29.2	11.2	96

Biobutanol is produced from renewable sources using strains of bacteria such as *Clostridium acetobutylicum* (*Cb*) or *Clostridium beijerinckii* (*Ca*) (Qureshi et al., 2007). Hydrolysis and fermentation can be performed simultaneously and the process is referred to as Simultaneous Saccharification and Fermentation (SSF). The motivation behind using these strains lies in the fact that they can exploit both hexoses and pentoses, which places them in disparity with their yeast (ethanol-producing) counterparts (Qureshi and Ezeji, 2008). Another bacterium used in the current study is *Clostridium thermocellum* (*Ct*), which is an important anaerobic thermophile, used in industrial conversion of cellulosic biomass into liquid biofuels.

In the current study, thermo-stable clostridial species were developed through protoplast fusion

between mesophilic and thermophilic strains. A significant challenge facing enzymatic hydrolysis is the lower temperature during SSF considering the optimal temperature for saccharification to be around 45^{o}C (Qureshi et al., 2007). It was demonstrated from previous experiments in SSF done at Nanotechnology and Bioengineering Research Lab at Ryerson University that there were less sugars released during SSF when the hydrolysis was carried out at 35^{o}C as opposed to 45^{o}C. Therefore, in our current study we have used 45^{o}C as optimal temperature for performing SSF for our protoplast fused strains. Since it's a simultaneous process, it is required that both hydrolysis and fermentation occur at the same temperature. However, during the fermentation segment of the SSF, addition of external enzymes to the newly introduced fused strains was found to have a negative affect on the butanol production. The reason for the negative affect is that the bacteria were able to produce their own enzymes at 45^{o}C and any supplementary enzymes would compete with the already existing ones thereby reducing the concentration of the final products. Cost reduction of raw materials (including enzymes and substrates) is an important factor in the profitability from industrialization of the ABE process. The profitability and sustenance of a cellulosic butanol plant will depend heavily on the cost of enzymes (cellulase, xylase etc) that is used to break the cellulosic biomass to fermentable sugars (Eveleigh et al., 2009). In our current study, we do not add any external enzymes during SSF. It is believed that the introduction of *Ct* in the fused strains is able to saccharify cellulose and cellobiose. Studies have revealed that *Ct* has immense potential of hydrolyzing cellulose by a different mode of action from the classical mechanism involving solubilization by cellobiohydrolase (Zhang and Lynd, 2005).

ABE fermentation is a two phase process involving acidogenis (production of acids like acetic and butyric acid) followed by solventogenesis (production of solvents by conversion to acetone, butanol and ethanol) (Dürre, 1998). This process is further discussed in the next section. In the present study, the fused protoplast strains were passed on to successive five growth cycles and the resulting strains from each cycle were later inoculated with substrate and examined for solvent productivity.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofuel Statistics and BioButanol History

Biofuels are an appealing substitute to current petroleum based fuels primarily because of their compatibility with current engine technologies. Bioenergy, in general, is an imperative option to mitigate greenhouse gas emissions and to substitute fossil fuels (Hamelinck and Faaij, 2006). According to the "International Energy Outlook 2011" report prepared by the Center for Strategic and International Studies, renewables are the world's fastest-growing energy source, at 2.8% per year and by 2035 they will grow to roughly 15% of the world energy market. The report also indicates an enormous futuristic switch towards modest use of liquid biofuels driven by increasing oil prices. By 2035, liquid biofuels will comprise 29% of the world energy consumption translating to approximately 5 million barrels per day of unconventional production (International Energy Outlook, 2011). This rapid increase in reliance on biofuels is associated with access to vast amounts of biomass. During the next century, biomass is believed to posses the potential in becoming a major global energy source. By 2022, over 75% of the advanced biofuels will eventually come from cellulosic materials to produce a combination of ethanol and other advanced alcohols (Solomon, 2010). Bioenergy systems will be a principal contributor to a future of sustainable energy and development in industrialized and developing nations. (Berndes et al., 2003). In the US, biofuels will be an important contributor to the industrial sector and are projected to grow from 0.2 quadrillion Btu in 2005 to 0.9 quadrillion Btu in 2030 (Khatib, 2011). Brazil has been extremely successful in becoming a world leader in biofuel production through government regulation and policy. Currently, all petrol sold in Brazil contains a 20-25% ethanol blend on volume basis (Lamers et al., 2011). The increased demand for biofuels is also reflected under the US Energy Independence and Security Act of 2007 which sets production targets to 15 billion gallons of corn ethanol by 2015 and an additional 21 billion gallons of cellulosic and other

advanced biofuels including biobutanol by 2022 (Wiens et al., 2011). Biofuels produced from first generation food crops are not potentially desirable on a commercial scale as an increase in oil prices would lead to reductions in agricultural output. Studies have shown that a 25% increase in oil prices from its baseline would reduce global food supply including processed foods by 0.7% in 2020. Regions like China, India, South East Asia and Eastern Europe could face severe food supply reductions of 3.5-6.1% from baseline if such a scenario would occur (Timilsina, 2011). Therefore, considerable emphasis is being made on biofuels from second generation sources like wheat straw, corn stover and other agricultural residues.

Biobutanol is a biofuel produced from anaerobic fermentation and has been the subject of immense interest since 1862. French Scientist Louis Pasteur reported butanol as a fermentation product of a culture containing *C. butyricum* or *Ca.* Microbial solvent formation was also used in industry with ABE fermentation leading the way in becoming the second largest biotechnological process. When prohibition was introduced in 1920s in the United States, there was an immediate shortage of amyl alcohol used to produce amyl acetate, a solvent used in quick-drying lacquers required by the growing automobile industry (Dürre, 2007). Butanol proved to a be a perfect alternative for amyl alcohol and several ABE production plants were opened in different countries to fulfill the industrial requirements. Therefore, until 1950 two thirds of world's butanol supply came from biological fermentation. However, a decline in fermentation was associated with increasing costs of substrates, low crude oil prices and high enzyme costs. From that point until 2005, butanol was an intermediate chemical in producing acrylate and methacrylate esters, glycol ethers, butyl acetate, butylamines and amino esters. However, BP and DuPont have proposed to restart an industrial ABE fermentation plant to provide biobutanol to Great Britain (Dürre, 2007). Cobalt biofuels began producing biobutanol at its pilot plant in California and expect to complete a 1 million - 2 million gallon per year demonstration scale plant by mid 2012 co-located with an existing pulp and paper mill. The company is currently working towards producing more effective microbes that convert both corn and other cellulosic feedstocks into

biobutanol. Denver based Gevo Inc is lining up financing for five more ethanol plants for conversion to biobutanol. These new biobutanol capacities could be up to 200 million gallons per year if the company succeeds in raising finances (Scotia Captial Inc, 2010).

2.2 Agricultural Biomass as Substrate

Biomass is a contemporaneous and complex biogenic, organic-inorganic solid product composed of three major components that include cellulose, hemicellulose and lignin (Vassilev, 2010). In terms of elemental analysis, biomass elements can be classified into major, minor and trace according to their elemental concentrations on a dry basis. Major elements include C, O, H, N, Ca and K, while minor elements include Si, Mg, Al, S, Fe, P, Cl, and Na. Trace elements may include Mn and Ti (Vassilev, 2010). Most biomass is composed of roughly 40% cellulose, 25% hemi-cellulose and 25% lignin by mass with ash and miscellaneous compounds making up the remaining 10%.

Biomass provides a potential source of value not only in the form of biofuels but also other chemicals such as reducing sugars, acids and furfural. Three important biomass conversion techniques include thermochemical and biochemical. Thermochemical conversion can be performed using a variety of processes like direct combustion to solid biomass, pyrolysis of biomass, distillation of biomass, gasification of biomass and hydro-gasification (Küçük and Demirbaş, 1997). Biochemical conversion is a process where biomass is converted to biogas, alcohols and other wastes and water. Biochemical processes can be aerobic or anaerobic or alcoholic fermentations that produce ethanol, butanol, carbondioxide, methane, water and wastes. They are characterized by low energy consumption and are the most promising, environmentally sustainable alternatives for reducing green house gasses. Several agricultural wastes are available for conversion into energy. Table 2.2 lists the amount of biomass generated from agricultural crop residues in Canada. Almost 14.6 Million tonnes of Carbon per year (Mt C/yr) with an energy value of 0.6 EJ (exajoule), is recoverable from the field at

the same time preserving soil integrity and accounting for losses during harvest process. However, traditional uses reduce the current availability to 8.6 Mt C/yr with an energy potential of 0.3 EJ (Wood et al., 2003).

The non-edible part of the biomass is considered to be the second-generation lignocellulosic biomass. Figure 2.1 shows the structure of the three components associated with lingo-cellulosic biomass intertwined with each other to form a fibre matrix. This complexity creates strength associated with biomass. The first component is cellulose and is a strong un-branched polymer of β (1,4)-linked glucose chains. Figure 2.1 shows the cellulose fibres as long cylindrical structures (Badal et al., 2011). On the contrary to cellulose, hemicellulose comprises of highly branched polymer structure of both glucose and other sugars containing primarily five carbon atoms in their ring. During dilute acid pretreatment, hemicellulose is easily hydrolyzed into xylose and other pentose sugars.

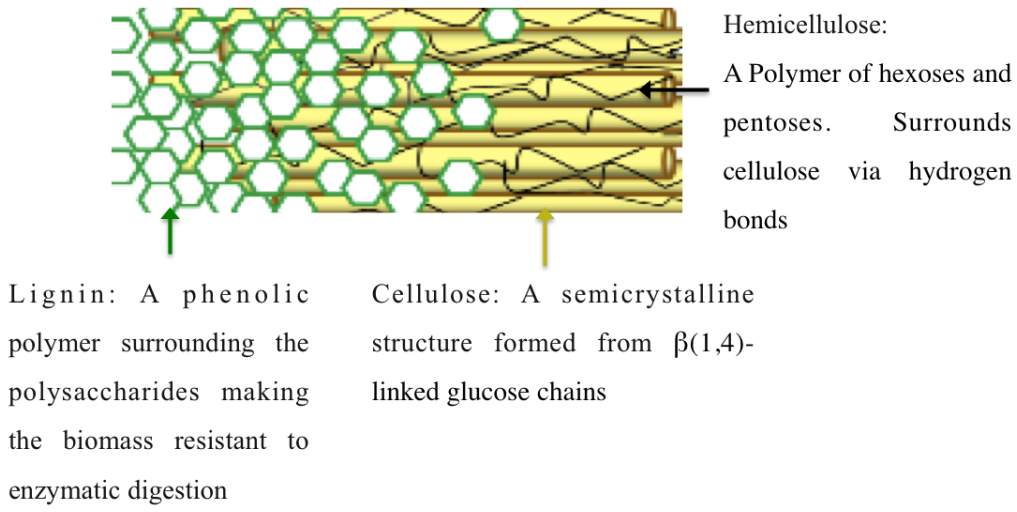

Figure 2. 1 Structure of lignocellulose with the components

Lignin is the third component in the lignocellulosic matrix and occupies the interstitial space of a plant cell. It interacts with hemicellulose chains through covalent bonding and is responsible for the strength and structure of the lignocellulosic matrix. This phenolic polymer surrounding the polysaccharide network makes the biomass resistant to enzymatic digestion unless it is pre-treated with

steam or acid. Therefore, the pretreatment should be able to break down the matrix and hydrolyse the cellulose and the hemicellulose into respective sugars. Sometimes, after pretreatment especially under high temperatures some inhibitory compounds like weak acids, furfural (from xylose), 5-hydroxymethyl furfural (from C6 monosaccharaides), and phenolic compounds from lignin can be produced (Qureshi et al., 2007). Table 2.1 shows the concentration and percentages of sugars in different agricultural wastes.

Table 2. 1 Composition of sugars for different sources of lignocellulosic biomass

Sugars	**WS**		**DDGS**		**CF**		**Molasses**	
	Actual (g/L)	wt %	Actual (g/L)	wt %	Actual (g/L)	wt %	Actual (g/L)	wt %
Glucose	28.9	48	23.6	44.9	37.2	53.4	14	25
Xylose	20.1	33.4	16.7	31.7	17.6	25.3	0	0
Arabinose	5	8.3	10.3	19.6	11.3	16.2	0	0
Galactose	3.5	5.8	1.2	2.3	3.6	5.1	0	0
Mannose	2.7	4.5	0.8	1.5	0	0	0	0
Sucrose	0	0	0	0	0	0	28	50
Fructose	0	0	0	0	0	0	14	25
Total sugar	60.2	100%	52.6	100%	69.6	100%	56	100%
References	(Qureshi et al., 2007)		(Ezeji and Blaschek, 2008)		(Qureshi and Ezeji, 2008)		(Shin et al., 1982)	

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

Table 2. 2 Yield of crop and crop residues in Canada

	Yield of Crops and Crop Residues						Currently Available Residues		
Crop	**Total Production**	**Straw/Stover**	**Sustainably Removable Residues (SRR)**	**Recoverable SRR**		**Energy Potential of SRR**	**Amount Available**	**Carbon content**	**Energy potential**
Units	M ODT/yr [a]	M ODT/yr	M ODT/yr	M ODT/yr	M tC/yr	EJ/yr [b]	M ODT/yr	Mt C/yr [c]	EJ/yr
Wheat	20.6	26.7	21.4	14.97	6.74	0.241	7.49	3.37	0.12
Barley	10.8	10.8	8.68	6.07	2.73	0.098	3.04	1.37	0.049
Oats	2.7	2.7	2.15	1.51	0.68	0.024	0.75	0.34	0.012
Grain Corn	8.3	8.3	6.65	3.33	1.5	0.054	3.33	1.5	0.054
Canola	4.9	4.9	3.94	2.76	1.24	0.044	2.76	1.24	0.044
Soybeans	1.6	1.6	0.33	0.16	0.07	0.003	0.16	0.7	0.003
Flaxseed	0.72	0.72	0.57	0.4	0.18	0.006	0.2	0.9	0.003
Rye	0.23	0.23	0.18	0.13	0.57	0.002	0.06	0.29	0.001
Fodder Corn	5.2	0	0	0	0.26	0.009	0.26	0.12	0.004
Tame hay	23.1	0	0	0	1.157	0.041	1.16	0.52	0.019
Totals	78.27	56.09	43.89	29.33	14.62	0.523	17.79	8.64	0.309

[a] M ODT/yr represents Million Oven dried tonnes per year [b] EJ/yr is Exajoule per year. 1EJ =10^{18} J [c] MtC/yr represents Million tonnes of Carbon per year

From the Table 2.2, (Wood et al., 2003) one can realize the immense energy potential associated with wheat straw in Canada. Out of a total production of 26.7 million oven-dried tonnes per year (M ODT/yr) of wheat straw, more than 50% of it is recoverable (after harvest) contributing to a potential energy contribution of 0.241 EJ/year. However, currently only 7.46 M ODT/yr is available, contributing to about 0.12 EJ/year, roughly half of its maximum potential. The reader might also realize from the table the need to invest in wheat straw as substrate in Canada due to large-scale production (of roughly more than a quarter of current crop production) coming from wheat crops.

2.3 Acetone-Butanol-Ethanol (ABE) Fermentation Metabolic Pathways for Butanol Production

Several studies have shown that butanol can be produced from numerous microorganisms belonging to the clostridium family like *Ca* or *Cb* (Schoutens et al., 1985) (Formanek et al., 1997). The enzymes produced by the strains can consume and metabolize sugars into acetone, butanol and ethanol as shown the Figure 2.2.

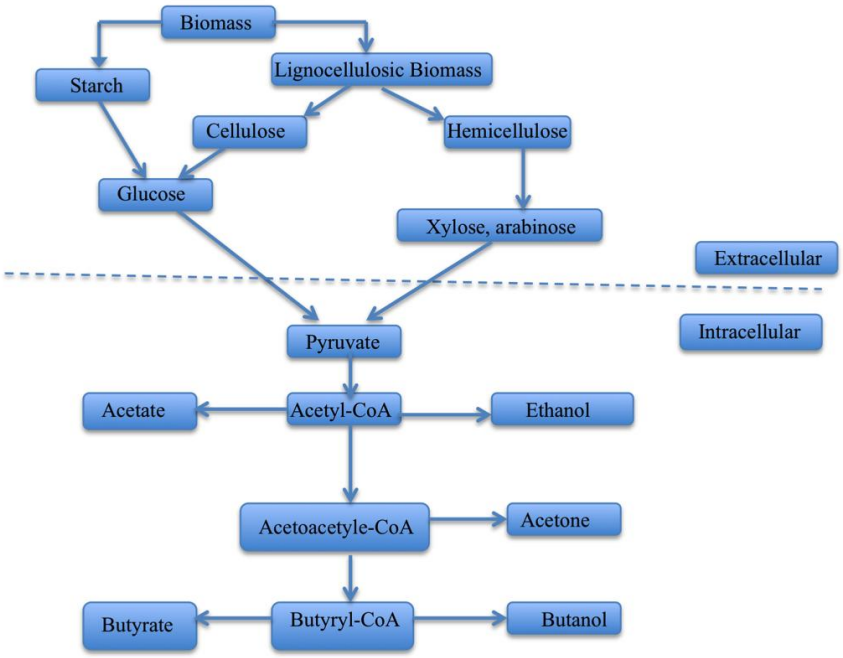

Figure 2. 2 Acetone-butanol-ethanol fermentation metabolic pathway (Nigam and Singh, 2011)

Clostridia are gram-positive, rod shaped anaerobes that use fermentative pathways to generate organic solvents like butanol and ethanol. Clostridia, unlike yeast, are able to metabolize and break down a wide range of saccharides including five carbon sugars. These type of solventogenic clostridia are of prime importance because of their ability to produce organic acids and alcohols by fermentation (Nölling et al., 2001). The process of production of the solvents is very complicated and difficult involving secretion of numerous enzymes by clostridia that facilitate the breakdown of polymeric carbohydrates into monomers of simple sugars. Furthermore, butanol toxicity is a major issue in ABE fermentation. A concentration of around 12 g/L of n-butanol is relatively toxic and will inhibit the bacterial cells from producing more solvents. (Nigam and Singh, 2011).

There are two metabolic units in the stages of organic acids formation. The first one is the unit of acetic acid formation from carbohydrate (glucose) and the second is the metabolic unit of butyric acid formation from glucose. Both process are shown in the Figure 2.2. For the first unit, Glucose is converted to pyruvate and then to acetyl-CoA, which is converted to acetyl-P using the enzyme phosphate transacylase and finally to acetate (acetic acid) using the enzyme acetate kinase. The second metabolic unit of this acidogenic stage is the conversion of glucose to butyrate. Glucose is converted to pyruvate through glycolysis, which is then converted to acetyl-CoA. It then uses the enzyme thiolase to convert to acetoacetyl-CoA. The enzyme 3-hydroxybutyryl-CoA dehydrogenase converts acetoacetyl-CoA to 3-hydroxybutyryl-CoA. Crotonase enzyme then converts the previous product to crotonyl-CoA. Butyryl-CoA dehydrogenase converts crotonyl-CoA to butyril-CoA. This product can be then converted to butyrate utilizing any of three possible pathways. These units comprise the acidogenesis sequence. The solventogenic sequence follows the same sequence as the first metabolic unit of the acidogenic sequence until the formation of acetyl-CoA. This product in the solventogenic stage is converted first to acetaldehyde using enzyme acetaldehyde dehydrogenase and is later converted to ethanol using ethanol dehydrogenase. The same acetyl-CoA is also converted to acetoacetyl-CoA using

thiolase and then converted to acetoacetate and finally to acetone using enzyme acetoacetate decarboxylase. The pathway for the generation of the butanol solvent is similar to the butyrate acidogenic pathway until the production of butyrl-CoA. While in the acidogenic pathway butyril-CoA is converted to butyrate, in the solventogenic pathway the product is converted to butyraldehyde and later to butanol using the enzyme butyraldehyde dehydrogenase and butanol dehydrogenase respectively (Jones and Woods, 1986).

2.4 Pretreatment and Hydrolysis

The complex intertwined network associated with the lignocellulotic biomass possesses a technological challenge in processing of the cellulose and hemicellulose. The process of breaking the bonds between the lignin and other carbohydrates using chemical or thermochemical operation is called pre-treatment. Several pre-treatment methods like steam explosion, ammonia fibre explosion (AFX), dilute acid hydrolysis, lime treatment, carbon dioxide explosion, alkaline hydrolysis, oxidative delignification, pulsed-electric-field pre-treatment, biological pre-treatment and many others have been employed in past studies (Kumar et al., 2009).

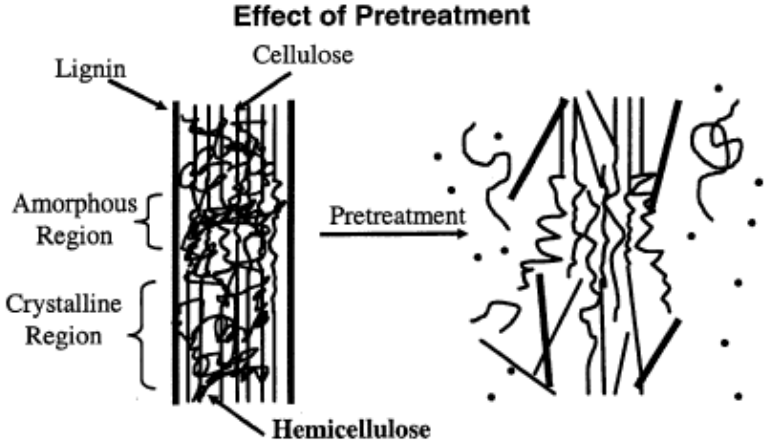

Figure 2. 3 Effect of pretreatment on lignocellulose (Harmsen and Hijgen, 2010)

During pretreatment, as shown in Figure 2.3, the biomass structure is altered so that enzymatic hydrolysis of cellulose and hemicellulose can be achieved more rapidly producing greater yields (Harmsen and Huijgen, 2010). If pretreatment were not performed, the hydrolysis enzymes would face difficulty accessing the polysaccharide chains embedded within the lignin polymer.

Steam pre-treatment coupled with dilute acid constitutes the best strategy to convert all hemicellulose into monosaccharides and oligosaccharides. While the wet-oxidation and alkaline methods are relatively more effective in solubilizing lignin, they leave behind insoluble hemicellulose in polymeric form (Bjerre et al., 1996). Hot water treatment is of particular interest because it does not involve any harsh chemicals and unlike the acid hydrolysed steam pre-treated lignocellulose, does not inhibit the yeast during fermentation (Allen et al., 2001).

However, dilute acid pretreatment is one of the most effective pre-treatment methods for lignocellulosic biomass. Dilute sulphuric acid is sprayed onto the raw material and mixture is held for a few minutes at temperature in the range of 100-220^{o}C. During this time, hemicellulose is hydrolysed to xylose releasing the monomer sugar and other soluble oligomers from the cell wall matrix into the hydrolysate. With the removal of hemicellulose, porosity within the lignocellulose increases thereby improving enzymatic digestibility (Kumar et al., 2009). The reader might wonder as to why the current study has chosen dilute acid treatment over other pretreatment processes. This method is the most cost effective method and produced outstanding results in comparison to other treatment methods (Mosier et al., 2005). Dilute acid pretreatment has the highest biofuel production of 56.1 MM gal/yr with 64% of the pretreatment taking place in the reactor itself and rest in other specialized unit operations. This distribution can significantly reduce costs associated with purchasing and maintaining equipment for other treatment methods. For example, for Lime pretreatment only 19% of the treatment occurs in the reactor and the rest in other sections of the plant. For AFEX, 26% of the treatment happens in the

reactor (Harmsen and Huijgen, 2010). Once pretreatment is accomplished, enzymatic hydrolysis can be done to convert remaining lignocellulose into sugars.

The conventional production of biofuel from cellulose involves a complex process of pretreatment, production of cellulose and other enzymes, hydrolysis of cellulose and hemicellulose using these enzymes, and finally fermentation of hexose and pentose sugars into biofuels. Cellulase are enzymes that are highly specific and are organized into three major group of enzymes including groups that attack areas of low crystallinity in cellulose, groups that degrade the molecule further by removing the cellobiose links between the monomers and groups which hydrolyze cellobiose to glucose (Coughlan, 1991). These three groups of enzymes are endocellulase, exocellulase and cellobioase (β-Glucosidase) in the order of their functionality as describe previously.

2.5 Fermentation Processes and Configurations for Butanol Production

To render ABE fermentation economically feasible, it is important to integrate pretreatment, hydrolysis, fermentation and recovery into one single stage (Hahn-Hägerdal et al., 2006). Technological improvements from developing efficient hydrolysis enzymes to process integration and economic feasibility in selecting agricultural residues as substrates has strengthened the competitiveness of fermentation to produce fuel as opposed to petroleum refining. Several integrated processes displayed in Table 2.3 have helped in boosting the ABE productivity to commercial standards.

