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SEQUENTIAL CO-CULTURE OF ANAEROBIC BACTERIA ON SWITCHGRASS IN A CONTINUOUS FLOW-THROUGH REACTOR FOR BIOFUEL PRODUCTION

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THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biosystems and Agricultural Engineering the College of Engineering at the University of Kentucky

By

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ABSTRACT OF THESIS

SEQUENTIAL CO-CULTURE OF ANAEROBIC BACTERIA ON SWITCHGRASS IN A CONTINUOUS FLOW-THROUGH REACTOR FOR BIOFUEL PRODUCTION

Solid substrate cultivation (SSC) using lignocellulosic non-food feedstock, such as switchgrass, is an alternative for advanced biofuel production. Acetone-Butanol-Ethanol (ABE) fermentation in two stages using a sequential culture of microorganisms from the class *Clostridia* is an approach proposed to increase the butanol production.

The goal was to test the efficacy of a sequential culture on high solid substrate cultivation in batch and continuous cultivation, and to evaluate conditions to optimize butanol production using switchgrass as substrate.

Initial batch experiments were used to determine particle size effect, choice of solvent producer and pretreatment evaluation: The effect of particle size on gas production was surface area-dependent, 2 mm particle size of switchgrass was better fermented by clostridia than the other particle sizes. *C. thermocellum* improved switchgrass fermentation by *C. beijerinckii*. Moreover, C. *saccharoperbutylacetonicum* produced the highest butanol yield on glucose as substrate. The Fenton reaction was studied as a potential pretreatment for switchgrass. *C. beijerinckii* grew better on Fenton-treated material, but solvent production was low.

The major conclusion of the continuous flow on SSC experiment was that there is no statistical difference in the effect of flow rate within the flow range tested.

KEYWORDS: Switchgrass, *Clostridium* co-culture, particle size, Fenton pretreatment, continuous fermentation

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Introduction

"*The time has come for every international body and agency to take strong and immediate action to limit greenhouse gas emissions and halt climate change. Global warming is rapidly accelerating beyond our best collective efforts to resist. If there was ever a time for the world's most successful environmental treaty to expand its efforts, that time is now*" (HFCs, the Montreal Protocol and the UNFCCC: Eliminating 1 of the 6 Kyoto gases Bangkok climate change conference, August-September 2012*).*

"We need to be prepared for nine billion people on this planet, as we all deserve a decent and secure life. By being creative, the world can reduce greenhouse gas emissions while creating jobs, promoting economic growth and ensuring better living standards. Where there is a will, there is a way!" (Minister Marcin Korolec, President of COP19/CMP9, Conference of Paris and Climate Change Conference, November 2013).

The limited stock of fossil fuels and the high demand of the population, together with climate change and the necessary limitation of greenhouse gas emissions have spurred research to look for clean and renewable sources of energy (Brown, 2003). In this scenario, biofuels are playing a crucial role.

Biofuels can be defined as a renewable resource derived from organic materials of recent biological origin (biomass). Thus, biofuel is any gas, liquid or solid fuel derived from biomass (Demirbas, 2012). The first biofuel used by humanity was wood about 500,000 years before the present. Moreover, oils and fats were also used as biofuels about 40,000 years ago. However, the use of coal (400 years ago) and petroleum (125 years ago) led to a decline in using biofuels. Currently, there are different motivations to return to a biobased economy such as environmental quality, national security and rural area development. Biofuels are a partial solution to address the challenge of reduction in petroleum supply and increased petroleum demand while simultaneously reducing greenhouse gas emissions.