Table 2. 3 Sugar and AB/ABE concentration for various product recovery systems

Fermentation and Product recovery Integrated system (substrate)	Substrate concentration (g/L)	AB or ABE produced (g/L)	Literature
Control (Batch, glucose, no recovery)	48.9	20.1	(Qureshi et al., 2007)
Gas stripping (Fed-batch, glucose)	500	232.8	(Ezeji, 2004)
Perstraction (Batch, whey permeate)[a]	227	99.3	(Qureshi and Maddox, 2005)
Gas stripping (Batch, WSH)[b]	128.3	47.6	(Qureshi et al., 2007)
Pervaporation (Fed-batch, glucose)[c]	342	119	(Qureshi and Blaschek, 2001a)
Pervaporation (Batch, glucose)	121.2	51.5	(Qureshi and Blaschek, 1999)
Gas stripping (Batch, whey permeate)	200	70	(Maddox et al., 1995)

[a] *Culture volume 1.38 L. Values in table are per liter broth (total sugar 313.3 g, total ABE 137.0 g)*
[b] *Total sugars include hexoses and pentoses*
[c] *Culture volumes 1-1.3 L. Values in table are per liter broth (total sugar 444.6 g, total ABE 154.7 g)*

Typically batch reactors yield low ABE productivities unless they are accompanied with use of novel fermentation technologies like free cell continuous fermentation, cell recycle and immobilized cell reactors. Studies have shown that continuous free cell systems offer higher productivity due to reduction in downtime associated with batch operations. However, a high cell concentration is difficult to achieve as retention of cells within the reactor is highly implausible and would result in cell washouts at higher dilution rates (Qureshi and Ezeji, 2008). To account for this technological obstacle, a “cell immobilization” and “cell recycle” systems were developed. Cell concentrations in the range of

50-70g/L were achieved using cell immobilization techniques. Moreover, such reactors can operate at high flow rates without experiencing any cell washout thereby increasing reactor productivities. Cell Concentrations in the range of 6.5-15.8g/L can be achieved using immobilized cell reactors (Qureshi and Ezeji, 2008). Cell recycle membrane reactors also increase cell concentration inside the reactors. The cells remain suspended in liquid broth and a membrane is used to prevent the cells from being washed out. While the reactor productivities of 6.5 g/L.h were achieved, major limitations associated with membrane fouling, membrane cost makes this technology less feasible.

Several studies have been implemented to produce ABE from lignocellulosic substrates such as wheat straw (WS). The substrates were hydrolysed using dilute acid and steam treatment. The WSH contained approximately 68.3 g/L of total sugars with a supplement of 60g/L glucose resulting in a total sugar concentration of 128 g/L. The substrates then produced solvents in a batch reactor with continuous recovery of solvents thereby reducing the product inhibition associated with butanol toxicity. A total of 47.6 g/L of AB was produced (Qureshi and Ezeji, 2008).

Similar to solvents, substrates also inhibit cell activity and therefore interfere in the metabolism of the cells. Substrate inhibition is very common in fermentations that produce solvents. In substrate inhibition studies (Qureshi and Blaschek, 2000), (Qureshi and Blaschek, 2001b) with *Cb* BA101, it was shown that high substrate concentration was toxic to *Cb* BA101. However, it was concluded that cell activity is severely affected for glucose concentrations only above 158g/L. Batch fermentation studies for butanol production using just *Cb* and wheat straw substrate under similar conditions as the current study (Liu et al., 2010) have shown that around 35-40 g/L of sugars (glucose, xylose, arabinose etc.) could be utilized by the strain during the batch fermentation process and that any supplemented glucose did not increase the butanol production. This was primarily due to butanol toxicity.

Studies have shown that toxicity of alcohols appears to increase with chain length with long chain alcohols being more toxic than short chain alcohols. (Ezeji et al., 2010). When solvent

concentration exceeds certain limit, it results in instantaneous inhibition of membrane-bound ATPase enzyme activity, which is essential to maintain an internal pH and an electrochemical gradient, and butanol in particular inhibits the activity of this enzyme by increasing the activation energy of the enzyme and disrupting the phospholipids of the cell membrane. This phenomenon was particularly observed by the addition of butanol to *Ca* (Ezeji et al., 2010).

The change in pH during SSF is also an important indicator of processes happening during fermentation. The phenomenon of "acid crash" is an occasional feature of batch fermentations which occur without any pH control (Maddox et al., 2000). During an acid crash excess acid production takes place without a significant switch to the solventogenic phase. Fermentation studies conducted in New Zealand and Austria have found that during ABE batch fermentation an acid crash is likely if the acidic concentration in the broth exceeds 60 mmol/L (Maddox et al., 2000).

2.6 Protoplast Fusion and Co-culture Technology

Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells. Protoplast can be readily isolated from bacterial cells by digestion of cell walls with the help of lysozyme in the presence of osmotic stabilizers (Gokhale et al., 1993). Chemical fusogens like polyethylene glycol (PEG) can help induce the fusion process. However, several other complex, efficient and expensive technologies like electrofusion reported higher fusion frequency than PEG induced fusion.

The use of a relatively simple technique of protoplast fusion as a means to produce novel strains and new products unobtainable by conventional methods is exciting and has aroused great interest in breeding intraspecies, interspecies and intergeneric hybrids for improving abilities of industrial microorganisms like that of the bacillus genera. Morphology and growth characteristics of the intergeneric hybrids are similar to that of the parental strains. Studies have shown that the hybrids

possess parental morphologies retaining common genes from both the parents (Puntambekar et al., 1995) (Chen, 1987) (Fujita and Saito, 1990).

Studies on industrial strain improvement through protoplast fusion has also resulted in improvement in glucose metabolising activity in *Brevicaterium* and enhanced lignin degradation activity (Gokhale et al., 1993). The problem often encountered with protoplast fusion technology is the stability of the fusion products. However, studies using PEG have shown that haploid (bacterial prokaryotic) recombinants can maintain genetic stability. Protoplast fusion between *Cellulomonas* and *B. Subtilis* showed that only four out of 11 hybrids are stable and did not segregate back into parental types even after multiple transfers to rich medium (Gokhale et al., 1993). Studies with protoplast fusion involving *Corynebacterium acetoacidophilum* and *B. Subtilis* were very stable even after growing them for 30 generations (Deb et al., 1990).

The mechanism of protoplast fusion is not fully understood and several explanations have been provided. Literatures show that when the protoplasts are closely adhered, the external fusogens (PEG) cause disturbance in the intramembranous proteins and glycoproteins. This increases membrane fluidity and creates a region where lipid molecule intermix, allowing coalescence of adjacent membranes. The negative charge carried by protoplast is mainly due to intramembranous phosphate groups and therefore the addition of Ca^{2+} ions causes a reduction in zeta potential of plasma membranes and under this situation the protoplasts fuse. The high molecular weight of PEG has been known to act as a bridge connecting the protoplasts (Peberdy, 1980).

Although they have been reports of protoplast formation and regeneration of certain strains of *Ca* (Reilly and Rogers, 1987)(Allcock et al., 1982) and for *Cb* (Birrer et al., 1989), there was no mention of protoplast fusion using PEG (Gokhale et al., 1993). The prospects of ABE solvent production will be greatly enhanced if this technology is adapted in feeding genetically modified strains to the fermentation process.

Once a protoplast-fused strain is obtained it is important to check for stability. In several protoplast fusion studies, (RN Krishnamoorthy, 2010 and Y Couteaudier, 1996) the protoplast fused strains were passed on to consecutive growth cycles and the strains obtained from each growth cycle were later inoculated with substrate and examined for products. Apart from this, PCR and Gel Electrophoresis were also used to check for genetic stability.

A coculture is defined as anaerobic or aerobic incubation of different specified microbial strains under aseptic condition. Degradation and metabolism of substrates occur by the combined metabolic activity of the known microbial strains (Bader, 2010). The microorganisms present in a coculture system can either defend their habitat against intravenous species by secreting of growth-inhibition substances or can exist in a symbiotic relationship with each other (Rikkinen et al., 2002). In terms of utilization of lignocellulosic biomass, coculture fermentation processes offer the possibility to implement all required enzymatic conversions in one step inside the bioreactor. The simultaneous conversion of glucose and xylose to ethanol by using a co-culture of *Z.mobilis* and *P.stipidis* has been reported (Maki et al., 2009). This study has reported co-culturing *Ct* together with other strains of *Clostridium sp.* or *Thermoanaerobacterium saccharolyticum* to improve the prospects of metabolizing both glucose and other hemicellulose derived pentoses. Production of butanol from crystalline cellulose was examined by developing a co-culture of *Ct* and *C.saccharoperbutylacetonicum* and a similar medium was used to undertake co-culture experiments in the current study (Nakayama et al., 2011).

2.7 Enzyme Activity

Employing pretreatment process is essential before enzymatic hydrolysis. The removal of lignin and lower cellulose crystallinity achieved during the pretreatment process can significantly improve the yield of enzymatic saccharification. There are three different enzymes needed for the enzymatic hydrolysis of cellulose. The long chains of cellulose are broken down into shorter chains (typically two

linked glucose chains) by endo-glucanase. Cellobiohydrolase then breaks down two linked glucose molecules (cellobiose) from the ends of cellulose chain. β-glucosidase then cleaves cellobiose into glucose molecules that can later be converted to acids and solvents. These three enzymes are a part of the bigger enzyme complex called cellulase (Eveleigh et al., 2009). Cellulase activity is primarily evaluated using reducing sugar assay to measure the end products of cellulose hydrolysis activity. In our case, the end products of cellulose hydrolysis are most primarily glucose, which is converted later to biobutanol by the bacterial strains. To measure and compare efficacy of cellulose activity between different strains and their secreted enzymes, a method to determine total cellulose activity is necessary (Dashtban et al., 2010). Filter Paper Activity (FPA) method was developed by Mandels in 1976 (Lin et al., 2010). The international unit of filter paper activity is defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions, where assay conditions refer to the conditions such as pH and temperature at which the enzymes are held at during the assay and depend largely on the properties of the enzyme, varying widely between cellulases and microorganisms (Dashtban et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Chemicals

Ca (ATCC 459*)* and *Cb* ATCC BA101 were purchased from American type culture collections. *Ct* was obtained from Dr. Wolfaardt's lab at Ryerson University. All the chemicals were purchased from Sigma-Aldrich Canada, and were used as received without any further purification. Table A1 in appendix A summarizes all chemicals that were used in the current study.

3.2 Experimental Procedure and Methodology

In the present work, ABE solvent production was examined for SSF using two protoplast fused strains of the wild clostridial species and were compared with co-culture fermentation of the corresponding species. Fused strains were further studied for their reproducible stability.

3.2.1. Culture Conditions and Medium Preparation

All Clostridium strains (wild and fused strains of *Ca*, *Cb*, and *Ct*) were maintained as cell suspension in medium containing 30% (v/v) sterile glycerol and Cooked Meat Medium (CMM) and stored in eppendorf tubes in -82^{o}C ultra low freezer (Thermo fisher Scientific, USA). Before working with frozen strains, they were heat shocked for 5 min at 80^{o}C (Qureshi and Blaschek, 2001a). Any anaerobic manipulations were carried out in an anaerobic chamber (i.e., Glove Box; Terra Universal, Canada) at a temperature of 25 ± 2°C. The elements of aseptic technique were implemented throughout this study, including routine cleaning through wiping the work surface of the anaerobic chamber with 70% ethanol before and after work, in addition to the utilization of ultraviolet light to sterilize the exposed work surfaces.

Culture mediums such as Clostridium Growth medium (CGM), Clostridium Basal Medium (CBM), Protoplasting Medium (PPM), Agar Based Regeneration Medium (RM), Cooked Meat

Medium (CMM) MT0350 and National Biological Research Center (NBRC) medium number 979 were used in the current study. Table 3.1 summarizes all the chemicals used to prepare the mediums for protoplast fusion and co-culture. CMM (obtained from the supplier) were used for culture activation/maintenance of *Ca*, *Cb* and fused strains (i.e., *CaCt*, *CbCt*) and were prepared by dissolving 12.5 g respectively in 100 mL distilled H_2O.

From Table 3.1, CBM is a medium used to grow wild and fused clostridium strains and was prepared by adding 2g Glucose, 0.04g $MgSO_4.7H_2O$, 0.002g of $MnSO_4.4H_2O$, 0.002g of $FeSO_4.7H_2O$, 0.0002g of Para-Amino benzoic acid (PABA), 0.004g of Biotin, 0.00002g of thiamin HCl and 0.8g of Casein hydrolysate in 200ml water. The CBM medium was used for growing clostridium cultures (Allcock et al., 1982). Two basic vitamins required to maintain active growth of the culture are biotin and PABA, with PABA being the limiting factor (Huang et al., 2004). PPM was prepared by adding to CBM, 0.3 M Sucrose, 50 mM $CaCl_2.2H_2O$ and 50mM $MgCl_2.6H_2O$. The solution was autoclaved (SANYO labo autoclave, USA) at 120^oC for 20 min (Qureshi et al., 2008). This medium along with lysozymes was used for generation of protoplasts during protoplast fusion (Reilly and Rogers, 1987). CGM was used as alternative medium to CBM for growing Clostridium bacteria for the co-culture process. CGM was found to have greater selectivity for butanol (Mpho Setlhaku 2009). It contains 0.75g/L KH_2PO_4, 0.982 g/L K_2HPO_4, 1 g/L NaCl, 0.01 g/L $MnSO_4$, 0.004 g/L **p**-amino benzoic acid, 0.348 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 2 g/L asparagine, 5 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$ and 40 g/L glucose (Cai and Bennett, 2011). RM is an essential medium for growing bacterial colonies and was prepared by the addition of stock solutions to a basal mixture. Stock solutions A to E were prepared for making RM. Stock solution A contained 1g/L biotin, 1g/L PABA, 0.1g/L thiamin-HCl, 1g/L $FeSO_4.7H_2O$, 1g/L $MnSO_4.4H_2O$ and 20g/L of $MgSO_4 .7H_2O$. 100ml of Stock solution A was prepared and filter sterilized and kept in N_2/CO_2 atmosphere. Stock solution B was made by adding 25 g glucose

in 100ml of H_2O. Stock solution C comprised of 2.5 M solution of $MgCl_2$. Stock D solution consists of 2.5 M solution of $CaCl_2$ and finally stock solution E consists of 7.0g of K_2HPO_4, and 3.0g of KH_2PO4, dissolved in 100 ml H_2O. Stock solutions B through E were autoclaved separately. Basal Mixture contained 50g gelatin, 15g Agar, 8g Yeast extract, 2.5g Casamino acids, 1g asparagine.

Table 3. 1 Chemical composition of culture mediums used in this study

Medium	Chemical Composition
CGM	KH_2PO_4, K_2HPO_4, NaCl, $MnSO_4$, **p**-amino benzoic acid (PABA), $MgSO_4$, $FeSO_4$, asparagine, yeast extract, $(NH_4)_2SO_4$ and glucose
CBM	Glucose, $MgSO_4$.7H2O, $MnSO_4$.4H2O, FeSO4.7H2O, **p**-amino benzoic acid (PABA), Biotin, thiamin HCl and Casein hydrolysate
PPM	CBM, Sucrose, $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$.
CMM (MT0350)	(Provided from supplier) Beef heart solids, casein/meat peptone, dextrose and sodium chloride
RM	Biotin, **p**-amino benzoic acid (PABA), thiamin-HCl, $FeSO_4.7H_2O$, $MnSO_4.4H_2O$ $MgSO_4$ $.7H_2O$, glucose, $MgCl_2$, $CaCl_2$, K_2HPO_4, KH_2PO4, water, gelatin, Agar, Yeast extract, Casamino acids, L-asparagine.
NBRC 979	$(NH_4)_2SO_4$, $MgCl_2 \cdot 6H_2O$, KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$, $CaCl_2 \cdot 2H_2O$, Sodium glycerophosphate, $FeSO_4 \cdot 7H_2O$, glutathione, yeast extract, Resazurin and cellobiose.

These ingredients were mixed in 930ml H_2O and the mixture was stirred and brought to boiling before autoclaving at 121^oC for 20 min (Birrer et al., 1989). Upon cooling, 10ml of stock solution A and 40ml of solution B was added to the basal mixture. To make the RM medium, 5ml of each of solutions C and D was added along with 10 ml of stock solution E. This viscous medium was then poured into petri dishes and allowed to set until they form a smooth solid surface for the bacteria to grow. These are Agar streak plates and are an essential tool in microbiology. They allow bacteria and

fungi to grow on a semi-solid surface to produce discrete colonies. NBRC Medium 979 is used for co-culture and contains 1.3g/L $(NH_4)_2SO_4$, 2.6 g/L $MgCl_2 \cdot 6H_2O$, 1.43 g/L KH_2PO_4, 7.2 g/L $K_2HPO_4 \cdot 3H_2O$, 0.13 g/L $CaCl_2 \cdot 2H_2O$, 6 g/L sodium glycerophosphate, 1.1 mg/L $FeSO_4 \cdot 7H_2O$, 0.25 g/L glutathione, 4.5 g/L yeast extract, 1 mg/L Resazurin and 5 g/L cellobiose. All ingredients except for cellobiose are mixed and autoclaved under N_2/CO_2 condition. Filter-sterile 10% cellobiose solution was added aseptically and anaerobically before inoculation (Nakayama et al., 2011). Cooked meat medium supplemented with 0.1% glucose (AY Kim, 1994) was used to maintain bacterial strains. CMM is composed of beef heart solids (100 g), casein/meat peptone (20 g), dextrose (2 g) and sodium chloride (5 g).

3.2.2 Protoplast Fusion Process

Protoplast Fusion was employed to produce genetically improved strains for ABE fermentation. In this method cell gene recombination occurs because the DNA of two kinds of non-divided cells co-exist inside a cell during fusion. The method is primarily used for cell function improvement and is well-known process to change the genetic characteristics of microorganisms without the need of complicated engineering techniques (Gokhale et al., 1993).

Three wild strains were used for the protoplast fusion process. This includes *C. beijerinckii* (*Cb*), *C. acetobutylicum* (*Ca*) and *C.thermocellum* (*Ct*). A total of two protoplast fusions were produced. The first fusion was from protoplast derived from *Ca* and *Ct* (i.e., *CaCt)* and the second was from the fusion of protoplast derived from *Cb* and *Ct* (i.e., *CbCt*). The first generation of the protoplast fused strains were referred to as the parent strains. They were passed through several growth cycles to check for the stability of the parent strain.

The fusion process consists of three steps; formation of protoplast, fusion and regeneration of cell wall around the fused protoplast (Birrer et al., 1989). To prepare for the first step, overnight cultures of the wild strains were harvested by centrifugation at 10600 x g for 10 min at room

temperature and then were diluted 1:4 with fresh and sterile CBM broth containing 0.4% or 0.8% glycine. After 45-60 min when the cultures became nearly 100% motile, the osmotic strength of each culture was increased by suspending in 5ml PPM. The pH was adjusted to 7.5. A 2.5mg/ml of Lysozyme (Chicken egg white grade1) was added to the cell suspension and incubated at 35^{o}C for 60min. After 60 min, the PPM cell suspensions were centrifuged at 3300g for 5 min to extract the protoplast pellets and were resuspended in fresh PPM medium without lysozyme. The protoplast pellets were directly used if the fusion is to be performed the same day or were stored by resuspending in fresh PPM without lysozyme. The protoplast remains intact in this medium. Protoplast formation was performed for *Ca, Cb* and *Ct* individually. Protoplasts of *Ca* were mixed with *Ct* and that of *Cb* were mixed with *Ct* before precipitation by centrifugation at 1500g for 10 min at 20^{o}C**,** and the pelleted protoplasts were gently resuspended in 1 ml polyethylene glycol (PEG) 4000 (40%, w/v) in PPM for 2-3min. Some of the common ways of mixing protoplasts are chemofusion, mechanical fusion and electrofusion (S Gleddie, 1989). During chemical induced protoplast fusion (chemofusion), both protoplasts that carry negative charges around the outside of the plasma membrane are mixed and treated with fusogens like PEG (molecular wt. of 1000). Dilutions of the fused protoplasts were plated onto RM and incubated at 34^{o}C for 2 days (Jones et al., 1985). A sterile inoculating loop was used to streak the fused protoplast onto the agar RM plates. The loop was sterilized by holding it under a flame until it is red-hot. The agar plates are placed in anaerobic jars (HP011, Thermo Scientific) and sealed tightly before incubating them at 34^{o}C for 2 days. The colonies were extracted and suspended in CBM medium with 30% (v/v) sterile glycerol at -82^{o}C in eppendorf tubes (Liu et al., 2010). The eppendorf tubes are labeled parent or first generation of the fused strains. Second generation of fused strains were produced from this parent strain by inoculating 100ml of CBM culture medium with 8ml of parent strain and incubating in an anaerobic environment for 3-5 days at 45^{o}C.

In the present study, the parent protoplast fused strains were passed on to successive growth

cycles and the cell culture obtained from each growth cycle were later inoculated with substrate and examined for products. Samples from five growth cycles were collected in eppendorf tubes and labeled for both *CbCt* (fused) and *CaCt* (fused) strains.

All microbial manipulations were carried out inside a sterile glove box. Anaerobic environment was created inside the glove box by initially purging any air out of the box using a vacuum pump for about 8-10 minutes and later was supplied with a constant flow of N_2 gas until all the containers were tightly sealed. Before protoplast fusion, the protoplast regeneration frequency for all the wild strains was calculated using the formula 100 x (b-c)/a, where 'a' is the CFU colony forming unit per ml of cells grown in CBM prior to protoplast formation and was determined from a viable count after 48 h growth at 35^{o}C on agar RM plates, 'b' is the protoplasts that were formed in PPM after treatment with lysozyme (2.5 mg/ml) for 60 min and plated on RM Agar plates. CFU per ml of regenerated protoplasts was determined after 48 h growth at 35^{o}C (Jones et al., 1985). 'c' is the number of non-protoplasted units, which were determined from a viable count of the protoplast suspension after dilution in CBM to bring about osmolysis of protoplasts. The percent regeneration results are discussed in Table 4.1 (Chapter 4). Figure 3.1 shows colony forming units after plating on RM plates.

Figure 3. 1 L-Colony growth after plating on RM plates

3.2.3 Pre-treatment of Wheat Straw (WS)

The source of the wheat straw (WS) used in our study comes from *Springridge Farm* located in Milton, Ontario and was stored at room temperature. Prior to using it as a substrate for fermentation, the wheat straw was grounded into fine particles using a 1 mm Sieve screen in a hammer mill (Retsch GmbH Inc., USA). The moisture content of the wheat straw was reduced through heating in a convention oven at 105°C for 10 h until no further weight loss was reported. Acidic pre-treated WS was obtained by suspending 4.5 g (dry) in 50 mL of 1% dilute sulphuric acid (H_2SO_4) in 250 ml Wheaton serum bottles (Qureshi et al., 2008). Dilute sulphuric acid (1%) solution was prepared by adding 1ml of 99.99% sulphuric acid to 99ml water. The WS-acid solution was autoclaved at 121°C for 60 minutes. Any decrease in water content was accommodated for after autoclaving.

The serum bottles were removed from the autoclave and allowed to cool down to room temperature (Qureshi et al., 2008). This pre-treated WS can now be used in the SSF process. Previous studies in the lab have shown that the fused strains developed more rapidly at high temperatures and produced the required enzymes for the saccharification of wheat straw (i.e., endoglucanase, exoglucanase, and β- glucosidase). This eliminates the need to add enzymes required to hydrolyze the agriculture substrates in feedstocks and reduces the total cost of operation.

3.2.4 Preparation of the Corresponding Clostridial Species Co-Cultures

The co-culture fermentation used in this research is based on co-culture techniques used by the Department of Fermentation Science and Technology at the Tokyo University of Agriculture in Japan (Nakayama et al., 2011). *Ct* (8%) was inoculated with 100 ml of NBRC (National Biological Resource Center) 979 (Table 3.1) medium and incubated at 60^{o}C in strictly anaerobic environment. *Ca* and *Cb* were cultured anaerobically at 35^{o}C in CGM (Table 3.1) in strict nitrogen medium without any outside

air contamination. Co-Culture experiments were performed in a 250ml serum bottles. *Ct* cells grown in NBRC medium were collected by centrifugation. Cell pellets were washed and resuspended in the same medium without any added carbon source. Cell suspension solution (6 ml) was then inoculated in 6ml of NBRC medium containing avicel cellulose and cultured at 60^{o}C in a serum bottle.

The butanol producing *Ca* or *Cb* strains that grew exponentially were collected after 24hours by centrifugation. They were washed and suspended back into CGM medium containing 4% glucose. A 1 ml of this cell suspension was then added to serum bottles containing *Ct* after the incubation temperature was decreased from 60^{o}C to 34^{o}C (Nakayama et al., 2011). The co-culture was then incubated at 35^{o}C for about 3 days. Samples were collected during the co-culture experiments in eppendorf tubes with 30% (v/v) sterile glycerol and stored at -82^{o}C.

3.2.5 Batch SSF Using Co-cultured or Fused Strains

ABE solvents were produced in batch SSF experiments that were performed in 250 ml sealed wheaton serum bottles. Profiles for ABE concentration, sugar consumption, pH changes (acid concentrations) and cell growths were thoroughly examined for all SSF experiments. Incubation temperature during fermentation for protoplast fusion strains was maintained at 45^{o}C and for co-culture strains was maintained at 35^{o}C and 45^{o}C. Before inoculation, 40 ml growth medium (i.e., CBM) was added to provide the strains with nutrients for growth. The pH was adjusted to 6.5 with 10M NaOH and the serum bottle was transferred to the anaerobic hood. The culture medium and WS was then inoculated with 7-8ml of actively growing cells (protoplast fused or co-cultured). When dealing with bacteria, manipulations were performed under a constant supply of N_2 gas. After the inoculation was complete, the fermentation broth was bubbled with N_2 gas for about 5-10 minutes. The blue neoprene rubber stopper along with a metallic cap was used to seal the bottles using a vial crimper (Cole Palmer Canada). The bottles are brought out of the glove box and transferred to the incubator. The temperature

of the incubator was adjusted to the right fermentation temperature (35^{o}C and 45^{o}C for co-culture strains and 45^{o}C for protoplast fusion strains). SSF experiments were carried out for all growth cycles of protoplast-fused strains and for co-culture strains.

3.2.6 Sampling

Sample collection for SSF experiments and culture inoculum was performed inside the anaerobic biosafety hood, which was left in UV light for 10 min prior to sampling and was cleaned with ethanol. Syringes, needles, spatulas or any equipment that comes in contact with the bacteria was washed with ethanol sterilized under UV light for 10 minutes. After 3-5 days of SSF in the incubator, the serum bottles were placed inside the anaerobic hood and samples were collected into eppendorf tubes. The eppendorf tubes were washed with ethanol and autoclaved for 10 minutes prior sampling. Sampling was performed by inserting a sterilized syringe-needle combination through the serum bottle's rubber stopper. While sampling, it was required to load only the liquid fermentation broth medium which contained the solvents, acids, sugars and cells. Samples were collected every 12 h and kept in 2 ml eppendorf tubes at -82°C in the ultra-low freezer until analyzed. All samples were analyzed for pH, cell growth, in addition to ABE and sugar concentration using HPLC. All results reported were the average reading of triplicate sampling.

3.2.7 Enzyme Assay

The FPA assay method is used in this study to quantify the enzyme activity and is adapted from the National Renewable Energy Laboratory in the US department of energy (Adney et al., 2008). The assay is performed so that 2 mg of reducing sugar as glucose is released from 50 mg of filter paper (4% conversion) in 60 min and is assigned as the intercept for calculating Filter Paper Cellulose Units (FPU). Reducing sugar is measured according to DNS assay method (Wood and Bhat, 1988). Since the reducing sugar yield is not a direct linear function of the enzyme concentration, the assay procedure

therefore involves finding a dilution of the original enzyme stock such that a 0.5 ml aliquot of the dilution will catalyze 4% conversion in 60 min. A DNS reagent was prepared by adding 10.6 g of 3,5-dinitrosalicylic acid and 19.8 g of sodium hydroxide in 1416 ml of distilled water. The above solution was dissolved and 306 g of Rochelle salt, 7.6ml of phenol melt and 8.3 g of sodium metabisulfite was added. 3ml of this solution was titrated with 0.1 N HCl to the phenolphthalein endpoint and stored for use in filter paper assay experiment.

Three categories of experimental test tubes were prepared as follows; assay mixtures, blanks and controls and glucose standards. The substrate used is 50mg of whatman No.1 filter paper strip. For preparing the enzyme assay tubes, a rolled filter paper strip was placed in test tubes. To these test tubes 1 ml of 0.05 M Na-citrate solution was added such that the buffer saturates the filter paper strip. The buffer and substrate were brought up to equilibrium temperature of 50^{o}C by keeping in a water bath. To each test tube 0.5 ml of enzyme sample (from SSF bottles) was added. Several dilutions of the original enzyme sample were made and added to the citrate buffer. At least two dilutions are required to make sure that one dilution releases more than 2mg of glucose and the other less than 2.0 mg of glucose. However, for our enzyme assay, three dilutions, as shown in Table B2, (Appendix B) were run to target the 2 mg glucose release target. The solutions were incubated at 50^{o}C for 60min. The enzyme reaction was then stopped immediately by adding 3 ml DNS reagent.

A 0.05 M citrate buffer solution with pH of 4.8 was prepared by adding 210 g of citric acid monohydrate in 750 ml deionized water. The solution was diluted to 1L and the pH adjusted to 4.5 using NaOH. A 1.5ml citrate buffer solution was used as a reagent blank. For each dilution a separate enzyme control was prepared by adding 1 ml citrate buffer to 0.5 ml enzyme dilution. To account for a substrate control a filter paper strip was added to 1.5 ml citrate buffer.

To prepare glucose standards, a stock solution of anhydrous glucose (10 mg/ml) was made. Dilutions of this stock solution in the ratio of 1:1.5, 1:2, 1:3 and 1:5 were made in citrate buffer.

Standards for glucose were made, as shown in Table B1 (Appendix B), by adding 0.5ml of each of the

previously mentioned ratios to 1ml of citrate buffer in test tubes. Finally, all the blanks, controls, glucose standards and enzyme assays were incubated in a 50^{o}C water bath for 60 min and were stopped by adding 3ml of the prepared DNS reagent. The test tubes were then boiled for exactly 5 minutes in a boiling water bath. All samples including controls, blanks, and glucose standards were boiled together and cooled in ice-water bath. The test tube contents were then diluted in water (0.2 ml of color-developed reaction mixture in 2.5 ml water). Color formation in terms of absorbance was measured using UV spectrophotometer against the reagent blank at 540 nm.

A linear glucose standard curve with concentration units of mg/0.5 ml was plotted against absorbance at 540 nm, A_{540}. This standard curve is used to determine the amount of glucose released from each sample. Calculation of FPA from the graph of dilution versus glucose concentration was done using equation 3.1.

$$Filter\ Paper\ Activity = \frac{0.37}{[enzyme] releasing\ 2mg\ of\ glucose} \left(\frac{units}{mL}\right) \quad (3.1)$$

3.2.8 Genetic Stability Study of the Fused Strain Using PCR and Gel Electrophoresis

Polymer chain reaction (PCR) was used to amplify small amounts of DNA from the fused strains. PCR is a technique that takes a specific sequence of DNA of small quantities and amplifies it to be used for further testing. PCR targets on a DNA sequence in a region of interest, which can be a complete gene or smaller sequences within genes. It involves three stages. Firstly DNA denaturing, where DNA strands are separated by heating to 95^{o}C, secondly primer annealing at 50-60^{o}C, which is the process of allowing two sequences of DNA to form hydrogen bonds, this is where primers bind to target sequence, and finally DNA polymerization or extension by a thermostable taq DNA polymerase at 72^{o}C. Taq (taq stands for *Thermus aquaticus*) and is a thermophilic bacteria found in hot springs which produces an enzyme called DNA polymerase that amplifies the DNA from the primers by the

polymerase chain reaction, in presence of magnesium (Sadeghi et al., 2010). Primers usually range from 15 to 30 single stranded nucleotides which are used as complementary building blocks of the target sequence. They are added in excess so that they will bind to the target DNA instead of the two strands binding back to each other. To determine the genetic stability of CbCt (fused) strains genomic DNA was extracted using the MO BIO UltraClean Microbial DNA Isolation Kit (Medicorp, Canada), for growth cycles 1-5 and 10. Two biomarkers β-glucosidase A gene (*bgl*A) and aldehyde dehydrogenase gene (*ald*) from *Ct* and *Cb*, respectively, were selected for the polymerase chain reaction (PCR), corresponding primer sequences can be found in Table 3.2. The biomarker genes were chosen based on their exclusive specificity to each respective strain. The *bglA* gene was chosen for *Ct* because of its prominence and involvement in glucose metabolism. Also, its full sequence was available and when the nucleotide sequence of the gene was BLASTed on the NCBI interface against the whole genome of *Cb* there was no similarity. Additionally, the *ald* gene was chosen for *Cb* because it is a prominent gene involved in the oxidation of aldehydes and ultimately production of butanol. As well, using the available sequence information there was no similarity in the sequence of this gene to the genome of *Ct* when a nucleotide BLAST was done. Primers for *bglA* and *ald* were designed using DNAMAN software to reduce primer dimers and ensure ~50% GC content (the number of Guanine's and Cytosine's in the primer as a percentage of the total bases). They were designed within the *bglA* and *ald* gene targets to amplify a ~538 bp and ~463 bp products, respectively.

bglA is the beta-glucoside gene found in *Ct* and is made of a sequence of nucleobases in the nucleic acid of DNA. The four nucleotide bases include adenine (A), cystosine (C), guanine (G) and thymine (T). The aldehyde dehydrogenase (*ald*) is a gene found in for *Cb* . Table 3.2 also shows the amount of base pairs (bp) in the product. Base pairs are the linkage between two nitrogenous bases on complementary DNA that are connected with hydrogen bonds. Adenine always pairs with thymine from the complementary DNA strand and guanine always pairs with Cytosine.

Table 3. 2 Primer sequence and product size for biomarker gene

Gene	Forward (5'-3')	Reverse (5'-3')	Product (bp)
bglA	ATCTGGACTCGGAGGTGTAT	TTGTGCCATACCAACCATG	538
ald	ATGTTGCATGCGACCACTATC	TCGGATGCGGGATAATGGT	463

The PCR reaction mixtures contained approximately 10 ng of genomic DNA individually from each generation, 10 pmol of both forward and reverse primers, 10x Taq thermol buffer with 25 mmol/L $MgCl_2$, 0.2 mmol deoxynucleoside triphosphate, and 5 U(units) DNA polymerase per 50 µl reaction. The PCR program was as follows: primary denaturation 4 minutes at 95°C, followed by 35 amplification cycles consisting of denaturing at 95°C for 30 seconds, annealing for 1 minute at 54°C, and extension at 68°C for 1 minute, upon completion of 35 amplification cycles a final extension step was done at 72°C for 10 minutes. The PCR products were then detected and viewed on a 1% agarose gel electrophoresis to confirm size, quantity and purity. Following PCR, Agarose gel electrophoresis was used to analyse DNA fragments. Samples from the PCR analysis are loaded into the wells of the agarose gel within the electrophoresis chamber. DNA samples are negatively charged (due to the phosphate molecule) and are loaded at the negative end of the electrophoresis chamber. Electric current is applied through the chamber and the negatively charged samples repel the negative end and move towards positive end. Different DNA bands (or fragments) with different sizes will travel different lengths based on the mesh-like pores in the agarose gel. To set up the electrophoresis experiment, the gel chamber is completely filled with TAE buffer to several millimeters over the gel. TAE is a buffer solution containing a mixture of tris base, acetic acid and EDTA. Once the samples are mixed with loading dye and loaded, they sink down to the bottom of the well in the gel. The lid of the chamber is now closed and electric current is applied by setting the voltage to 140 V and the timer for 30 min.

3.3. Analytical Techniques

3.3.1. High Performance Liquid Chromatography (HPLC)

The eppendorf tubes that were used to store sample size of 1 ml after each time interval were used for quantitative analysis of ABE (Acetone, Butanol, and Ethanol), acids (Acetic acid and Butyric acid) sugars (Glucose, xylose, arabinose, mannose, galactose) and inhibitor concentrations. These samples were stored at -80°C until analyzed. Sugar concentrations were measured using High Performance Liquid Chromatography (HPLC-Perkin Elmer) equipped with an automatic sample injector and a refractive index detector (2414, Waters).

Three HPLC columns were used as follows Shodex KC811 for measuring sugars, Shodex SP0810 to measure inhibitors, and Aminex HPX-87H to measure ABE solvent and acids concentrations. The samples were centrifuged at 15000g for 15 min and double filtered through 0.2 μm PTFE- filters (Whatman, USA). The solvent (mobile phase, 5 mM H_2SO4) was filter sterilized and autoclaved at 121°C for 15 min. Following that, solvent was degassed using vacuum filtration. A blank sample with only double distilled water was applied in the first sample vial tray of HPLC. This blank was used to increase the flow rate of the solvent from 0 to 0.6 ml/min. The flow rate was maintained at 0.6 ml/min for 1 hour while increasing the temperature of the HPLC column from 20°C to 60°C. This also fixes the pressure at a constant value and cancels most of the noise generated during the analysis. Then, each sample vials were arranged in a sequence and 0.1 μl of sample was extracted by the automatic sample injector. Each sample was analysed through the HPLC for 30 min. Data was processed by the computer software (Turbochrom Navigator). It was important to fill the HPLC testing vials to a minimum headspace to reduce the loss of solvents in vapor phase. The reliability of HPLC column, and testing parameters were confirmed by running solvents, acids, and sugars standards in triplicate.

3.3.2. Haemocytometer

A Qiujing XB.K.25 haemocytometer with dimensions 0.1mm x 1/400 mm^2 was used for counting cells and reporting cell concentration in number of cells per ml. The haemocytometer was cleaned with 75% ethanol before use. Diluted samples of 10 µL were injected on both sides of the haemocytometer and were observed under an optical microscope (Zeiss Axio ObserverA1). Each side has four quadrants and therefore cells in a total of 8 quadrants need to be counted. Number of bacterial cells in each of the eight quadrants were counted and recorded. The number of cells in each quadrant divided by the number of quadrants (which is 8) will give the average cell count in each quadrant. This number corresponds to the number of cells present in 0.1mm x 1/400 mm^2 volume of each quadrant. Cell density in cells/ml was then calculated for protoplast fused and co-culture strains of wheat straw fermentation. The only drawback of this technique is that without tagging the bacterial cells, there was no way of quantifying the dead cells and so were assumed to be negligible.

A second, more traditional approach was also employed to count cells for a part of the project. Dilutions in the range of 10^5 of the sample were prepared and 0.1 ml of the final dilution was plated onto agar plates. The plates were marked with the appropriate strain and the dilution rate. The plates were left overnight for colonies to form. Each colony represents one cell in the original sample. The number of colonies were counted and multiplied by the dilution rate and expressed in cells /ml.

3.3.3 UV-Vis Spectrophotometer

Enzyme activity was measured using pre-calibrated UV/VIS scanning spectrophotometer. A glucose standard calibration curve was generation by measuring the absorbance of different glucose standard solutions (mg/0.5ml) with a wavelength setup of 540 nm (Figure B1, Appendix B). Enzyme samples were analysed for absorbance against a reagent blank at the same wavelength. Preceding any analysis, 0.2 ml of color developed reaction mixture for sample assays, blanks, standards and controls

were diluted in 2.5 ml water and were placed in the spectrophotometer cuvette and analysed for absorbance.

3.4 Reproducability of Experimental Data and Error Analysis

SSF was repeated three times for each strain and samples were collected and stored for analysis. A total of 12 SSF were performed using all co-cultured and fused strains.

Table C1, C2, C3 and C4 in Appendix C show the raw data for concentration of total sugars including average concentration, standard deviation for triplicates and Percent Relative Standard Deviation (%RSD).

Table F1 and F2 in Appendix F show results that were obtained from three SSF experiments performed for each strain examined in this study. The tables show production of ABE and acids at the end of all the three SSF experiments.

Table G1, G2, G3 and G4 represent the results for biobutanol concentration during all three SSF experiments performed for any particular strain. Table E1, E2, E3 and E4 display the results for bacterial cell concentration during the SSF experiments for co-culture and fused strains. Results for three SSF experiments performed using each strain are tabulated. Averages and standard deviation were calculated for data from the three replicates. Percent Relative standard deviation (%RSD) was calculated by taking a ratio of the standard deviation with the mean and multiplying it with 100.

From Table C1, C2, C3 and C4, the percent error for total sugar concentration is in the range of 1% to 2.5% for all strains used in this study. From Table F1 and F2, the standard deviation range for ABE and Acids concentrations is in the range of 0.05 to 0.3 and 0.02 to 0.1 respectively with percent error in the range of 0.5 to 1.5% and 0.5 to 3% respectively. From Table G1, G2, G3 and G4, the standard deviation range for biobutanol concentration profile for *CaCt* (fused), *CaCt* (co-culture), *CbCt*

(fused) and *CbCt* (co-culture) were in the range of 0 to 0.5, 0 to 0.1, 0 to 0.15 and 0 to 0.1 respectively. The percent RSD following the same order was 0 to 12%, 0 to 9%, 0 to 17% and 0 to 14% respectively.

Equations 3.2 and 3.3 were used to calculate standard deviation and %RSD respectively.

$$\sigma = \sqrt{\frac{\sum(X-\bar{X})^2}{(n-1)}} \qquad (3.2)$$

Where,

σ = Standard deviation Error

$\bar{X}$= sample mean value; X = data point n = sample size;

%RSD was calculated from the equation shown below.

$$RSD\ (\%) = \frac{STDEVX100}{\bar{X}} \qquad (3.3)$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Protoplast Formation and Regeneration of Wild Strains on Agar Plates

Regenerative capability of protoplasts each individual wild strain was tested before performing their fusion. At each step of the protoplast formation, the culture was plated on the regeneration medium and the colony forming units (CFU) were determined. Table 4.1 shows the regeneration percentage for *Cb, Ca* and *Ct.*

From Table 4.1, the wilds strains *Cb* and *Ca* had the highest percentage of regenerated protoplasts after treatment with lysozymes and the lowest was observed for *Ct.* The frequency of regenerated protoplast for all wild strains ranged from 5% to 15% with the highest being 14.6% for *Cb,* followed by 11.3% for *Ca* and finally 5.4% for *Ct*. Previous protoplast isolation studies done on *Ca* grown on different regeneration mediums have shown regular regeneration frequencies of 8-25% when (Reilly and Rogers, 1987). This is in agreement with the regeneration frequency observed in the current study. Several studies have also concluded that protoplast formation and stabilization in minimal media with $CaC1_2$, $MgC1_2$ and sugars were crucial to subsequent regeneration on soft yeast extract agar. Therefore, RM medium consists of calcium and magnesium salts used in the present work are considered essential for regeneration on agar based yeast extract medium.

According to the results presented above, the frequency of regeneration for the wild strains is reasonable enough for the wild strains to be utilized for a protoplast fusion. A weaker frequency of regeneration would have resulted in weaker strength of the protoplast fusion.

Table 4. 1 Table showing bacterial colony forming units per ml (CFU/ml) and percent regeneration on RM plates

Wild Strains	Cells (CFU/ml) [a]	Regenerated protoplasts (CFU/ml) [b]	Non-protoplasted units (CFU/ml)[c]	Percent regeneration [d]
Cb	7.5×10^6	2.3×10^6	1.2×10^6	14.6
Ct	6.4×10^5	1×10^5	6.5×10^4	5.4
Ca	8.8×10^5	2.7×10^5	1.7×10^5	11.3

[a] CFU/ml of cells grown in CBM prior to protoplast formation was determined from a viable count after 48 h growth at 35°C on agar plates.
[b] Protoplasts were formed in PPM by treatment with lysozyme (2.5 mg/ml) for 60 min, centrifuged, suspended in PPM and plated on RM Agar plates. CFU/ml of regenerated protoplasts was determined after 48 h growth at 35°C for mesophiles and 60°C for thermophiles
[c] Determined from a viable count of the protoplast suspension after dilution in CBM to bring about osmolysis of protoplasts.
[d] Calculated as described in Materials and Methods. (Reilly and Rogers, 1987)

4.2. Production of Solvents

4.2.1 *CaCt* as Fused and Co-Cultured Bacterial Strains

Final ABE concentration was determined for the SSF experiments using *CaCt* (fused) and *CaCt* (co-culture) strains. The concentrations were calculated from HPLC detector response graphs as shown in Appendix H. Table 4.2 shows the concentration of ABE solvents and acids production achieved by both classes of strains.

Table 4. 2 Concentration of ABE and acids, sugars consumed and average cell proliferation rate for all protoplast fused and Co-culture strains

Fused and Co-culture strains	ABE (g/L)			Acids (g/L)		Total sugars consumed (g/L)	Average cell proliferation rate ($x10^5$ cells/ml.h)
	Acetone	Butanol	Ethanol	Acetic acid	Butyric acid		
CaCt (fused) at 45^{o}C	6.11	12.08	2.01	1.78	0.92	35	3.25
CaCt (Co-culture) at 35^{o}C	2.15	4.53	0.76	2.43	1.28	26.59	2.95
CbCt (fused) at 45^{o}C	6.89	13.81	2.28	1.75	0.87	40.21	3.46
CbCt (Co-culture) at 35^{o}C	2.77	5.79	0.96	2.21	1.17	28.08	3.16

From Table 4.2, final biobutanol concentration achieved using the parent *CaCt* (fused) strain was 12.08g/L which is comparable to previous research studies (Qureshi et al., 2007) where butanol production was examined from wheat straw using clostridium strains with initial sugar concentration of 62 g/L. Several processes were examined in Qureshi et al (2007) study including two that utilize SSF at 35^{o}C. However, one particular process that employed SSF coupled with gas stripping to remove butanol from the batch system recorded the highest butanol production of 12.7 g/L. The parent *CaCt* (fused) strain used in the current study measured almost same maximum productivity without utilizing gas stripping. Removal of butanol by gas stripping has been reported to be an essential procedure to avoid toxicity that suppresses further butanol production (Qureshi et al., 2007). From Table 4.2, the total solvent concentration for *CaCt* (fused) was found to be 20.2g/L. While many papers have reported higher solvent concentrations than reported by the current study, their main substrate happens to be pure glucose or food grade cornstarch, soy molasses and peanuts (Ezeji, et. al, 2004).

Furfural inhibitor production during the SSF was quantified to be in the range of 0.5 to 0.6 g/L for all strains. These concentrations were significantly lower than the inhibitory levels of 1 g/L, above which furfural activity is known to negatively affect fermentation (Modig et al., 2002). The low concentration of furfural can be explained by the lower temperature of 120^{o}C at which pretretment was performed. Research studies have shown that the concentration of inhibitors increases with increase in pretreatment temperature (Wei et al., 2012). Therefore, this study focussed on the affect of other inhibitors and toxins like acids and butanol while neglecting the affect of insignificant concentrations of furfural.

Results from Table 4.2 show that *CaCt* (co-culture) strains produced only 36.8% of the ABE production obtained when using the corresponding *CaCt* (fused) strain. Studies done to examine butanol production from crystalline cellulose by co-culturing *Ct* with another mesophillic strain (C.*saccharoperbutylacetonicum*) reported a final butanol concentration of 4.5 g/L after 5 days of

fermentation. (Nakayama et al., 2011). This result is comparable to the 4.53 g/L of butanol obtained by using *CaCt* (co-culture) strains.

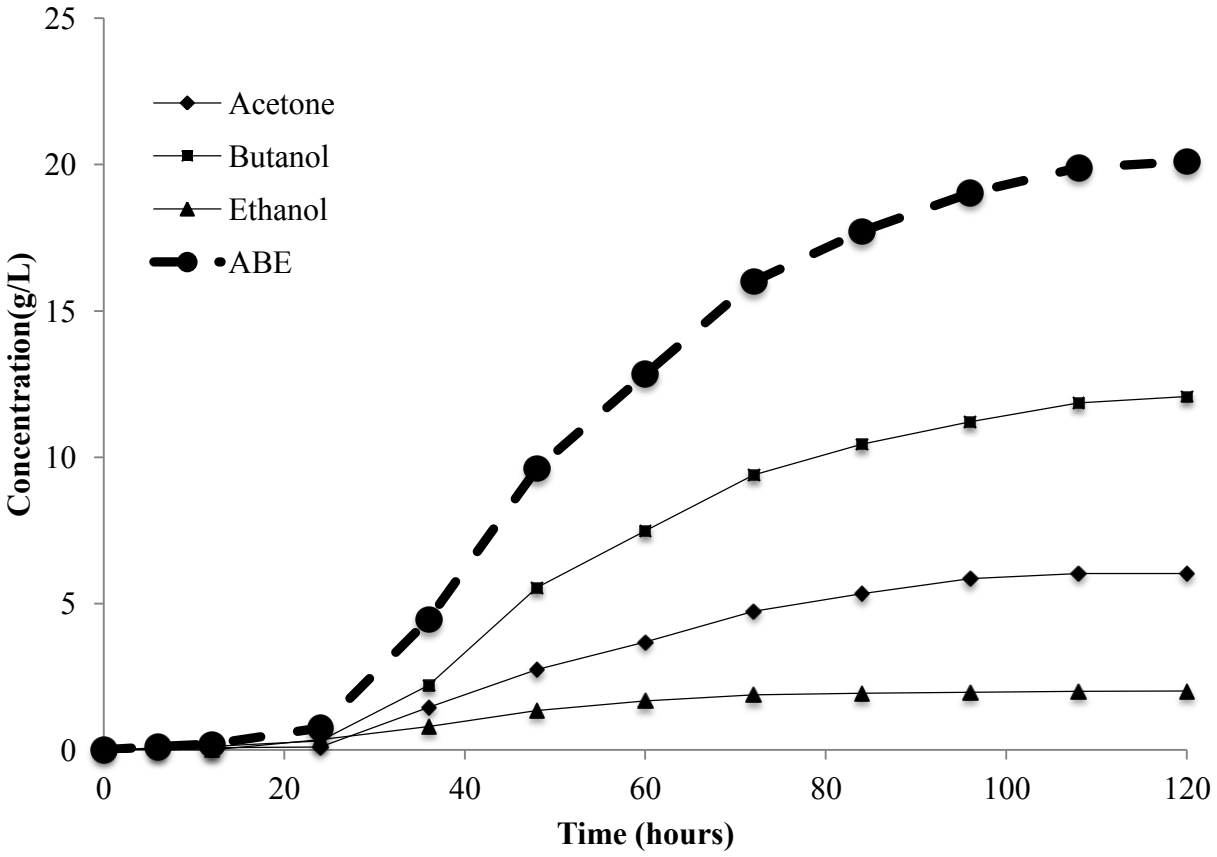

Figure 4. 1 ABE solvents concentration profile during SSF at 45°C using *CaCt* (fused) strains (Average RSD: 3%)

Figure 4.1 shows the ABE solvents concentration profile during SSF of wheat straw using *CaCt* (fused) strains. As shown in Figure 4.1, the *CaCt* (fused) strains produced 20.13 g/L of total ABE after 120 hours of SSF consuming 35 g/L sugars (Table 4.2). The concentrations of individual solvents such as acetone, butanol and ethanol at the end of fermentation were 6.05 g/L, 12.07g/L and 1.85 g/L respectively. It can be observed from Figure 4.1 that no solvent production was observed for the first 24 h into SSF. Although the bacterial cells are out of their lag phase and well into the exponential phase

between 6 to 24 h, enzyme production and utilization is mostly devoted towards breaking down of cellulose and hemicellulose rather than fermentation into solvents. From Figure 4.1, the production of solvents was most predominant between 24 h and 84 h with the production of 5.73 g/L, 10.15 g/L and 1.58 g/L of acetone, butanol and ethanol between those time periods. Solvent toxicity is often a cause of concern while monitoring product formation during ABE fermentation. During the final phases of SSF, it was observed that the cell metabolism proceeds until the solvent reaches a concentration of 19.8 g/L at 108 h. For the next 12 h, the percent increase in total solvent concentration was just about 1.5% increasing from 19.8 g/L at 108 h to 20.1 g/L at 120 h. Among the three solvents, butanol renders the most toxic affect on cell activity and metabolism.