Biofuels

The first generation biofuels are those that have been derived from starch, vegetable oil, animal fat or sugar using conventional technology (Demirbas et al., 2012). The most popular biofuels from this generation are biodiesel and ethanol. Biodiesel is derived from oil seed crops such as soybeans, rape, sunflowers, jatropha, among others. Also, it can be derived from oil discarded as waste frying oil or lard. The American Society for Testing and Materials (ASTM) defines biodiesel is a "molecule composed of methyl or ethyl esters of fatty acids produced by the transesterification of plant oils and animal fats with simple alcohols" (Demirbas, 2002). Because of biodiesel's similarities with petroleum derived fuels up to 20% biodiesel can be blended with gasoline without requiring engine modifications. Bioethanol is derived from the fermentation of monosaccharides (Demirbas and Demirbas, 2010). Sugars from corn, rice or wheat starch or sugarcane, sugarbeets and sweet sorghum are fermented by microorganisms obtaining ethanol. These first generation fuels compete with the food supply and are thought to increase food prices (Godfray et al., 2010). In contrast, second-generation biofuels, also known as next generation biofuels, are produced from lignocellulosic non-food biomass. Fuels derived from biomass such as bioethanol and biobutanol are examples of this generation. Wood, vegetable waste, sawdust, municipal organic wastes, non-edible crops are examples of feedstock for these fuels (Kim and Dale, 2004). Biomass is an attractive feedstock because it is abundant globally. Lignocellulosic biomass is a plentiful renewable resource and it could be a primary fuel (Monique et al., 2003). Another attractive feature of this feedstock is that perennial biomass takes less energy to grow and cultivate since it requires less fertilizer and herbicides (Schmer et al., 2008). Furthermore, perennial grasses help to sequester carbon and reduce the erosion improving the soil quality. Biomass crops have the potential to provide additional energy sources such as recalcitrant residues that can be burned and produce heat or electricity. Biomass energy can play an important role in reducing greenhouse gas emissions; since CO_2 that would originally have been released into the air is fixed into the biomass (Lynd, 1996). Fossil fuels contain carbon that has been sequestered for millions of years; when it is used, carbon dioxide is released to the atmosphere.

Biobutanol is one of the second generation fuels (Durre, 2008). It is an alcohol with similar properties to gasoline. The butanol is produced by conventional chemical processes for chemical synthesis of esters, ethers, plasticizers, and solvents. However, butanol was already produced by the acetone-butanol-ethanol fermentation in the early 1900s to obtain butanediene for rubber (Spivey, 1978). During the First World War, many fermentation plants were built to produce acetone for the manufacture of explosives and butanol was used in different manners. For instance, butyl acetate derived from butanol was an excellent solvent for nitrocellulose lacquers, also, rayon, detergents and brake fluid, among others. By the end of the Second World War, the fermentation industry was displaced by petroleum and natural gas (Lenz and Moreira, 1980). Currently, butanol is attractive as a fuel for having certain advantages over ethanol. Until now, ethanol has been the primary biofuel, because it is economically favorable to produce and easy to manufacture (Andersen et al., 2010). However, compared to ethanol, butanol has more energy per gallon, thus more miles per gallon. Butanol has 110,000 BTUs per gallon, while bioethanol only has 84,000 BTUs per gallon. Butanol can also be blended with gasoline at much higher levels than ethanol without engine alterations, because its physical attributes are more closely aligned with gasoline. Compared with gasoline, butanol use results in a reduction of the greenhouse gases emission burden (32-48% less) and a substantial fossil energy savings (39-56%) when it is produced by ABE fermentation (Wu et al., 2008). It is less hydroscopic and less corrosive (Dürre, 2008). Butanol has lower vapor pressure than gasoline, which makes it safer to store and handle (Wallner et al., 2009).

Swana et al. (2011) compared these biofuels in a life cycle assessment (LCA) and found that the net energy return from biobutanol (6.53 MJ/L) was higher than ethanol (0.40MJ/L). All these features favor biobutanol as the second generation biofuel of choice (Table 1.1).

Table 1.1. Properties of different fuels. Source: Lee et al., 2008.

Physical property	Ethanol	Butanol	Gasoline
Chemical formula	C_2H_5OH	$CH_3(CH_2)_3OH$	C_4–C_{12}
Energy density (MJ/L)	19.6	29.2	32
Heat of vaporization (MJ/kg)	0.92	0.43	0.36
Octane Number	129	96	91-99
Motor octane Number	101	78	81-89
Miscibility with:			
Gasoline	Fair	Good	
Diesel	Poor	Good	
Water	High	Low	Low

Biorefineries

The biorefinery parallels the petroleum refinery because it utilizes raw material from renewable polysaccharides and lignin that pass through different processes, then it is fractionated and converted into a mixture of products. Biorefineries have the potential to enhance the use of feedstocks via proper modification (Kamm and Kamm, 2004). Biomass carbohydrates provide alcohols, carboxylic acids and esters, which are usually produced using expensive chiral catalysts and complex processes. There are a broad variety of biobased products. For instance, polylactic acid resin (PLA) is the most well-known renewable biodegradable plastic (Bhardwaj and Mohanty, 2007). It is obtained from the fermentation of dextrose derived from plant starch. In the process of milling corn, starch is separated, hydrolyzed to glucose which is anaerobically fermented to lactic acid with *Bacillus dextrolacticus* or *Lactobacillus delbrueckii*. Lastly, esterification with ethanol is used to obtain ethyl lactate which is converted in polylactic acid resin. It is used as buffering agent, acidulant and bacterial inhibitor in processed foods, among others (Jacobsen and Fritz, 1996). It is currently industrially produced and commercialized around the world. Cargill produced PLA by fermentation and it is economically competitive with the chemical synthesis. Gluconic acid is produced by oxidation of glucose in air or oxygen (Anastassiadis and Morgunov, 2007). It is converted in the chelating agent sodium gluconate, which is used in metal cleaning and