According to previous experimental studies (Jones and Woods, 1986), production in the range of 7-13 g/L of butanol has resulted in cell growth inhibition. It can be seen that butanol concentration increased from 12 h to 108 h reaching to about 11.86 g/L. However, after 108 h the *CaCt* (fused) strains were still exposed to butanol toxicity due to the hydrophobic nature of butanol. Based on this observation, butanol toxicity is most likely the factor behind sluggish production of ABE solvents after 108 h where the results show that the total change in butanol concentration was just 1.8% going from 11.86 g/L at 96 h to 12.08 g/L at the end of fermentation.

Acetone and ethanol toxicity are not so much of a concern when compared to butanol. Cell growth inhibition was previously observed by other researchers for acetone at 70 g/L and for ethanol at 50 to 60 g/L (Costa, J. M, 1983 and Leung, J. C. Y, 1981). In our current study, ethanol and acetone concentrations do not reach above 10 g/L and therefore do not contribute to solvent toxicity and cell inhibition. However, in the case of butanol, we can conclude that genetic improvement has provided the strain with some butanol tolerance and has thus resulted in relatively high biobutanol production (12.08 g/L) in the case of *CaCt* (fused) strain. Still, this strain does experience toxicity from butanol for concentrations above 11.86 g/L (reached at 108 h) as demonstrated by Figure 4.1.

To further understand the affect a protoplast fusion between a butanol-producing mesophilic strains with a thermophile, it is important to compare the solvent production by the fused strains to that of a co-culture between the two wild strains. Thus, a co-culture was prepared and used to perform SSF of wheat straw and compare solvent production.

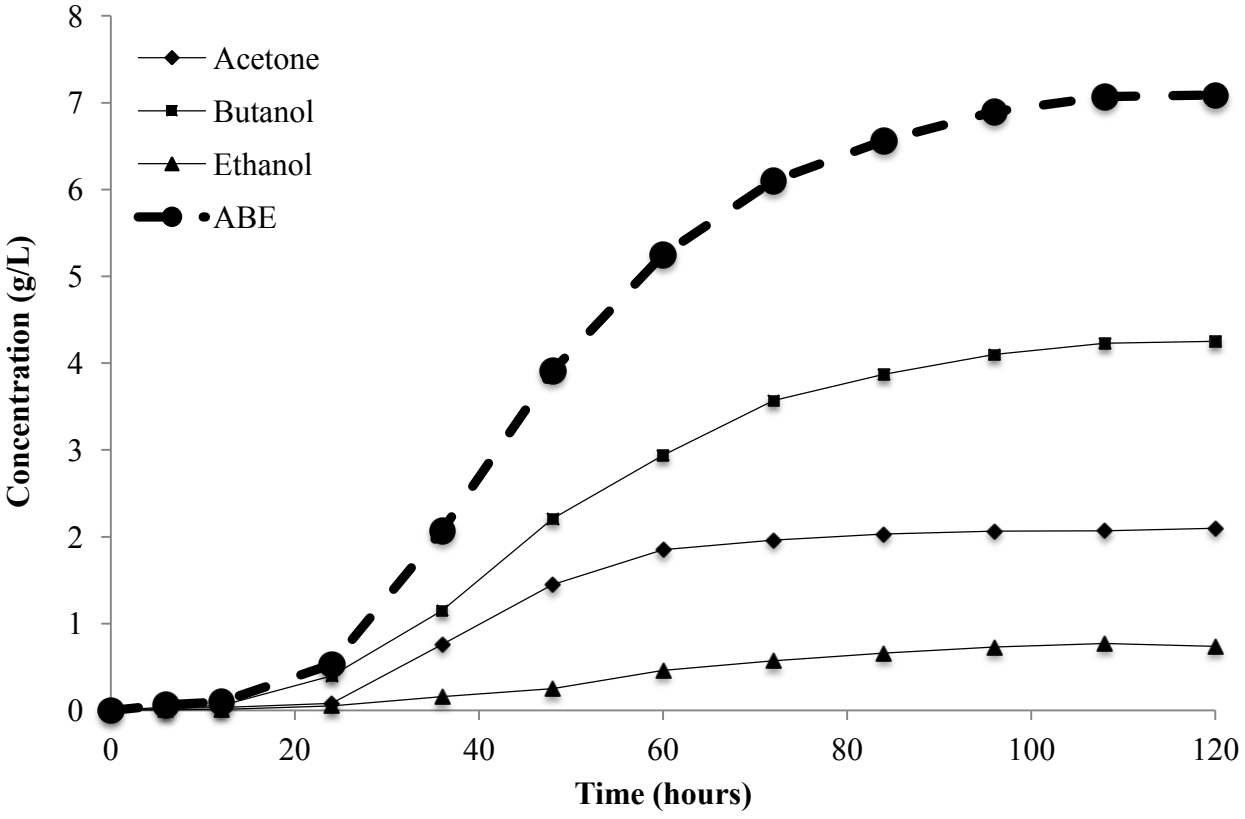

Figure 4. 2 ABE solvents concentration profile during SSF at 35°C using *CaCt* (co-culture) strains (Average RSD: 2.2%)

SSF was performed at two different temperatures of 35°C and 45°C for *CaCt* (co-culture). Although *Ca* is mesophilic with optimal temperature in the range of 10-65°C, it is able to produce solvents only in the range of 33-37°C, unless it is genetically modified to produce solvents at a higher temperature (Nölling et al. 2001). Figure 4.2 shows the ABE solvents concentration profile during 120 h of SSF using *CaCt* (co-culture) strains. The first 24 h is characterized by a sluggish growth as bacteria multiply

and produce enzymes essential to saccharify polysaccharides into sugars. The concentrations of acetone, butanol and ethanol at the end of fermentation were 2.15, 4.4 and 0.76 g/L respectively. Again, butanol toxicity does not seem to be a major concern here, as the concentration of biobutanol after 120 h of fermentation does not reach the inhibition limit of 7-13 g/L. Inhibition caused by acetone and ethanol production is also not of any concern as their levels are far below the solvent toxicity range. The objective to conduct SSF using *CaCt* (co-culture) strains was to compare the solvent production from these strains to that of *CaCt* (fused) strains. Comparing the biobutanol productivity after 120 h of SSF for *CaCt* (co-culture) and *CaCt* (fused) strains, it can be concluded that *CaCt* (fused) strains were able to produce 174% more butanol than their co-culture counterparts. When the SSF temperature for *CaCt* (co-culture) was increased to 45^{o}C, the solvent activity of the *Ca* was ineffective and the thermophilic strain did not provide any tolerance towards high temperature to the butanol producing strains. However, insignificant amount of ethanol was produced due to the ethanogenic nature of *Ct*.

4.2.2 *CbCt* as Fused and Co-Cultured Bacterial Strains

As shown in Table 4.2, the highest biobutanol concentration of 13.82 g/L was recorded for parent *CbCt* (fused) strain. This strain recorded an 8.8% increase in biobutanol production compared to SSF gas stripping procedure of Qureshi et al. (2007). One of the issues associated with industrializing fermentation technology for biobutanol production is the lower solvent production achieved during this process. Parekh et al. (1999) used the same strain of *Cb* (BA101) from our study to set up a pilot plant to produce biobutanol from corn steep water. The results obtained in this pilot study reported a solvent concentration of 24 g/L. When we compare this to our solvent concentration of around 23g/L obtained for *CbCt* (fused) in laboratory scale batch fermentations, one can realize the extreme potential of this fusion in reaching much higher levels when incorporated into a pilot plant.

The substrate used in the current study is also of significant importance. While butanol concentrations of around 19.6 g/L (from glucose), 15.8 g/L (from corn) have been reported in many studies (Ezeji et. al, 2004) using *Cb*, the main source of substrate was either added sugars in the form of food source such as corn or soy. However, the biobutanol concentration achieved from agricultural wastes in the same study was 9.8 g/L, 30% less than biobutanol achieved from *CbCt* (fused) strains used in the current study.

Figure 4.3 shows the ABE solvents concentration profile for 120 h of fermentation using *CbCt* (fused) strains.

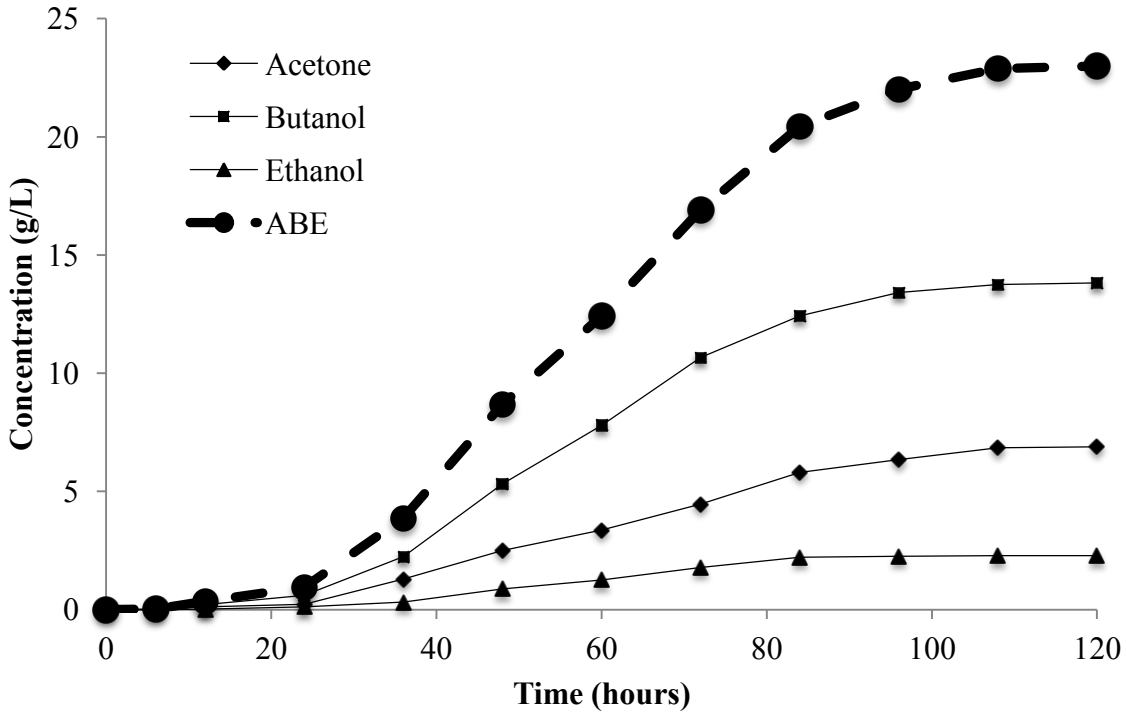

Figure 4. 3 ABE solvents concentration profile during SSF at 45°C using *CbCt* (fused) strains (Average RSD: 2.3%)

As shown in Figure 4.3, the *CbCt* (fused) strains produced 23 g/L of ABE after 120 hours of SSF by consuming 40.21 g/L sugars. In this run, concentrations of acetone, butanol and ethanol at the end of fermentation were 6.89, 13.82 and 2.29 g/L respectively (see Table 4.2). It can also be observed

from Figure 4.3 that production of solvents was most predominant between 24 h and 96 h with the production of 6.75 g/L, 13 g/L and 2.2 g/L of acetone, butanol and ethanol produced in the system between those times. During the later SSF stage, it was observed that the cell metabolism proceeds until the solvent reaches a concentration of 22.02 g/L at 96 h. For the next 24 h, the percent increase in total solvent concentration was just about 4% increasing from 22.02 g/L at 96 h to 23 g/L at 120 h.

This strain has undoubtedly shown the strongest tolerance particularly towards butanol toxicity. From Figure 4.3, it can be seen that butanol concentration steadily increased from 24 h to 96 h reaching to about 13.42 g/L. After 96 h, the total change in butanol concentration was just 2.75% going from 13.42 g/L at 96 h to 13.8 g/L at the end of fermentation corresponding to an increase in concentration of 0.38 g/L. As mentioned before, acetone and ethanol toxicity is not a crucial concern when compared to butanol. In our current study with *CbCt* (fused), ethanol and acetone concentrations do not reach above 10 g/L and therefore do not contribute to solvent toxicity and cell inhibition. However, in the case of butanol, we can conclude that genetic improvements accomplished through protoplast fusion has provided the strains with some butanol tolerance and has thus resulted in relatively high biobutanol production (13.8 g/L) in the case of *CbCt* (fused) strains. Still, this strain does experience toxicity from butanol for concentrations above 13.42 g/L (reached at 96 h).

Similar to *CaCt* (co-culture), a SSF of wheat straw was performed using *CbCt* (co-culture) strains at 35^{o}C and 45^{o}C and the results were compared to the corresponding *CbCt* (fused) strains. Figure 4.4 shows the ABE solvents concentration profile for SSF using *CbCt* (co-culture) strains. Nakayama et al. (2011) reported around 2g/L of butanol using only crystalline cellulose as substrate. In the current study, *CbCt* (co-culture) strains produced 5.8 g/L of butanol (9.52 g/L of ABE) after 120 hours of SSF. In this run, the concentrations of acetone, butanol and ethanol at the end of fermentation were 2.77, 5.8 and 0.96 g/L respectively (Table 4.2). Similar to *CaCt* (co-culture), neither butanol toxicity nor acetone, ethanol toxicity was a major concern.

The co-culture studies were performed to compare the solvent production from these strains to that of protoplast fused strains. After 120h of SSF, *CbCt* (fused) strains were able to produce 138% more butanol than *CbCt* (co-culture) strains. When the SSF temperature of the co-culture was increased to 45^{o}C, the activity of the *Cb* was ineffective and the thermophilic strain did not provide any tolerance towards high temperature for the butanol producing strains. However, insignificant amounts of ethanol were produced due to the ethanogenic nature of *Ct*.

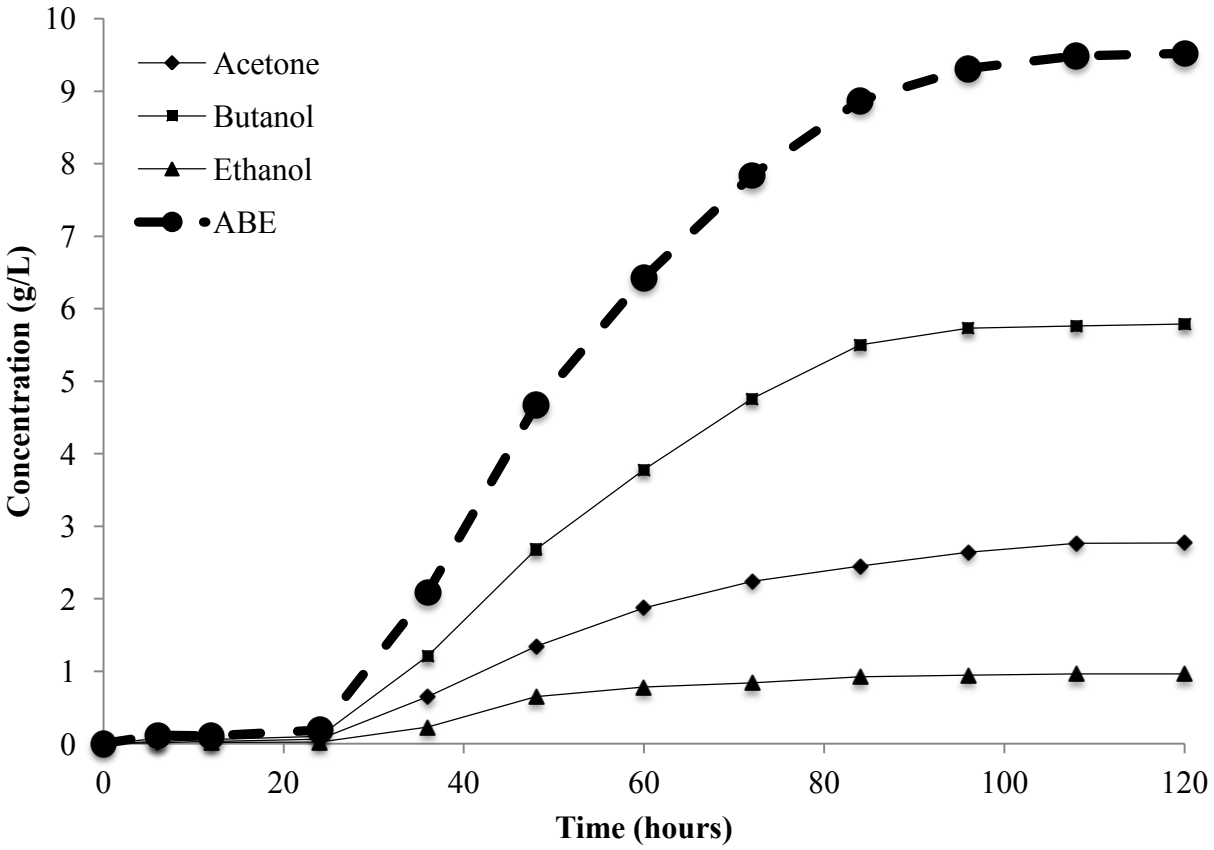

Figure 4. 4 ABE solvents concentration profile during SSF at 35^{o}C using *CbCt* (co-culture) strains (Average RSD: 3%)

4.3 Production of Acids and pH Changes during SSF

The two acids generated during SSF for all strains were butyric acid and acetic acid. These are produced during the acidogenic phase of the ABE metabolic pathway (Figure 2.2). *CaCt* (co-culture) produced the highest acids of all strains of about 2.43 g/L of acetic acid and 1.28 g/L of butyric acid. Figure 4.5 shows the change in the pH during batch SSF for all strains used in this study. It shows that pH values during SSF for *CaCt* (co-culture) strain decreased the most in accordance with acid generation (pH 4.34, 24 h). This was followed by *CbCt* (co-culture) strains that produced 2.21 g/L of acetic acid and 1.17 g/L of butyric acid. In general, co-culture strains produced more acid than fusion strains (Table 4.2). Parent *CbCt* (fused) strains produced about 1.75 g/L of acetic acid and 0.92 g/L of butyric acid with the pH dropping from 6.5 to 5.2 in the first 12 h of fermentation before increasing to 5 after 36 h. During the period from 48h to 120 h, pH of the system was in the range of 5 to 6.3 (Figure 4.5). Parent *CaCt* (fused) strains producing around 1.78 g/L of acetic acid and 0.92 g/L of butyric acid (Table 4.2) with the pH dropping from 6.5 to 4.8 in the first 24 h before returning back to 5.0 at 48h.

It is known that the pH of the system is an important indicator of the biological synthetic processes since major changes can inhibit production (Maddox et al., 2000). While the reduction in pH at the beginning of fermentation can be attributed to the production of acids (i.e. acetic and butyric acids), the increase in pH following that is due to the conversion of acids to the ABE solvents (i.e., acetone, butanol, and ethanol) during the solventogenesis stage (Maddox et al., 1995). The phenomenon of acid crash was discussed earlier and was cited as a concern if acidic concentration in the broth exceeds 60 mmol/L. The highest acidic molar concentration for *CaCt* (fused), *CbCt* (fused), *CaCt* (Co-culture) and *CbCt* (Co-Culture) are 40.4mmol/L (both acetic and butyric acid), 48.9mmol/L, 55mmol/L and 50.1mmol/L respectively. Therefore, it can be concluded after examining the total acid concentration in our system that acid crash was not a major cause of concern. However, deviation from

the optimum pH impacts production in general, which demonstrates potential to further enhance biobutanol production using the fused strains developed in the present work.

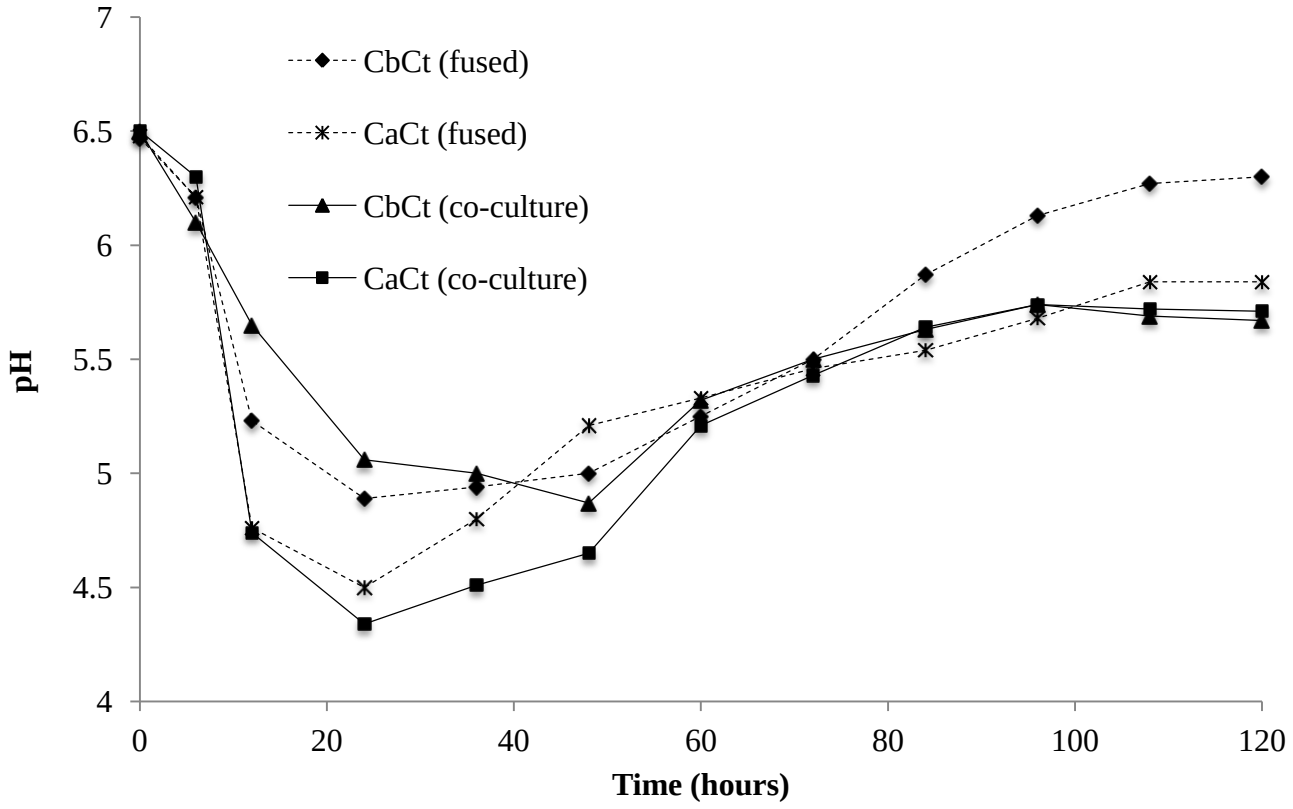

Figure 4. 5 Changes in pH during SSF for all fused and co-culture strains (Average RSD :2.3%)

4.4 Sugar Consumption during Batch SSF

4.4.1 *CaCt* as Fused and Co-culture Strains

Table 4.3 shows individual and total sugar concentration during SSF for *CaCt* (fused) strains. Figure 4.6 shows the corresponding change in the percentages of individual sugars concentrations (i.e., glucose, xylose, arabinose, galactose and mannose) during SSF with respect to the initial concentration of total sugars at the beginning of fermentation (after pre-treatment) using *CaCt* (fused) strains.

Examining this Figure shows that generally sugar concentrations increased in the first day of fermentation. This can obviously be observed for glucose, xylose, and arabinose, while the increase in the concentration of galactose was relatively minor. The increase in the total sugar is as a result of saccharification of unhydrolyzed polysaccharides that release monomers like glucose, xylose, mannose, galactose and arabinose by action of the enzymes released from the fused strains (*Ct* in particular).

From Table 4.3, the concentration of total sugars (including added glucose) after pretreatment was 29.8 g/L. However, this concentration increased to about 45.3 g/L at 24 h in accordance with hydrolysis enzymes generated by *CaCt* (fused) strains. This accounted for a 60% increase in sugar concentration within 24 hours of enzymatic saccharification (Figure 4.6).

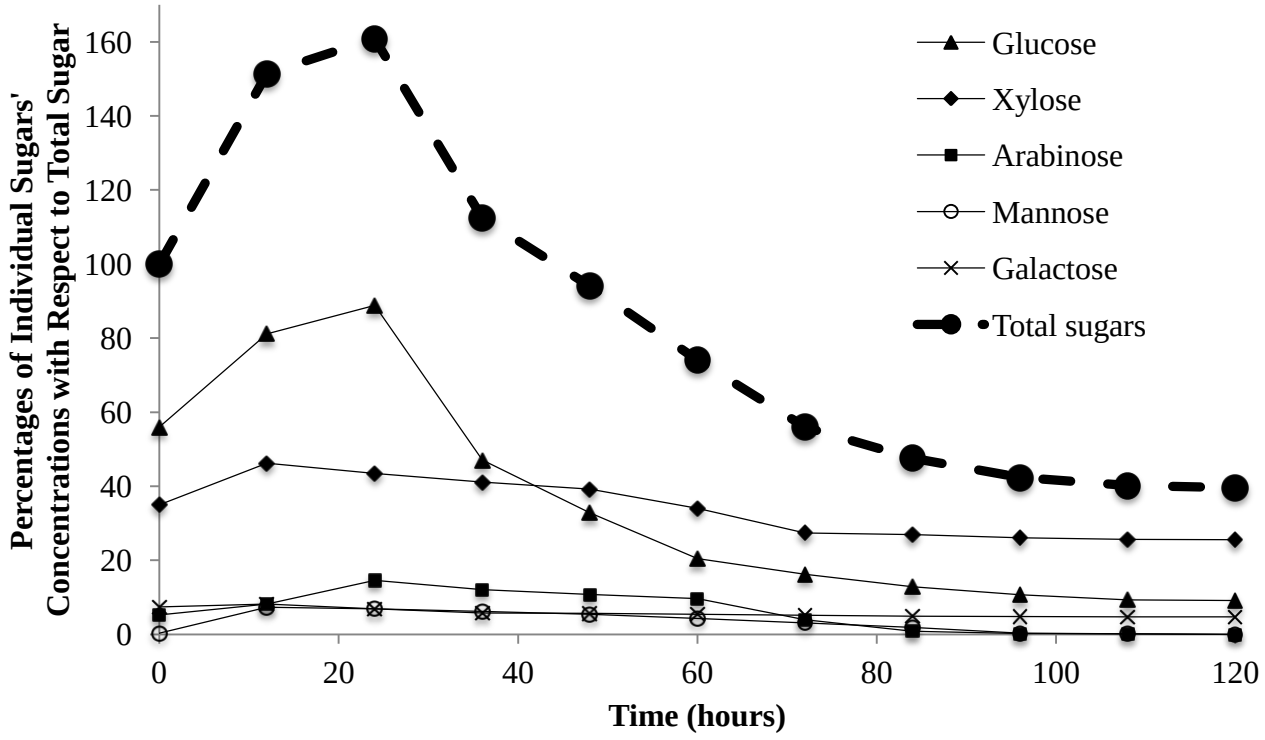

Figure 4. 6 Percentage of Individual Sugars' Concentrations with Respect to Total Sugars during SSF (45^{o}C) for *CaCt* (fused) Strains (Average RSD: 1.46%)

Several papers have studied the extraordinary capability of cellulolytic *Ct* to convert cellulose and cellobiose to glucose (Tsai et al., 2009). It is important to note that no external enzymes were

added during the SSF and all enzymes were generated after inoculation with the fused strains. As reported from Table 4.2, butanol concentration for *CaCt* (fused) strains has reached concentrations of almost 12g/L. The increased resistance of the strains towards butanol toxicity has contributed to the increase in sugar consumption. From Figure 4.6, glucose formed 60% of the total sugars at the beginning of fermentation and after pretreatement. Maximum glucose consumption is noticed between 24 h to 36 h where the percent glucose remaining in the system dropped from 88% (25.4 g/L) to 47% (13.46 g/L). At the end of SSF, only 4.7% (2.61 g/L) of glucose remained. In other studies that examine *Ca* wild strain, it was found that in either batch or fed-batch cultures xylose utilization was inhibited at higher glucose concentrations (Fond, 1986, El Kanouni et al., 1998). This phenomenon is evident from Figure 4.6. It was noticed that the presence of higher percentages of glucose in the batch between 12 h to 36 h has inhibited utilization of xylose leading to invariable percentages of xylose left during that time period. As the concentration of glucose goes significantly below 15g/L (at 36 h, Table 4.3), there is a rapid increase in the consumption of xylose, which is evident from the decrease in percent xylose remaining from 41% (36 h) to 27% (72 h). Still, xylose is not completely consumed and at the end of the fermentation about 25% (7.31 g/L) remains in the system. There were no traces of arabinose or mannose at the end of SSF and they were the only two sugars that were completely consumed. It can be observed that in the initial stages of SSF the bacteria utilize glucose rapidly before transferring to xylose.

Table 4. 3 Concentration of Individual and Total Sugars during SSF for *CaCt* (fused) strains

Time	Glucose	Xylose	Arabinose	Mannose	Galactose	Total sugars
0	16	10	1.5	0.1	2.2	29.8
12	23.21	13.21	2.34	2.1	2.45	43.31
24	25.4	12.42	4.16	1.98	2.05	46.01
36	13.46	11.76	3.45	1.76	1.73	32.16
48	9.38	11.21	3.07	1.56	1.68	26.90
60	5.86	9.74	2.75	1.24	1.61	21.2
72	4.64	7.84	1.12	0.87	1.54	16.01
84	3.67	7.7	0.23	0.54	1.47	13.61
96	3.06	7.45	0.07	0.08	1.44	12.11
108	2.67	7.34	0.04	0.03	1.42	11.49
120	2.61	7.31	0	0	1.40	11.32

Figure 4.7 shows the change in the percentages of individual sugars concentrations (i.e., glucose, xylose, arabinose, galactose and mannose) during SSF with respect to the initial concentration of total sugars at the beginning of fermentation (after pre-treatment) using *CaCt* (co-culture) strains. Table 4.4 shows the corresponding concentrations. From Figure 4.7, 61% of total sugars remained in the system after 120 h. Xylose inhibition can be observed for higher glucose concentrations (above 15g/L) between 12 h and 36 h where percent xylose remaining decreased from 44% to 41.7% representing a preferential consumption of glucose over xylose by the bacteria. During the subsequent 24 h, when lower percent of glucose remains in the system, the xylose in the system drops from 41.7% to 27.2%.

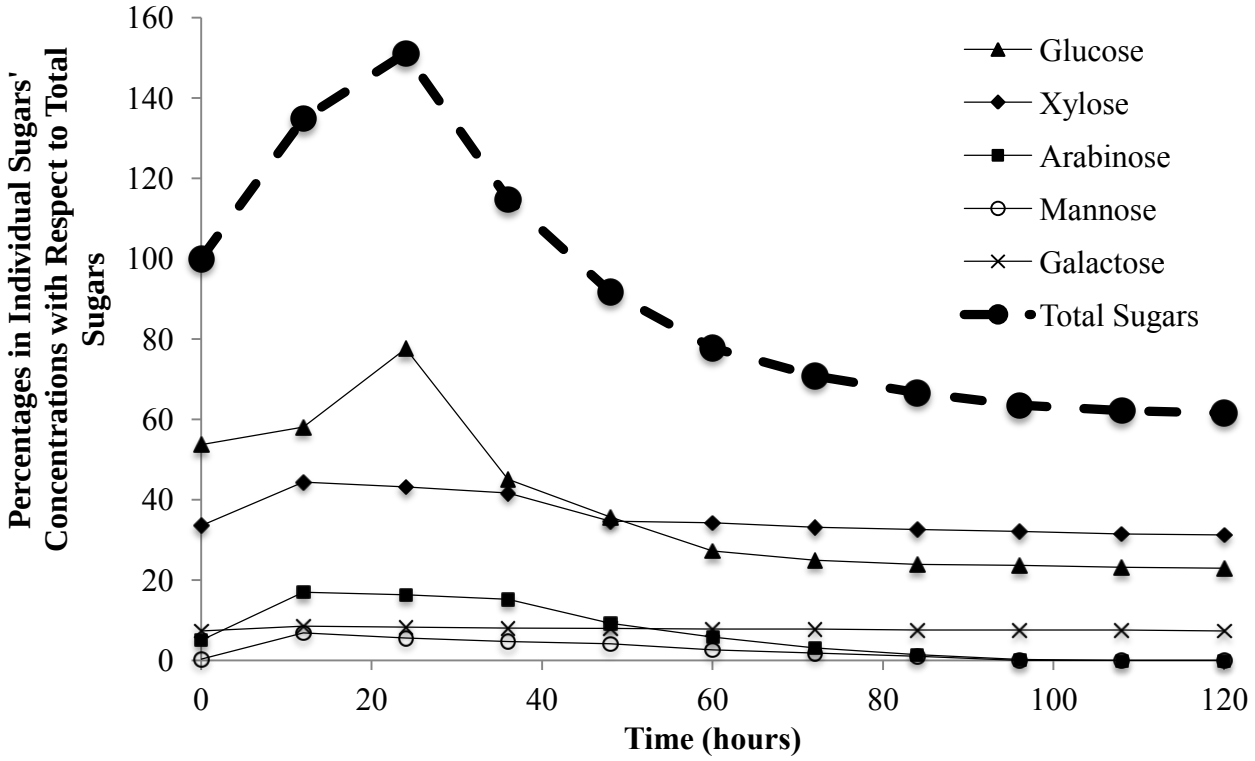

Figure 4. 7 Percentage of Individual Sugars' Concentration with Respect to Total Sugars during SSF (35^{O}C) for *CaCt* (co-culture) Strains (Average RSD: 1.76%)

From Table 4.4, arabinose and mannose were completely consumed at 108 h and 96 h respectively. At the end of SSF, 6.85 g/L of Glucose, 9.32 g/L of xylose, 2.18 g/L of Galactose and 18.35 g/L of total sugars remained unconsumed in the system. The percent total sugar consumed was 59% for *CaCt* (co-culture) strains registering a 26.3% decline in sugar consumption compared to their *CaCt* (fused) counterparts.

Table 4. 4 Concentration of Individual and Total Sugars during SSF for *CaCt* (Co-culture) Strains

Time	Glucose	Xylose	Arabinose	Mannose	Galactose	Total sugars
0	16	10	1.5	0.1	2.2	29.8
12	17.32	13.23	5.05	2.04	2.54	40.18
24	23.12	12.87	4.87	1.67	2.48	45.01
36	13.43	12.43	4.54	1.4	2.41	34.21
48	10.64	10.32	2.76	1.23	2.38	27.33
60	8.12	10.21	1.75	0.78	2.33	23.19
72	7.43	9.87	0.94	0.54	2.32	21.1
84	7.12	9.72	0.43	0.32	2.27	19.86
96	7.05	9.56	0.06	0	2.25	18.93
108	6.91	9.38	0	0	2.25	18.54
120	6.85	9.32	0	0	2.18	18.35

4.4.2 *CbCt* as Fused and Co-culture Strains

Figure 4.8 shows the percent sugars remaining in the system (including glucose, xylose, galactose, mannose and arabinose) with respect to the initial concentration of total sugars using *CbCt* (fused) strains. Table 4.5 shows individual and total sugar concentration during SSF for this strain. The percent total sugar consumption for this strain was around 84% (40.21 g/L, Table 4.5) and is the highest among all strains analysed in this study. The sugar consumption of the current strain is also higher than the one reported by Liu et al. based on studies done with *Cb* and wheat straw as substrate. Therefore, it can be concluded that the parent-fused strains have produced better results than their wild strains.

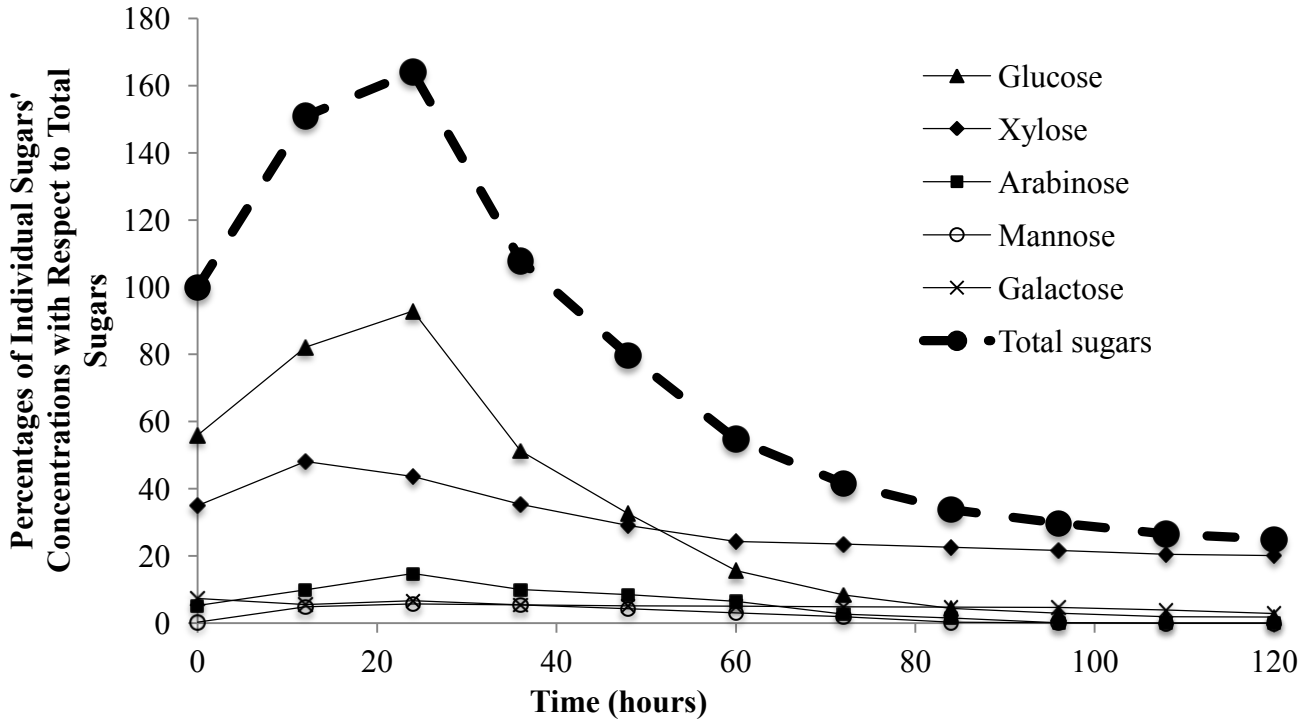

Figure 4. 8 Percentage of Individual Sugars' Concentration with Respect to Total Sugars during SSF (45^{O}C) for *CbCt* (fused) Strains (Average RSD: 2.65%)

In comparison to *CaCt* (fused) strains, the percent total sugars remaining in the system decreased by 36.8%. Fewer sugars remained in the system at the end of SSF for *CbCt* (fused) strains as compared to the others. Similar to other studies arabinose and mannose were completely consumed. From Figure 4.8, between 0 to 24 hours the percent sugars in the system increased 60% in accordance with hydrolysis enzymes helping in breaking the polysaccharides and releasing free sugars into the system. The 60% increase in sugars translates to a 17 g/L of sugars being released into the system (Table 4.5). A characteristic of *Cb* is that it is more capable than *Ca* in terms of breaking down pentose sugars such as xylose even in high glucose concentrations. Hence we see a steady decline in the percent xylose remaining even at glucose concentrations that inhibit xylose consumption. For example, from Figure 4.8, between 24 h to 36 h, the percent glucose is sufficiently high (above 15 g/L), however the percent of xylose remaining has also decreased from 44% to 35% (from 12.48 g/L at 24 h to 10.12 g/L at 36 h) suggesting that this strain might not be affected by xylose inhibition as much as its *CaCt* (fused) counterpart.

Table 4. 5 Concentration of Individual and Total Sugars during SSF for *CbCt* (fused) Strains

Time	Glucose	Xylose	Arabinose	Mannose	Galactose	Total sugars
0	16	10	1.5	0.1	2.2	29.8
12	23.5	13.76	2.85	1.4	1.65	43.16
24	26.56	12.48	4.23	1.65	2	46.92
36	14.67	10.12	2.87	1.56	1.63	30.85
48	9.31	8.31	2.42	1.24	1.54	22.82
60	4.47	6.95	1.87	0.87	1.51	15.67
72	2.41	6.73	0.76	0.54	1.46	11.9
84	1.27	6.45	0.45	0.08	1.42	9.67
96	0.85	6.18	0.04	0.04	1.41	8.50
108	0.56	5.86	0	0	1.18	7.6
120	0.52	5.76	0	0	0.87	7.15

Table 4.5 indicates that hemicellulotic pentose sugars such as xylose are not completely consumed at the end of the fermentation. At 120 h, the concentration of xylose stood 5.76 g/L. However very small traces of glucose (0.56 g/L) were found suggesting a nearly complete consumption of glucose unlike *CaCt* (fused) strain that still had 9% (2.61 g/L) of glucose remain in the system. Other hemicellulotic sugars like arabinose and mannose were completely consumed.

Figure 4.9 shows the percent sugars remaining in the system (including glucose, xylose, galactose, mannose and arabinose) with respect to the initial concentration of total sugars using *CbCt* (Co-culture) strains. Table 4.6 shows individual and total sugar concentration during SSF for this strain. The percent total sugar consumption for this strain was around 65% (28.76 g/L, Table 4.6) and is the highest among the two co-culture strains examined.

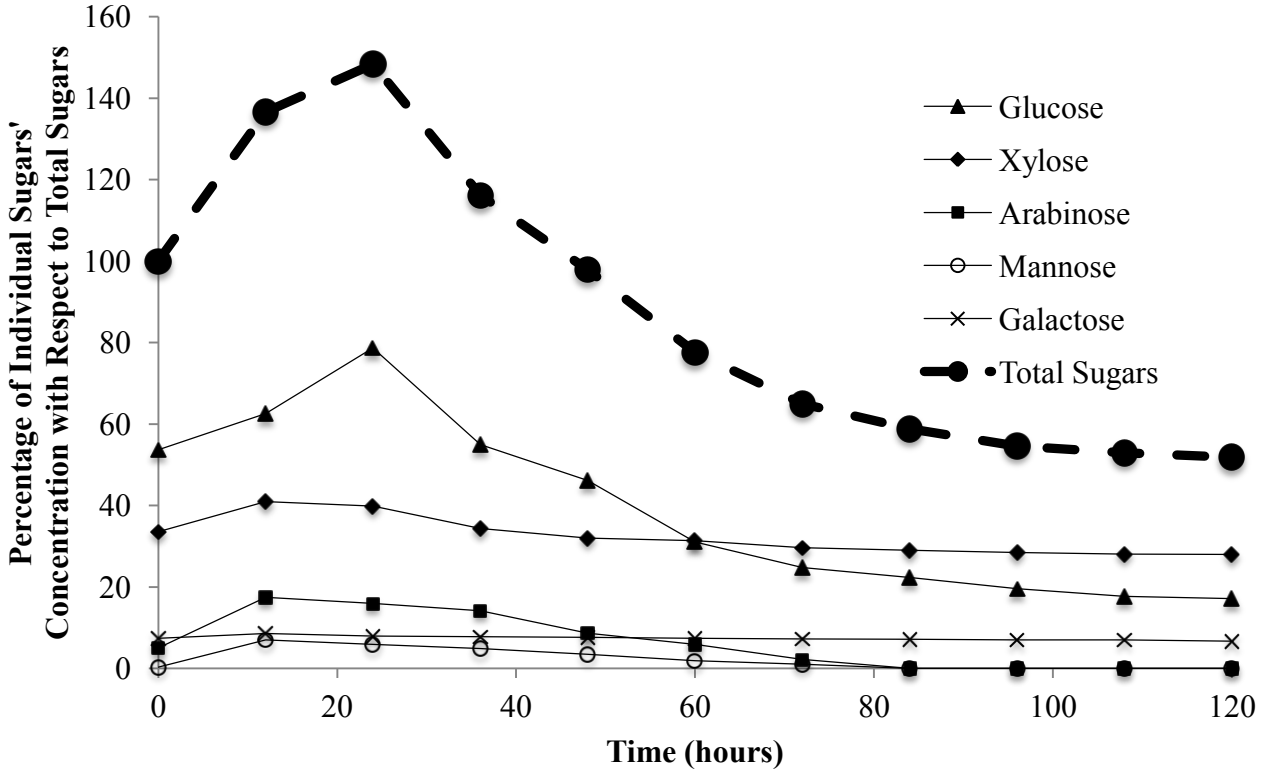

Figure 4. 9 Percentages of Individual Sugars' Concentration with Respect to Total Sugars during SSF (35^{O}C) for *CbCt* (co-culture) Strains (Average RSD: 1.7%)

In comparison to *CbCt* (fused) strains, twice the percent total sugars remained in the system for this strain. During the saccharification phase between 0 h to 24 h an additional 14 g/L of sugars were released into the system (Table 4.6). At the end of SSF, 52% of total sugars (i.e. 28% xylose, 17% glucose, 6.7% galactose) remained in the system. Arabinose and mannose were completely consumed.

Table 4. 6 Concentration of Individual and Total Sugars during SSF for *CbCt* (Co-culture) Strains.

Time	Glucose	Xylose	Arabinose	Mannose	Galactose	Total sugars
0	16	10	1.5	0.1	2.2	29.8
12	18.65	12.2	5.2	2.1	2.56	40.71
24	23.45	11.87	4.76	1.76	2.37	44.21
36	16.38	10.25	4.23	1.45	2.32	34.63
48	13.75	9.53	2.58	1.03	2.28	29.17
60	9.26	9.34	1.76	0.56	2.21	23.13
72	7.39	8.84	0.65	0.32	2.15	19.35
84	5.38	8.65	0	0	2.13	16.16
96	5.18	8.48	0	0	2.1	15.76
108	5.12	8.37	0	0	2.09	15.58
120	5.1	8.35	0	0	2	15.45

4.5 Cell Growth during SSF for Fused and Co-Culture Strains (*CaCt* and *CbCt*)

Figure 4.10 and Figure 4.11 show the changes in cell concentration observed for both parent fused strains and corresponding co-culture strains. Several parameters contribute to cell growth. All strains exhibit a lag phase where the cell concentration is almost constant. In this phase, the strains acclimatize to culture medium, pH levels and the surrounding temperature. The lag phase for *CbCt* (fused) and *CaCt* (fused) strain was between 0 h and 6 h. Co-culture strains exhibited similar lag phases. After this phase, the cells grew exponentially as they fed on the nutrients in the culture and entered the solventogenic phase. The exponential phase ended around 72-84 h into the SSF. For the *CaCt* (fused) strains the butanol concentration at 84 h was 10.45 g/L (Figure 4.1), which corresponds to the concentration range in which cell inhibition is initiated due to butanol toxicity. The concentration of cells decreases after 84 h and enters the decay phase.

A similar analysis of *CbCt* (fused) strains show that the concentration of cells increases exponentially between 12 h and 60 h after which the cells reach a stationary stage for the next 36 h. Due to relatively higher butanol tolerance of this strain, even concentrations as high as 12.5 g/L observed at 84 h do not initiate a cell decline. This strain maintains a wider stationary phase from 60 h to 96 h during which the butanol concentration varies from 7.8 g/L to 13.42 g/L. The strain enters the decay phase at 96 h when the butanol concentration reaches around 13.42 g/L.

Co-culture strains produce relatively lower butanol (not in the range of toxic levels) than their corresponding protoplast fused strains. The growth phase was recorded for *CbCt* (coculture) between 12 h and 72 h with a butanol concentration going up to 4.76 g/L. The stationary phase was observed for the rest of the SSF time without a distinct change into a decay phase.