equipment cleaning in the dairy and food industries. Other examples are xylitol and sorbitol. The former is obtained by fermentating xylose into xylitol by *Pichia guilliermondii.* It is used as a sweetener and humidity agent control. The latter is derived from catalytic hydrogenation of sucrose. It is used in cosmetics, toiletries, sweetener, bulking agent and flavoring agent, among others. Moreover, 1,4-butanediol (BDO) is an important commodity derived from oil and natural gas used in a variety of areas such as athletic apparel, automotive applications and electronics (Yin et al., 2011). However, BDO is currently produced from renewable carbohydrate feedstocks. Genomatica successfully produces BDO on commercial scale using a metabolically engineered strain of *Escherichia coli*. Moreover, Genomatica together with Versalis signed an agreement in April 2013 to produce butadiene, one of the seven basic chemicals in chemical industry which is used as raw material in production of tires, engineering polymers and latex products. Gevo Inc is producing isobutanol by combination of biology and chemistry (Gevo Inc).

Overall, sugars derived from biomass in a biorefinery can be the building blocks of chemicals which are synthetically produced. The key building blocks are ethanol, C3 to C6 carboxylic acids, glycerol and sorbitol (Briens et al., 2008). The development of these "green chemicals" relies on finding appropriate biomass feedstocks and competitive processes including microorganisms, enzymes, separation processes, among others.

Biomass as feedstock

Lignocellulose Structure

The wall cell of these plants contains carbohydrates in the form of hemicellulose and cellulose that can be used to produce biofuel (Olsson et al., 2005). The structure of plant cells varies in different plants, but in general the cell wall consists of cellulose fibers, which reinforce a matrix of hemicelluloses, lignin and pectin. It is known as lignocellulose (Figure 1.1). Cellulose is a high molecular weight, insoluble, linear homopolymer of glucoses residues linked by β-1, 4-glycosidic bonds. Cellulose exits in amorphous and crystalline forms. In nature, it is not completely crystalline nor

completely amorphous, there is a fraction of paracrystalline cellulose (Larsson et al., 1997). Cellulose that is highly ordered is called crystalline; otherwise, it is amorphous. The consequence of this difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose (Nisizawa, 1973). It is the principal chemical component in most lignocellulosic biomass (35-50 w-t %). Hemicelluloses are the second major component (20-35 w-t %) of lignocellulosic biomass. Hemicelluloses are alkali-soluble, linear and branched heteropolymers of D-glucose, D-xylose, L-arabinose, D-mannose, D-galactose and D-glucuronic acid (Jeffries, 1994; van Soest and Wine, 1967). Lignin encloses the cellulose and fortifies the plant cell wall (Olsson et al., 2005). Lignin (5-30 wt %) is a non-sugar based, non-fermentable hydrophobic and aromatic macromolecule. Lignin is a random polymer of three major monomer species: p-hydroxyphenyl, guaiacyl and syringyl produced by reduction of CoA thioesters of coumaric, ferulic and sinapic acids respectively (Davin and Lewis, 1992). Moreover, the lignin type varies in softwood and hardwoods (Sjöström, 1993). Then, lignin degradation depends on the type and amount of lignin. There is a natural resistance of cell walls to being degraded by microbial enzymes. This natural resistance is known as "biomass recalcitrance" (Himmel et al., 2007). Thus, lignin content does not give fermentable sugar and interferes in the enzymatic hydrolysis of cellulose and hemicellulose (Zhang and Lynd, 2004).

Biomass to biofuels

In general, there are three basic steps to obtain biofuels from lignocellulosic biomass: pretreatment of lignocellulosic biomass, saccharification and fermentation.

Firstly, biomass is pretreated in order to increase bacterial enzyme access to polysaccharides and increase surface area for hydrolysis, remove lignin and reduce cellulose crystallinity (Lynd, 1996; Chang and Holtzapple, 2000). Pretreatment options are crucial to reduce cost and energy consumed in the biomass conversion. Pretreatment process represents one third of the cost of the overall process. Also, it has been shown that pretreatment increases sugar yields by 90 %, but enzymatic hydrolysis alone increases sugar yields only by 20 % (Zhang and Lynd, 2004).