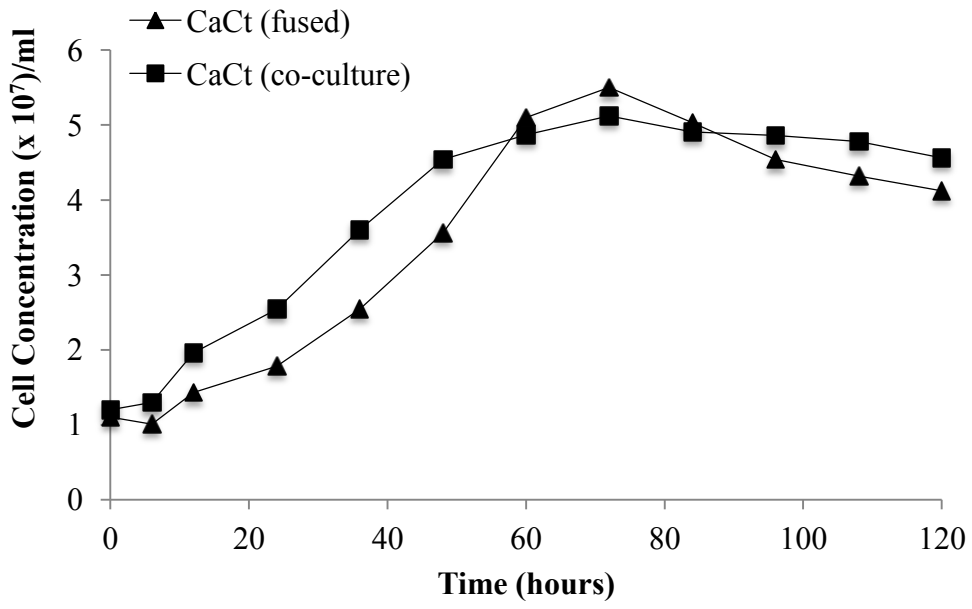

Figure 4. 10 Change in cell concentration for *CaCt* (fused) and *CaCt* (Co-culture) strains during SSF (Average RSD: 3.4% and 2.85% respectively)

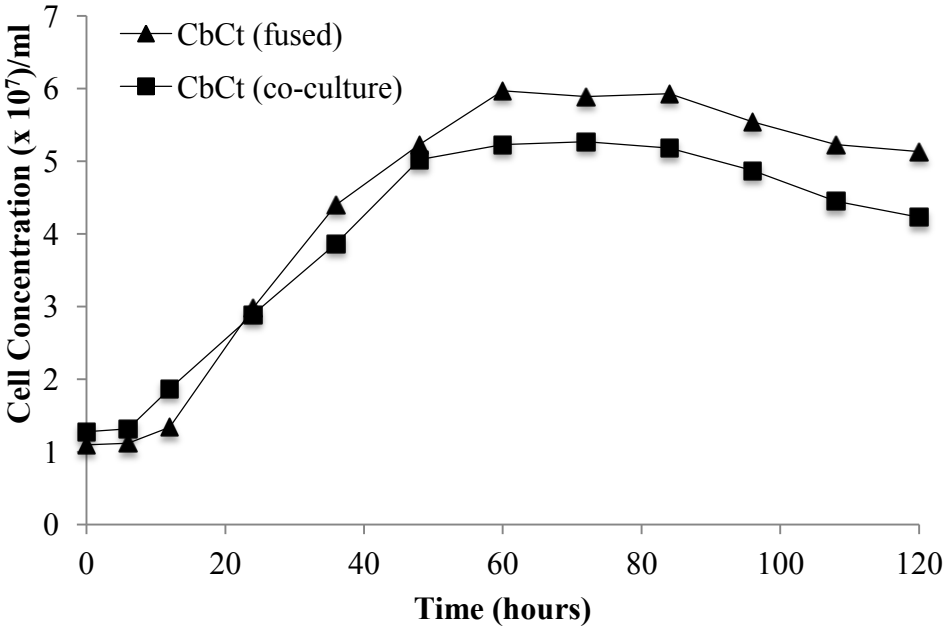

Figure 4. 11 Change in cell concentration for *CbCt* (fused) and *CbCt* (Co-culture) strains during SSF (Average RSD: 4.52% and 2.85% respectively)

4.6 Enzyme Assay Analysis

In our current study, the production of three enzymes cellulose (endoglucanase and exoglucanase), and β-glucosidase was studied in terms of filter paper activity (FPA). Table 4.7 summarizes the amount of glucose released from the filter paper for each sample and the corresponding enzyme activity in filter paper units per ml (FPU/ml). Appendix B describes the calculations involving amount of glucose released from each sample.

CbCt (fused) strain has demonstrated a capability to produce higher biobutanol. Enzymes were generated by both thermophilic and mesophilic parts of the fusion. Cellulosome producing *Ct* and its ability to ferment sugars offers a promising future in biofuels. Distinct from the fungal cellulases, the *Ct* cellulase has a very high enzyme activity on cellulose (Maki et al., 2009)

Table 4. 7 Concentration of glucose released from samples including calculated enzyme activity for *CbCt* (fused) and *CaCt* (fused) strains

Dilution #	*CbCt* (fused)		*CaCt* (fused)	
	Abs 540 nm	Glucose (mg / 0.5mL)	Abs 540 nm	Glucose (mg / 0.5mL)
1	0.586	2.551	0.563	2.451
2	0.456	1.985	0.403	1.754
3	0.282	1.228	0.247	1.075
Enzyme activity (FPU/ml)	68.52		58.73	

Previous studies have demonstrated higher biobutanol productivity for *CbCt* (fused) strains without addition of any external enzymes. Thermophilic and mesophillic enzymes are able to digest cellulose and hemicellulose into sugars and finally into solvents and acids. *Ct*, an anaerobic thermophile that is reported to produce both soluble, cellulose degrading enzymes and cellulosomes cellulases, has been a subject of study for decades (Dashtban et al., 2010). Therefore, the fused strains should be able to produce all the enzymes associated with cellulose hydrolysis (endoglucanase, exoglucanase and β-gluocosidase) during the SSF experiments. From Table 4.7, *CbCt* (fused) strains produced cellulolytic enzymes in the order of 68.52 FPU/ml and *CaCt* (fused) produced enzymes in the order of 58.73 FPU/ml. These values can be compared with the activity of many cellulase enzymes derived from other organisms like *Trichoderma reesei* ATCC 26921 whose activity is 85.1 FPU/ml (Yang, 2010) and accellerase 1500 with an activity of 43.21 FPU/ml (Pessani, 2011). The enhanced enzyme activity of *CbCt* (fused) strains and its relative similarity to cellulase activity from commercial sources represents a breakthrough in enzymatic hydrolysis associated with SSF of lignocellulose.

4.7 Examination of Several Growth Cycles of the Fused Strains

In the current study, parent protoplast fused strains (i.e., 1st generation of the fusants) were passed on to five successive growth cycles (i.e., five generations). Fused strains of each growth cycle were later inoculated with substrate, examined for solvent production. Figure 4.12 shows ABE (total and individual solvent) concentration after 120 hours of SSF for the first five growth cycles of *CbCt* (fused) strains.

From Figure 4.12, it can be seen that the 1st growth cycle (parent generation) of the *CbCt* (fused) strains produced a total ABE concentration of 23g/L (i.e., 6.89 g/L acetone, 13.81g/L butanol and 2.29g/L ethanol). The second growth cycle of *CbCt* (fused) strain produced 22g/L of ABE (i.e., 6.52 g/L acetone, 13.34g/L butanol and 2.17g/L ethanol). The decline in concentration of ABE and biobutanol from 1st to 2nd growth cycles was 4.34% and 3.4% respectively. The next cycle of *CbCt* (fused) strains recorded an increase and showed the strongest expression of genes acquired from both wild strains (*Ca* and *Ct*). Genetic stability will be discussed further in section 4.7 below. This cycle of *CbCt* (fused) strains produced total ABE of 23.26 g/L (7g/L acetone, 13.89g/L butanol and 2.35g/L ethanol). The increase in concentration of ABE and biobutanol from 2nd to 3rd growth cycles is 6.2% and 4.1% respectively. The subsequent growth cycle of *CbCt* (fused) strains (4th cycle) produced total ABE of 23.07 g/L (7g/L acetone, 13.85g/L butanol and 2.31g/L ethanol). This represents further increase in concentration of ABE and biobutanol from 3rd to 4th generation of 0.817% and 0.29% respectively. Finally, the 5th growth cycle produced total ABE of 22.95g/L (6.84g/L acetone, 13.8g/L butanol and 2.3g/L ethanol), which represents a decline in concentration of ABE and biobutanol from 4rd to 5th cycle of 0.52% and 0.361% respectively.

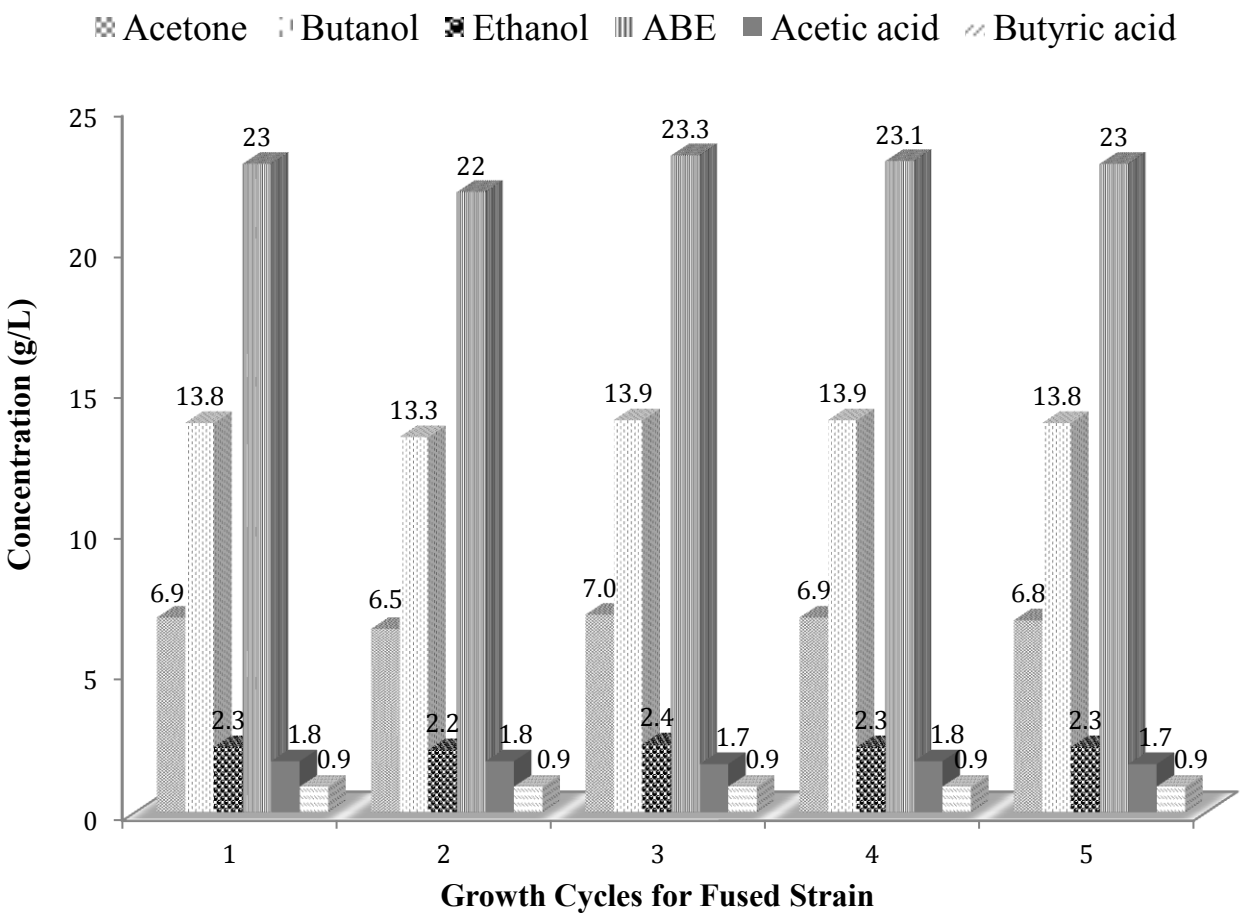

Figure 4. 12 ABE and acid concentration in SSF recorded for the five growth cycles of *CbCt* (fused) strains.

Figure 4.13 shows the final ABE concentrations after 120 hours of SSF for the first five growth cycles for *CaCt* (fused) strains. As shown in Figure 4.13, the 1st growth cycle of the *CaCt* (fused) strains produced a total ABE of 20g/L (6.12 g/L acetone, 12.07g/L butanol and 1.85g/L ethanol). The second cycle produced a total ABE of 16.83g/L (4.88 g/L acetone, 10.3g/L butanol and 1.65g/L ethanol). The decline in concentration of ABE and biobutanol from 1st to 2nd generation is 15.85% and 14.66% respectively. The next cycle of *CaCt* (fused) strains (3rd cycle) produced a total ABE of 15 g/L (4.13 g/L acetone, 9.52 g/L butanol and 1.3g/L ethanol). The decline in concentration of ABE and biobutanol from 2nd to 3rd generation was calculated to be 10.87% and 7.57% respectively. The

decrease in productivity of solvents over five growth cycles clearly demonstrates the inability of these strains to remain in a fused state and therefore unable to maintain the solvent levels observed while using the parent strains.

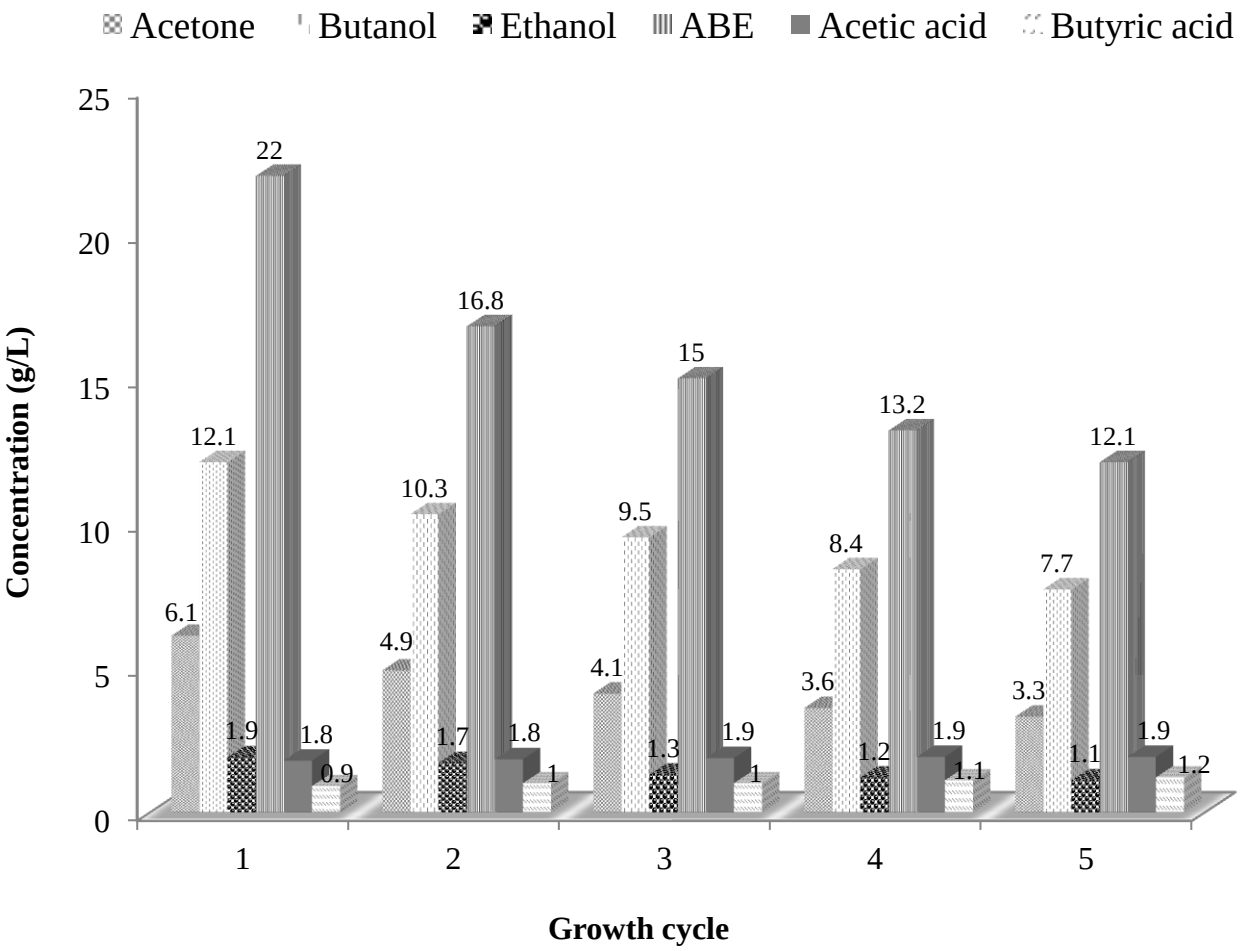

Figure 4. 13 ABE and acid concentration in SSF recorded for five growth cycles of *CaCt* (fused) strains.

The subsequent growth cycle for *CaCt* (fused) (4th cycle) produced a total ABE of 13.16 g/L, (3.55g/L acetone, 8.4g/L butanol and 1.2g/L ethanol). The decrease in concentration of ABE and biobutanol from 3rd to 4th generation is 12.27% and 11.76% respectively. Finally, the last cycle (5th) produced an ABE of 12.06 g/L (3.33g/L acetone, 7.65g/L butanol and 1.08g/L ethanol). This represents

a decline in concentration of ABE and biobutanol from 4rd to 5th cycle by 11.32% and 8.3%, respectively. The overall concentration of the solvents and biobutanol by *CaCt* (fused) strains declined by 39% and 36% respectively as compared to the parent fused strains.

Table 4.8 and Table 4.9 compare the average cell proliferation rate, sugar consumption (percentage and absolute) and ABE productivity over 120 h of SSF of wheat straw using *CbCt* fused and *CaCt* fused strains, respectively. Average cell proliferation rate was calculated based on an average increase in the number of cells per hour as a result of cell growth and cell division. It is well known that the macromolecular composition (e.g., the mass fractions of protein, RNA, and DNA) of bacterial cells under exponential growth depends on the growth medium, predominantly due to the growth rate allowed by the nutritional content of the medium (M Scott, 2010). Cell proliferation rate was calculated for all growth cycles for *CbCt* (fused) and *CaCt* (fused) strains. From Table 4.8, parent generation *CbCt* fused strains are able to proliferate by an average of 3.46 (x10^5) cells every hour. The proliferation rate after five generations goes down to 3.44 (x10^5) cells per hour. Therefore it can be concluded that after five growth cycles, this strain is able to maintain a stable kinetic behavior. However, the average proliferation rate for parent *CaCt* (fused) strain was calculated to be 2.8 (x10^5) cells every hour and decreases to 0.87 (x10^5) cells every hour for the fifth cycle. This significant decline in the average cell proliferation rate is a representative of slower cell growth kinetics associated with *CaCt* (fused) strain and could explain the substandard performance of this strain in terms of solvent production for the five growth cycles.

In terms of sugar consumption, total sugar uptake registered an overall increase from growth cycles 1 to 5 for *CbCt* (fused) strain from 85.3% consumption of sugars in the first cycle (parent fusion) to 85.6% in the last. The second growth cycle recorded the lowest sugar consumption of 39.3 g/L or 83.75%. The reason for this can be understood based on genetic analysis of this strain which will be discussed later.

The growth cycle for parent *CaCt* (fused) strain reported a sugar consumption of 34 g/L, which was 15% lower than parent CbCt (fused) strains. This strain reported a decline in sugar consumption from 75% sugar consumption in first growth cycle to 67.7% in the last. Solvent and sugar toxicity are important factors that contribute to the decline in sugar consumption.

ABE productivity is an important factor during scale up of this process to an industrial reactor. Higher ABE productivity represents higher profits with low processing time. The rate at which ABE is produced remains constant at 0.19 g/L.hr throughout all growth cycles for *CbCt* (fused) strains. However, butanol productivity decreased from 0.12 g/L.hr to 0.11 g/L.hr after five growth cycles. Passing parent *CaCt* (fused) strain through five growth cycles have resulted in loss of ABE productivity from 0.17 g/L.hr to 0.1 g/L.hr marking a 41% decline in productivity. This significant decline is undesirable in industry and therefore this fusion would be unsuccessful at the industrial scale.

Table 4. 8 Comparison of cell proliferation, sugar consumption and ABE productivity for growth cycles 1-5 of *CbCt* (fused) strains

Growth cycles	Average Cell proliferation rate ($x10^5$ cells/ml.h)[a]	Total Sugar Consumed		ABE Productivity (g/L.hr)			Total ABE (g/L.hr)
		Concentration (g/L)	Percentage (%)	Acetone (g/L.hr)	Butanol (g/L.hr)	Ethanol (g/L.hr)	
1st	3.46	40.05	85.3	0.06	0.12	0.02	0.19
2nd	3.37	39.3	83.75	0.06	0.11	0.02	0.19
3rd	3.48	40.3	85.6	0.06	0.11	0.02	0.19
4th	3.43	39.7	84.6	0.06	0.12	0.02	0.19
5th	3.44	40.16	85.6	0.06	0.11	0.02	0.19

[a] *Average cell proliferation was calculated by taking an average of the change in cell concentration over 120 h of fermentation*

Table 4. 9 Comparison of cell proliferation rate, sugar consumption and ABE productivity for cycles 1-5 of *CaCt* (fused) strains

Growth cycles	Average Cell proliferation rate ($x10^5$ cells/ml.h)[a]	Total Sugar Consumed		ABE productivity (g/L.hr)			Total ABE (g/L.hr)
		Concentration (g/L)	Percentage (%)	Acetone (g/L.hr)	Butanol (g/L.hr)	Ethanol (g/L.hr)	
1st	2.8	34	75	0.05	0.1	0.02	0.17
2nd	2.08	33.4	73.73	0.04	0.09	0.01	0.14
3rd	1.54	32.1	70.86	0.03	0.08	0.01	0.12
4th	1.32	31.3	69.1	0.03	0.07	0.01	0.11
5th	0.87	30.7	67.7	0.03	0.06	0.01	0.1

[a] *Average cell proliferation was calculated by taking an average of the change in cell concentration over 120 h of fermentation*

4.8 Genetic Stability Tests

The products formed at the end of PCR reactions were examined using agarose gel electrophoresis to study the genetic stability of *CbCt* (fused) and *CaCt* (fused) strains. Figure 4.14 shows the electrophoresis result for all growth cycles of *CbCt* (fused) strains. G1 represents the parent fusion or first growth cycle; G2 represents the second growth cycle and so on. A G10 strain was grown and analysed to ensure reproducible stability. The biomarker control genes that were selected for investigation in the *CbCt* (fused) strains of G1 - G5 and G10 were chosen to be β-glucosidase A gene (*bgl*A) and aldehyde dehydrogenase gene (*ald*) from the wild strains of *Ct* and *Cb*, respectively. The primer sequence for these biomarkers is listed in Table 3.2.

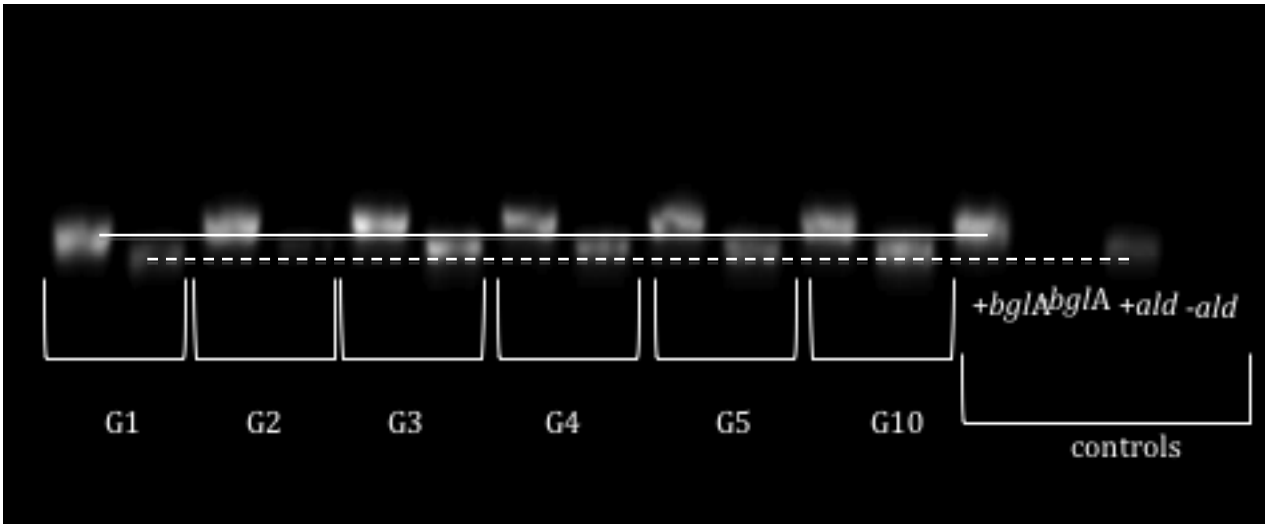

Figure 4. 14 Agarose Gel Electrophorosis of PCR products for biomarkers. From left, growth cycle 1 to 5 and 10 (G1-G5 and G10); first band (solid line) and second band (dotted line) are *bglA* and *ald* gene respectively. Controls as designated

To obtain the DNA profile for growth cycle G1 to G5 and G10 of *CbCt* (fused) strains as shown in the Figure 4.14 (note only the specific biomarker profile is shown), the DNA fragments from PCR are transferred to the negative terminal of the electrophoresis equipment (Figure 3.4) where they move down the gel at different speeds based on their molecular weight. DNA molecules of different lengths

separate, the shortest moving the farthest. For example the *ald* gene has travelled farther (Figure 4.14) than the *bglA* gene.

All DNA fragments are then transferred onto a gel document imaging system (G:Box from SynGene) for further examination. The results from the imaging system are shown in Figure 4.14. The existence of the biomarker control genes in G1-G5 and G10 not only confirm the presence of genes from both wild species in the protoplast-fused strains but also gives an idea about the quantity of genetic information that was transferred to the fusion from the wild strains. For example, from Figure 4.14, it can be observed that the aldehyde dehydrogenase gene (*ald*) responsible for ABE production is represented less in G2. This could explain the feeble decline in ABE productivity that was observed in G2 for *CbCt* (fused) strains. However the presence of both the genes from the wild species confirms that the protoplast fusion has been successful and is also stable. The stability of the fusion is confirmed by the strong expression of both biomarker genes found in G10 contributing to the reproducable stability of the fusion. The results from genetic test are in correlation with solvent productivity examined for *CbCt* (fused) strains in Figure 4.12.

However similar analysis performed using *CaCt* (fused) strains did not produce expected results. While G1 showed strong expression of both biomarker genes, the *bglA* biomarker gene faded from G2-G5 and was almost invisible in G10. The results indicated that *CaCt* (fused) parent strains were not able to maintain reproducible stability over successive generations. This phenomenon is reflective of the decline in solvent productivity observed in Figure 4.13. One way of understanding the lower affinity of *Ca* towards *Ct* can be associated with the presence of DAM methylated DNA in *Ca* (Azeddoug and Reysset, 1991). The phenomenon of DAM methylation is when an enzyme called DAM methylase adds a methyl group to the adenine part of the sequence 5'-GATC-3' in DNA. These methylases are of special interest. It was found that some or all sites for a restriction endonuclease maybe resistant to cleavage when isolated from strains expressing the DAM methylasis. This occurs due to the fact that DNA is protected from cleavage when a particular base in the recognition site of a

restriction endonuclease is methylated. Restriction endonucleases are enzymes that cleave DNA at specific nucleotide sequences. The recognized sequence is often four to six nucleotides long. They occur naturally in some bacteria and they break up foreign DNA. In the absence of DNA, they form a relatively open (less constricted) structure and when encountered with DNA they wrap around it and slide up and down the DNA molecule to search for a particular sequence. Studies have shown that *Ct* undergoes DAM methylation in which any foreign DNA entering the cell must be methylated or restriction endonucleases will digest the DNA at the site 5'-GATC-3 (Azeddoug and Reysset, 1991). It might be possible that when exposed to the methylated DNA from *Ca* the restriction endonucleases in *Ct* will not digest the methylated DNA evading transfer of sufficient genetic information. However, a lot of studies have not been conducted on *Cb* with respect to restriction endonucleases.

4.9 Yield Calculations

Table 4.10 compares overall ABE, acetone, butanol and ethanol yield with respect to sugar consumption. Genetic modification of butanol producing strains (*Cb, Ca*) has allowed for lower solvent inhibition, enhanced productivity and yield. Total ABE yield for *CbCt* (fused) strains remained almost constant for all the five growth cycles at around 0.57-0.58, with acetone yield of 0.17, butanol yield in the range of 0.34-0.35 and ethanol yield of 0.06. The ABE yield obtained from this strain is significantly higher than that reported by (Ezeji, 2004). In the case of *CaCt* (fused) strains, the total ABE yield dropped from 0.57 for the first growth cycle to 0.51. Acetone yield dropped from 0.17 to 0.14, butanol yield changed from 0.34 to 0.32 and ethanol yield was at 0.05. *CbCt* (Co-culture) strains recorded an ABE yield of 0.33 and *CaCt* (Co-culture) strains with an ABE yield of 0.28. It can be concluded that *CaCt* (fused) and *CbCt* (fused) strains were to produce higher yields of biobutanol as compared to their co-culture counterparts.

Table 4. 10 ABE and individual solvent yield for all growth cycles of protoplast fused and co-culture strains

Strains	Growth cycles	ABE Yield ($Y_{ABE/S}$)[a]	Acetone Yield ($Y_{A/S}$)[b]	Butanol Yield ($Y_{B/S}$)[c]	Ethanol Yield ($Y_{E/S}$)[d]
CbCt (fused)	1st	0.57	0.17	0.34	0.06
	2nd	0.57	0.17	0.35	0.06
	3rd	0.58	0.17	0.35	0.06
	4th	0.57	0.17	0.34	0.06
	5th	0.57	0.17	0.34	0.06
CaCt (fused)	1st	0.57	0.17	0.34	0.05
	2nd	0.52	0.15	0.32	0.05
	3rd	0.52	0.14	0.33	0.05
	4th	0.52	0.14	0.33	0.05
	5th	0.51	0.14	0.32	0.05
CbCt (Co-culture)		0.33	0.10	0.20	0.03
CaCt (Co-culture)		0.28	0.08	0.17	0.03
Cb BA101		0.40 (Ezeji, 2004)	-	0.26 (Ezeji, 2004)	-
Cb P260		0.42 (Qureshi et al., 2007)	-	0.2 (Qureshi et al., 2007)	-

[a] *$Y_{ABE/S}$ was calculated by dividing final ABE concentration with total sugars consumed during SSF*
[b] *$Y_{A/S}$ was calculated by dividing final Acetone concentration with total sugars consumed during SSF*
[c] *$Y_{B/S}$ was calculated by dividing final Butanol concentration with total sugars consumed during SSF*
[d] *$Y_{E/S}$ was calculated by dividing final Ethanol concentration with total sugars consumed during SSF*

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Simultaneous saccharification and fermentation (SSF) was performed using the batch approach based on renewable green substrates (wheat straw) and by utilizing novel clostridia protoplast fused strains. This novel development has been successful in eliminating the requirement to add external enzymes to help in the saccharification process and perform SSF at an elevated temperature of 45^{o}C to amplify activity of internal enzymes (from within the fused strains) eventually leading to higher concentration of butanol in the fermentation broth. The strains also showed immense level of tolerance towards butanol toxicity. These characteristics will be of prime importance while commercializing this technology as lower enzyme costs leads to more profitability.

Protoplast fused strains in general produced higher concentration of biobutanol and other solvents when compared to co-cultures. This confirms the superiority of the process as opposed to a co-culture. The *CbCt* (fused) strains produced 13.81 g/L of butanol (yield of 0.34) as compared to their co-culture counterpart which only produced about 5.79 g/L (yield of 0.2). A similar conclusion can be derived from *CaCt* (fused) strains with butanol concentration of 12 g/L (yield of 0.34) as compared to 4.53 g/L (yield of 0.17) achieved when using the co-culture strains.

In the current study, the parent protoplast fused strains were passed on to five successive growth cycles and the fused strains of each growth cycle were later inoculated with substrate and examined for solvent production and compared to the parent generation. Growth cycles for *CbCt* (fused) strains maintained strong butanol productivity throughout all growth cycles whereas the corresponding *CaCt* (fused) strains declined in biobutanol concentration by 36% over five growth cycles. Genetic stability studies using Polymerase Chain Reaction Technique (PCR) and Agarose Gel Electrophoresis demonstrated a reproducable stability of *CbCt* (fused) strains.

Evidently, *CbCt* (fused) exhibited immense potential not only in terms of butanol production but also from enzyme activity. The perfect combination (through protoplast fusion) of a thermophile (*Ct*) which can produce enzymes that can degrade cellulose and cellobiose into sugars and that of a mesophile (*Cb)* that is known to hydrolyse hemicellulose to pentose sugars and finally to butanol holds an immense potential to revolutionize and bring back commercial/industrial levels of butanol production.

5.2 Recommendations

To truly understand the potential of the *CbCt* (fused) strains, the author suggests performing SSF studies using this strain on a scale up reactor that would mimic industrial production. Pilot scale batch reactors pose additional advantages such as continuous removal of toxic butanol, pH and acid control during SSF when compared to laboratory scale batch processes. Hence, the author believes that the true potential of the strains developed in this study can be thoroughly examined in a pilot scale reactor.

While performing SSF in a batch reactor using the novel strains, the author also suggests the use of gas stripping and pH control to further reduce the affect of butanol toxicity on these strains. A combination of gas stripping, pH control and genetically improved protoplast fused strains has the potential to produce high levels of butanol.

In terms of genetic improvement, the author would like to suggest mutagenesis (UV or chemical) on the fused strains to study the effect of DNA mutations on ABE metabolism.

APPENDICES

Appendix A: Chemicals Used in the Current Study

Table A 1 List of chemicals used in the current study

Product	Company	Catalogue No.
Reinforced Clostridial Medium (RCM)	Oxoid Ltd. (Basingstoke, Hampshire, UK)	CM0149
Cooked Meat Medium (CMM)	Oxoid Ltd. (Basingstoke, Hampshire, UK)	MT0350
Glucose	Sigma-Aldrich (St. Louis, MO)	G8769
D-biotin	Sigma-Aldrich (St. Louis, MO)	B4501
PABA	Sigma-Aldrich (St. Louis, MO)	6930
Thiamine- HCl	Sigma-Aldrich (St. Louis, MO)	T4625
$FeSO_4.7H_2O$	Sigma-Aldrich (St. Louis, MO)	F8048
$MnSO_4.4H_20$	Sigma-Aldrich (St. Louis, MO)	M7634
$MgSO4._7H_20$	Sigma-Aldrich (St. Louis, MO)	63138
H_2SO_4	Sigma-Aldrich (St. Louis, MO)	339741
KH_2PO_4	Sigma-Aldrich (St. Louis, MO)	322431
K_2HPO_4	Sigma-Aldrich (St. Louis, MO)	GO139
NaOH	Sigma-Aldrich (St. Louis, MO)	S5881
DNS acid	Sigma-Aldrich (St. Louis, MO)	D0550
Sodium potassium tartrate tetrahydrate	Sigma-Aldrich (St. Louis, MO)	217255
Fluka-Yeast extract	Sigma-Aldrich (St. Louis, MO)	70161
Sucrose	Sigma-Aldrich (St. Louis, MO)	S0389
Poly ethylene glycol (PEG)	Sigma-Aldrich (St. Louis, MO)	X2753
Casein hydrolysate	Sigma-Aldrich (St. Louis, MO)	22090
$(NH_4)_2SO_4$	Sigma-Aldrich (St. Louis, MO)	7783202
Asparagine	Sigma-Aldrich (St. Louis, MO)	A0884
Agar	Sigma-Aldrich (St. Louis, MO)	05038

Product	Company	Catalogue No.
Glutathione	Sigma-Aldrich (St. Louis, MO)	G4251
Sodium glycerophosphate	Sigma-Aldrich (St. Louis, MO)	S579297
Casamino acid	Cole palmer	S79955
NaCl	Sigma-Aldrich (St. Louis, MO)	S7653

Appendix B: Filter Paper Aassay Calculations and Glucose Standards:

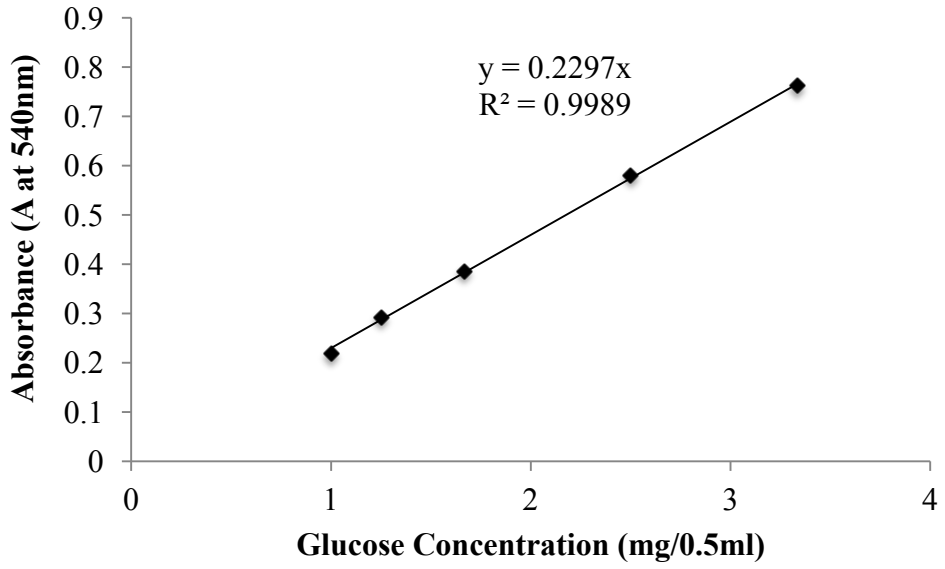

Figure B 1 Calibration curve of glucose concentration used for filter paper assay analysis using UV-vis at 540 nm

Table B 1 Dilution of glucose standards and UV-vis absorbance reading

Glucose stock (mL)	Citrate buffer (mL)	Dilution	Concentration	Abs. 540 nm
1	0.5	01:01.5	3.35 mg/0.5 mL	0.765
2	1	1:02	2.50 mg/0.5 mL	0.579
3	2	1:03	1.65 mg/0.5 mL	0.384
4	3	1:04	1.25mg/0.5ml	0.292
5	4	1:05	1.00 mg/0.5 mL	0.22

Table B 2 Enzyme dilutions made in sodium citrate buffer of 0.05 M pH 4.8

Dilution No.	Citrate buffer (mL)	1:20 Enzyme (mL)	*Concentration
1	17	3	0.0075
2	18	2	0.005
3	18.5	1.5	0.00375

**The term "concentration" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture.*

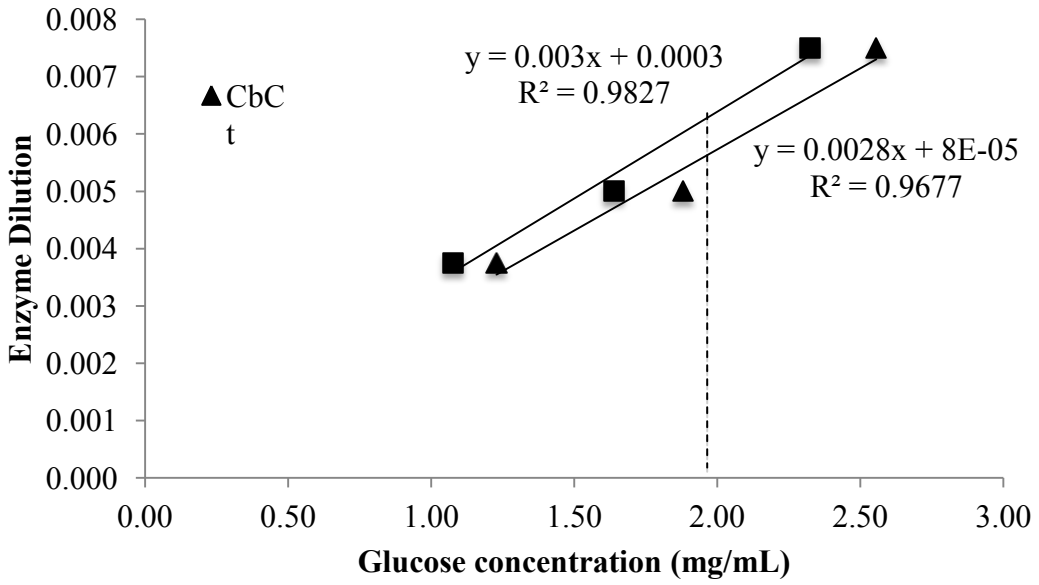

Figure B 2 Determination of concentration of enzyme that would release exactly 2 mg of glucose by plotting glucose liberated against enzyme dilution

Calculation of FPU from graph of dilution vs. glucose concentration using the formula

$$Filter\ Paper\ Activity = \frac{0.37}{[enzyme] releasing\ 2mg\ of\ glucose} \left(\frac{units}{mL}\right)$$

In the above formula, [*enzyme*] represents the proportion of original enzyme solution present in the directly tested enzyme dilution.

For *CbCt* (fused) = 0.37/0.0054 = 68.52 FPU/ml

For *CaCt* (fused) = 0.37/0.0063 = 58.73 FPU/ml

Appendix C: Raw Data for Total Sugars

Table C 1 Raw Concentration Data for Total Sugars during SSF for *CbCt* (fused) Strains

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	30.00	29.70	29.70	29.80	0.17	0.58
12	42.86	43.02	43.60	43.16	0.39	0.90
24	45.54	46.80	48.42	46.92	1.44	3.08
36	29.60	30.30	32.65	30.85	1.60	5.18
48	22.60	22.80	23.06	22.82	0.23	1.01
60	15.53	15.20	16.28	15.67	0.55	3.53
72	11.52	12.38	11.80	11.90	0.44	3.69
84	9.75	9.83	9.43	9.67	0.21	2.19
96	8.73	8.30	8.50	8.51	0.22	2.53
108	7.50	7.60	7.70	7.60	0.10	1.32
120	7.10	7.25	7.10	7.15	0.09	1.21

Table C 2 Raw Concentration Data for Total Sugars during SSF for *CbCt* (Co-culture) Strains.

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	30.00	29.70	29.70	29.80	0.17	0.58
12	40.42	41.25	40.46	40.71	0.47	1.15
24	44.01	44.50	44.12	44.21	0.26	0.58
36	34.50	34.53	34.86	34.63	0.20	0.58
48	29.74	29.56	28.21	29.17	0.84	2.87
60	21.54	22.06	20.00	21.20	1.07	5.05
72	19.46	19.37	19.21	19.35	0.13	0.65
84	16.25	16.30	15.94	16.16	0.20	1.21
96	15.45	16.18	15.65	15.76	0.38	2.39
108	15.33	16.01	15.40	15.58	0.37	2.40
120	15.52	15.43	15.40	15.45	0.06	0.40

Table C 3 Raw Concentration Data for Total Sugars during SSF for *CaCt* (fused) Strains

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	30.00	29.70	29.70	29.80	0.17	0.58
12	44.20	44.34	43.19	43.91	0.63	1.43
24	45.54	45.20	45.16	45.30	0.21	0.46
36	32.30	31.80	31.66	31.92	0.34	1.05
48	26.78	27.13	26.80	26.90	0.20	0.73
60	21.54	22.06	20.00	21.20	1.07	5.05
72	16.63	16.76	16.80	16.73	0.09	0.53
84	14.50	14.90	15.03	14.81	0.28	1.87
96	12.55	12.60	12.56	12.57	0.03	0.21
108	11.56	11.40	11.57	11.51	0.10	0.83
120	11.14	11.50	11.32	11.32	0.18	1.59

Table C 4 Raw Concentration Data for Total Sugars during SSF for *CaCt* (Co-culture) Strains

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	30.00	29.70	29.70	29.80	0.17	0.58
12	40.03	40.21	40.30	40.18	0.14	0.34
24	45.66	44.16	45.21	45.01	0.77	1.71
36	33.98	34.15	34.50	34.21	0.27	0.78
48	27.37	27.34	27.28	27.33	0.05	0.17
60	23.30	23.14	23.13	23.19	0.10	0.41
72	21.30	21.15	20.85	21.10	0.23	1.09
84	19.67	19.53	20.38	19.86	0.46	2.29
96	18.64	19.14	19.01	18.93	0.26	1.37
108	18.56	18.32	18.74	18.54	0.21	1.14
120	18.57	18.35	18.13	18.35	0.22	1.20

Appendix D Sugars, ABE, and Acids Standards (HPLC)

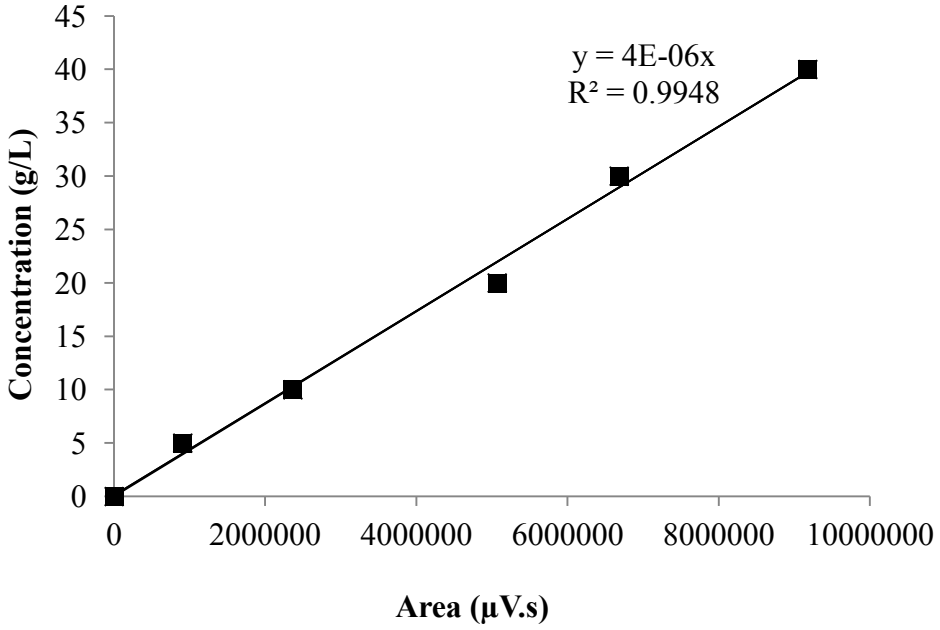

Figure D 1 HPLC standard curve for glucose with retention time of t = 12.92 min

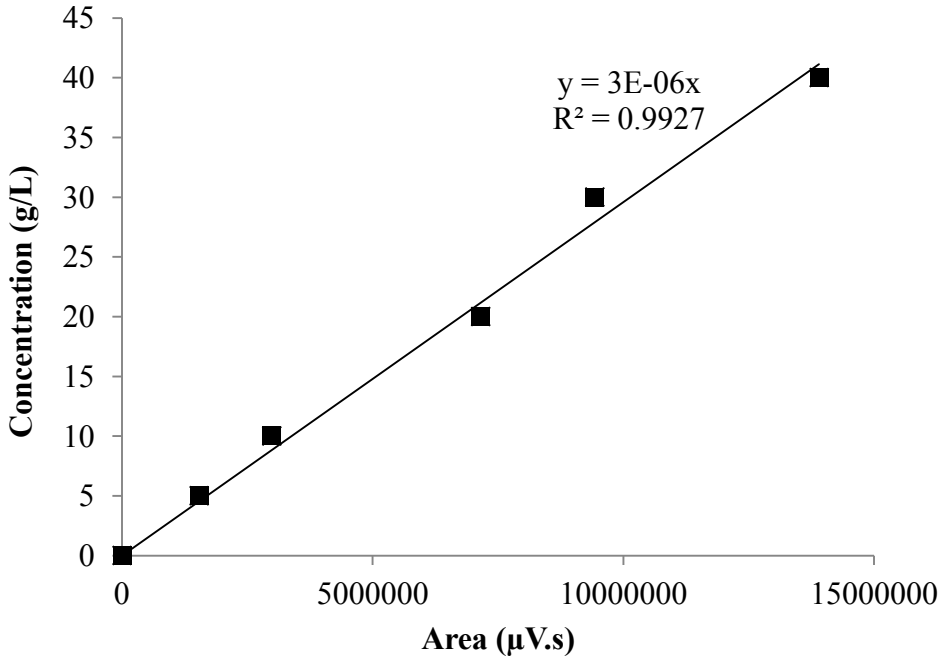

Figure D 2 HPLC standard curve for xylose with retention time of t = 14.02 min

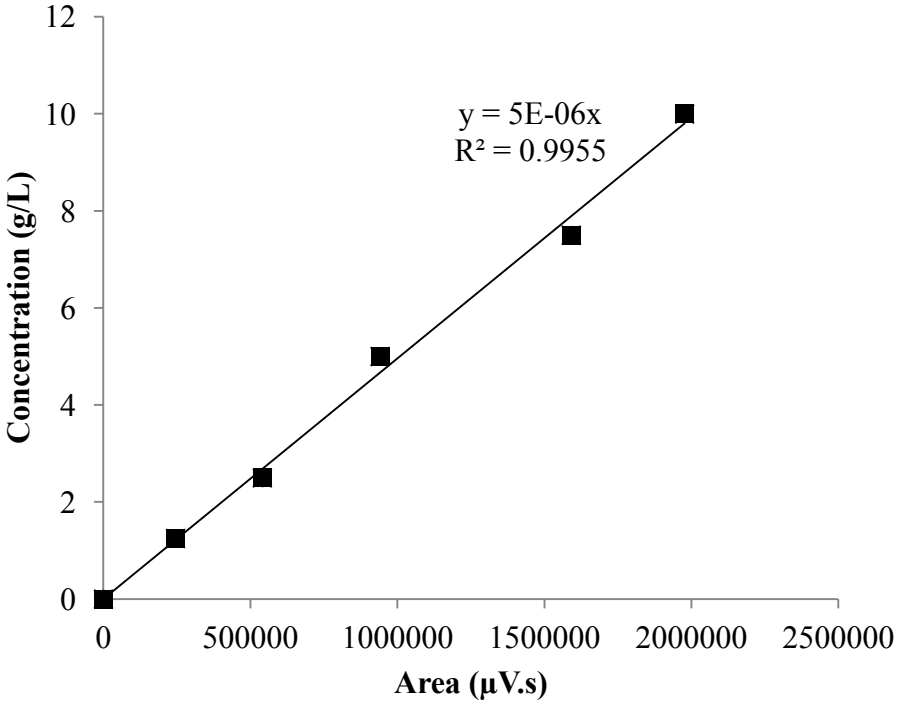

Figure D 3 HPLC standard curve for Mannose with retention time of t = 9.38 min

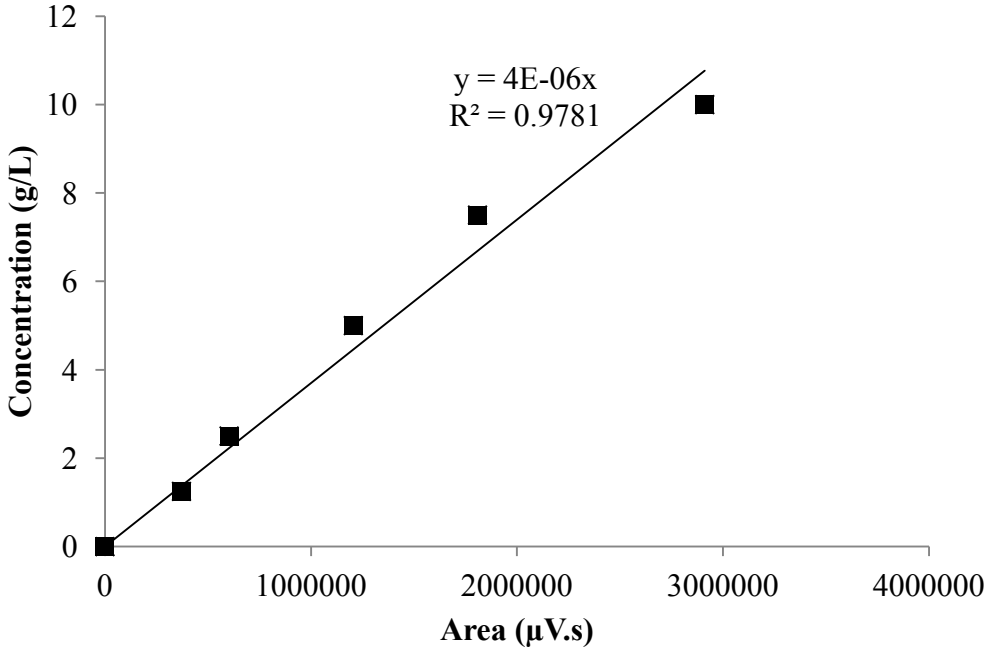

Figure D 4 HPLC standard curve for arabinose with retention time of t = 21.61 min

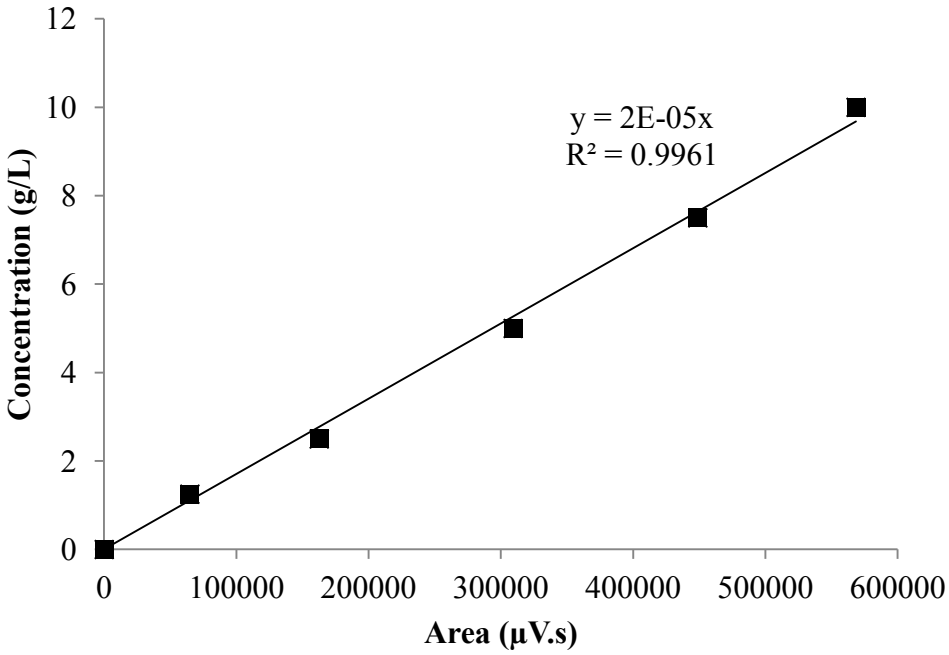

Figure D 5 HPLC standard curve for acetone with retention time of t = 15.03 min

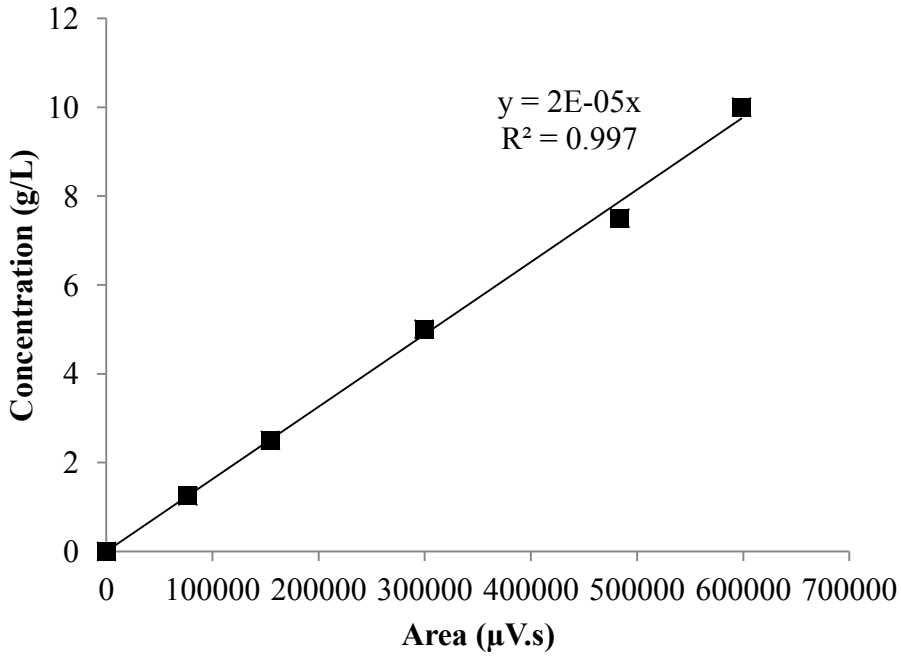

Figure D 6 HPLC standard curve for butanol with retention time of t = 15.54 min

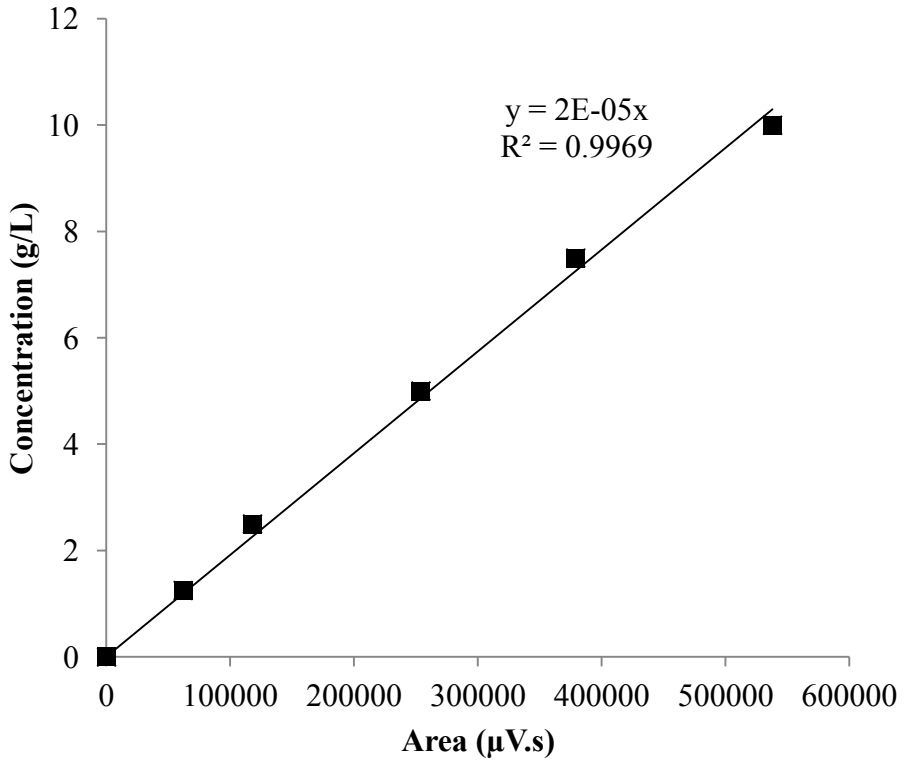

Figure D 7 HPLC standard curve for ethanol with retention time of t = 13.69 min

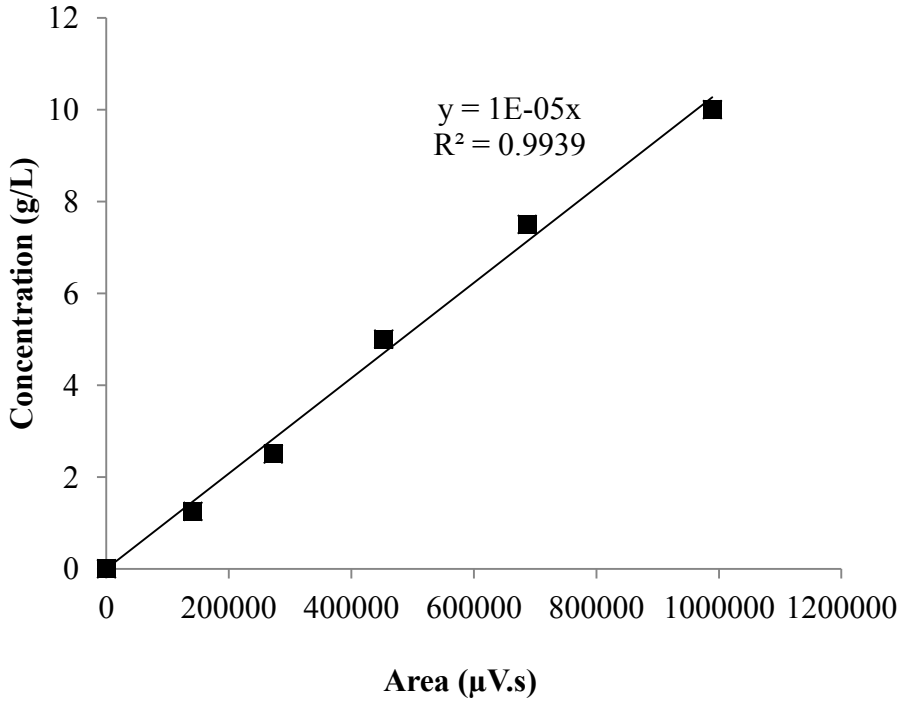

Figure D 8 HPLC standard curve for Acetic acid with retention time of t = 11.71 min

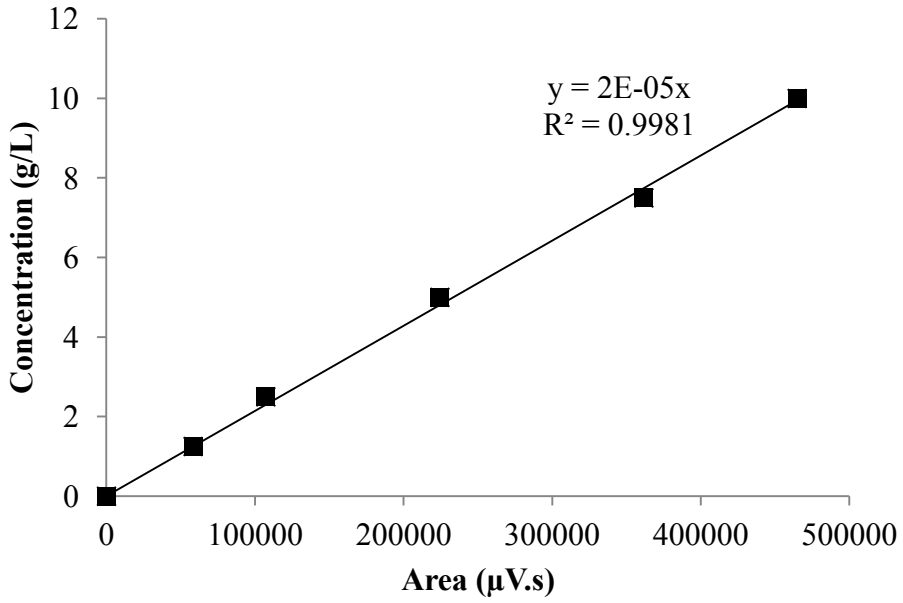

Figure D 9 HPLC standard curve for butyric acid with retention time of t = 12.38 min

Appendix E : Cell Growth Profiles for all Protoplast Fusion and Co-culture Strains

Table E 1 Cell growth profile for *CbCt* (fused)

Time (h)	Plate. # 1 (x 10^7)	Plate. # 2 (x 10^7)	Plate. # 3 (x 10^7)	Mean (x 10^7)	STDEV (x 10^7)	%RSD
0	1.10	1.20	1.00	1.10	0.10	9.09
12	1.30	1.27	1.30	1.29	0.02	1.3
24	3.00	3.20	3.40	3.2	0.2	6.25
36	4.40	4.30	4.50	4.40	0.10	2.27
48	5.20	5.40	5.10	5.23	0.15	2.92
60	6.00	5.80	6.10	5.97	0.15	2.56
72	5.80	5.90	5.80	5.83	0.05	9.90
84	6.00	6.30	5.50	5.93	0.40	6.81
96	5.50	5.70	5.40	5.53	0.15	2.76
108	5.20	5.40	5.10	5.23	0.15	2.92
120	5.10	5.00	5.30	5.13	0.15	2.98

Table E 2 Cell growth profile for *CaCt* (fused)

Time (h)	Plate. # 1 ($x 10^7$)	Plate. # 2 ($x 10^7$)	Plate. # 3 ($x 10^7$)	Mean ($x 10^7$)	STDEV ($x 10^7$)	%RSD
0	1.00	1.10	0.9	1.00	0.10	10
12	1.30	1.30	1.40	1.33	0.05	3.75
24	2.50	2.60	2.40	2.50	0.10	4
36	3.80	3.60	4.00	3.80	0.20	5.26
48	5.00	5.00	5.10	5.03	0.05	1.15
60	5.50	5.60	5.50	5.53	0.05	1.04
72	5.50	5.70	5.30	5.50	0.20	3.64
84	5.40	5.30	5.59	5.43	0.15	2.71
96	5.30	5.20	5.31	5.27	0.06	1.15
108	5.20	5.10	5.33	5.21	0.2	2.21
120	5.10	5.00	5.26	5.12	0.13	2.56

Table E 3 Cell growth profile for *CbCt* (co-culture)

Time (h)	Plate. # 1 (x 10^7)	Plate. # 2 (x 10^7)	Plate. # 3 (x 10^7)	MEAN (x 10^7)	STDEV (x 10^7)	%RSD
0	1.30	1.20	1.30	1.27	0.05	4.56
12	1.90	2.00	1.70	1.87	0.15	8.18
24	2.90	2.80	3.00	2.90	0.10	3.45
36	3.80	3.80	4.00	3.87	0.12	2.99
48	5.00	5.00	5.10	5.03	0.05	1.15
60	5.20	5.20	5.30	5.23	0.05	1.10
72	5.20	5.20	5.40	5.27	0.12	2.19
84	5.10	5.00	5.20	5.10	0.10	1.96
96	4.90	5.00	4.70	4.87	0.15	3.14
108	4.40	4.40	4.50	4.43	0.05	1.30
120	4.20	4.20	4.30	4.23	0.05	1.36

Table E 4 Cell growth profile for *CaCt* (co-culture)

Time (h)	Plate # 1 ($x 10^7$)	Plate # 2 ($x 10^7$)	Plate # 3 ($x 10^7$)	Mean ($x 10^7$)	STDEV ($x 10^7$)	%RSD
0	1.30	1.20	1.30	1.27	0.05	4.56
12	1.90	2.00	1.70	1.87	0.15	8.18
24	2.90	2.80	3.00	2.90	0.10	3.45
36	3.80	3.80	4.00	3.87	0.12	2.99
48	5.00	5.00	5.10	5.03	0.05	1.15
60	5.20	5.20	5.30	5.23	0.05	1.10
72	5.20	5.20	5.40	5.27	0.16	2.19
84	5.10	5.00	5.20	5.10	0.10	1.96
96	4.90	5.00	4.70	4.87	0.15	3.14
108	4.40	4.40	4.50	4.43	0.05	1.30
120	4.20	4.20	4.30	4.23	0.05	1.36

Appendix F : ABE and Acids Raw Data from three SSF Experiments

Table F 1 ABE concentration from three SSF experiments for all strains

Strains	ABE (Acetone, Butanol, Ethanol) Concentration (g/L), STDEV and %RSD					
	SSF 1	SSF 2	SSF 3	Mean	STDEV	% RSD
CaCt (fused)	19.87	20.43	20.3	20.2	0.29	1.45
CaCt (co-culture)	7.45	7.36	7.51	7.44	0.08	1.01
CbCt (fused)	22.83	23.05	23.12	23	0.15	0.66
CbCt (co-culture)	9.62	9.44	9.5	9.52	0.09	0.96

Table F 2 Final acid concentration from three SSF experiments for all strains

Strains	Total Acid Concentration (g/L), STDEV and %RSD					
	SSF 1	SSF 2	SSF 3	Mean	STDEV	%RSD
CaCt (fused)	2.72	2.68	2.7	2.7	0.02	0.74
CaCt (co-culture)	3.6	3.73	3.8	3.71	0.10	2.74
CbCt (fused)	2.6	2.61	2.65	2.62	0.03	1.01
CbCt (co-culture)	3.3	3.4	3.44	3.38	0.07	2.13

Appendix G : BioButanol Concentration Profile Raw Data from three SSF Experiments

Table G 1 Biobutanol concentration profile data for *CaCt* (fused) strains from three SSF experiments

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	0.00	0.00	0.00	0.00	0.00	0.00
12	0.08	0.09	0.08	0.08	0.01	6.93
24	0.29	0.27	0.34	0.30	0.04	12.02
36	2.33	2.00	2.30	2.21	0.18	8.26
48	5.40	5.70	5.52	5.54	0.15	2.73
60	7.60	7.50	7.34	7.48	0.13	1.75
72	9.56	9.43	9.21	9.40	0.18	1.88
84	10.30	10.65	10.40	10.45	0.18	1.72
96	11.54	12.19	12.06	11.93	0.34	2.88
108	11.89	12.12	12.17	12.06	0.15	1.24
120	12.01	12.10	12.10	12.07	0.05	0.43

Table G 2 Biobutanol concentration profile for *CaCt* (co-culture) strains from three SSF experiments

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	0.00	0.00	0.00	0.00	0.00	0.00
12	0.05	0.05	0.05	0.05	0.00	0.00
24	0.36	0.43	0.41	0.40	0.04	9.01
36	1.20	1.12	1.13	1.15	0.04	3.79
48	2.20	2.20	2.23	2.21	0.02	0.76
60	3.04	2.85	2.93	2.94	0.10	3.24
72	3.53	3.68	3.50	3.57	0.10	2.70
84	3.86	3.85	3.90	3.87	0.03	0.70
96	4.00	4.17	4.13	4.10	0.09	2.17
108	4.21	4.30	4.18	4.23	0.06	1.48
120	4.26	4.26	4.23	4.25	0.02	0.41

Table G 3 Biobutanol concentration profile data for *CbCt* (fused) strains from three SSF experiments

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	0.00	0.00	0.00	0.00	0.00	0.00
12	0.08	0.09	0.09	0.08	0.00	4.12
24	0.12	0.09	0.09	0.10	0.02	17.58
36	2.20	2.32	2.23	2.25	0.06	2.78
48	5.24	5.30	5.42	5.32	0.09	1.72
60	7.76	7.85	7.80	7.80	0.05	0.58
72	10.58	10.74	10.69	10.67	0.08	0.77
84	12.32	12.50	12.47	12.43	0.10	0.78
96	13.35	13.41	13.50	13.42	0.08	0.56
108	13.91	13.73	13.64	13.76	0.14	1.00
120	13.76	13.84	13.83	13.81	0.04	0.32

Table G 4 Biobutanol concentration profile data for *CbCt* (co-culture) strains from three SSF experiments

Time (h)	SSF 1	SSF 2	SSF 3	MEAN	STDEV	%RSD
0	0.00	0.00	0.00	0.00	0.00	0.00
12	0.05	0.06	0.05	0.05	0.01	10.83
24	0.12	0.09	0.11	0.11	0.02	14.32
36	1.24	1.22	1.18	1.21	0.03	2.52
48	2.65	2.74	2.65	2.68	0.05	1.94
60	3.82	3.77	3.75	3.78	0.04	0.95
72	4.74	4.74	4.80	4.76	0.03	0.73
84	5.48	5.54	5.48	5.50	0.03	0.63
96	5.77	5.77	5.65	5.73	0.07	1.21
108	5.72	5.77	5.80	5.76	0.04	0.70
120	5.83	5.71	5.80	5.78	0.06	1.08

Appendix H : HPLC response graphs for a SSF sample

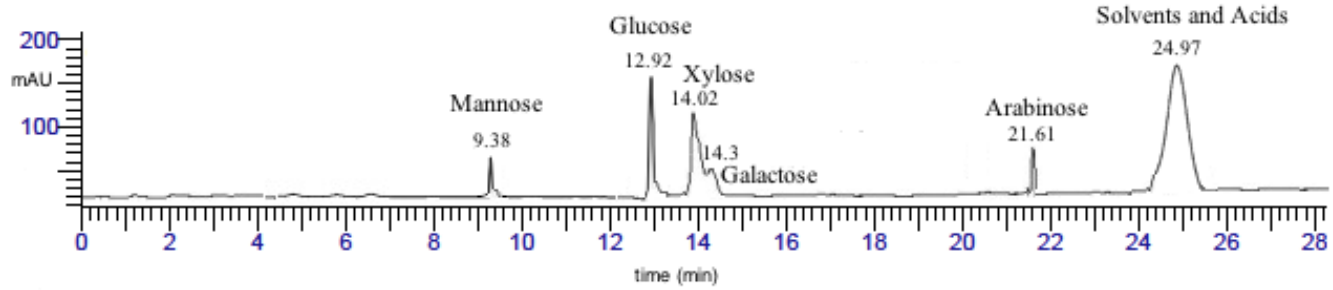

Figure H 1 HPLC detector response using Shodex KC811 sugar column for a SSF sample

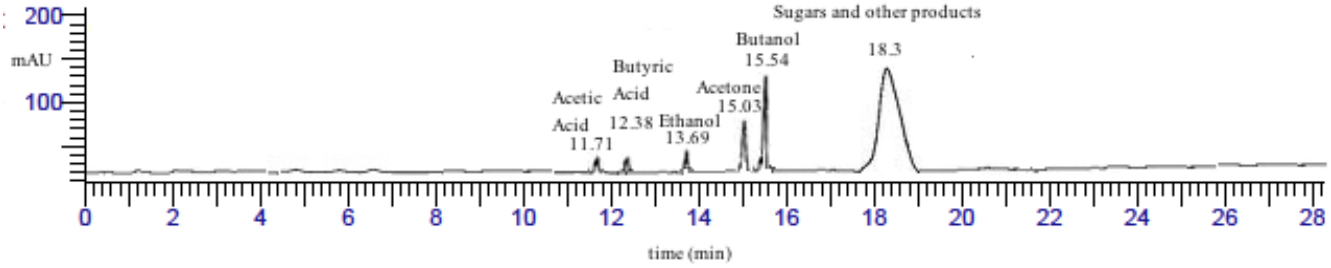

Figure H 2 HPLC detector response using Aminex HPX-87H acids and solvent column for a SSF sample

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