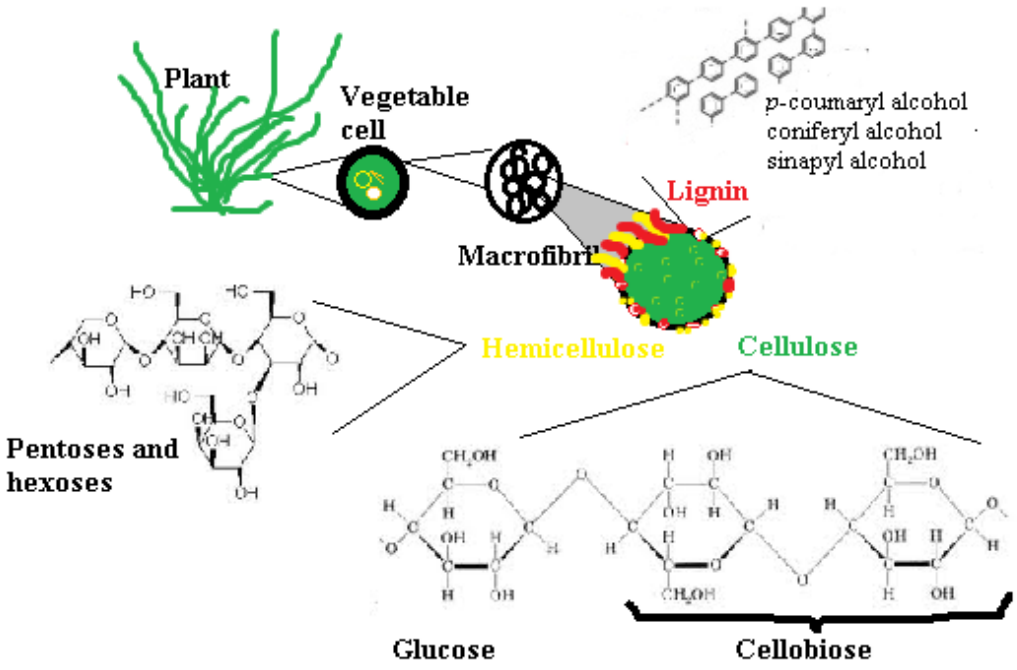


Figure 1.1. Schematic of lignocellulose composition. Lignin, cellulose and hemicellulose form the macrofibril of the vegetable cell.

At the beginning, it is needed to reduce the particle size not only to facilitate the transportation of the biomass but also to increase the surface area for the hydrolysis step (Vidal et al., 2011). Comminution is a mechanical process, which consists of reducing the particle size by pulverization (Himmel et al., 1985). Grinding is other process to reduce to powder or fine particles by friction. There are different machines to reduce the particle size such as hammer mills or knife mills, which consist of hammers or knives mounted on a rotating shaft which reduces particle size by impacting of the hammer bars or knifes with the material. The hammer mill and the knife mill have been compared in terms of energy consumption by Sun and Cheng, 2002 (Table 1.2). The smaller the particle size the bigger the energy consumption.

Table 1.2. Energy consumption comparison between hammer mill and knife mill (Sun and Cheng, 2002).

Final size (mm) Energy Consumption (kW/ton) **Hardwood**		
	Hammer Mill	Knife Mill
1.6	130	130
2.54	120	80
3.20	115	50
6.35	95	25

After reducing the particle size, other processes may be applied to further prepare the biomass for sacharrification and fermentation (Chang et al., 2001) Steam explosion consists of applying high-pressure saturated steam and the pressure is rapidly reduced causing the biomass to undergo an explosive decompression (Vidal et al., 2011). Another method is ammonia fiber expansion, in which, biomass is treated with ammonia at high pressures and then rapidly reduced to atmospheric pressure, boiling the ammonia and exploding the lignocellulosic material (Alizadeh et al., 2005). Among chemical pretreatments, acid or alkaline hydrolysis is commonly used not only to pretreat the lignocellulose but also for hydrolysis of cellulose (Sills and Gossett, 2011). Alkali metal hydroxide breaks the lignin-hemicellulose bond. However, there are many disadvantages of chemical pretreatment, which include: obtaining long cellulose fibers, destruction of cellulose at high concentrations of chemicals, destruction of hemicelluloses and the use of a large amount of chemicals (Brown, 2003). Mixtures of various solvents such as water, ammonia, carbon dioxide and/or ionic liquids have also been successfully used as a biomass pretreatment. However, these processes must be able to eliminate or reduce the use of chemical catalyst and water, be useful for different types of biomass, and increase the sugar yields (Luterbacher et al., 2009). Biological pretreatments involve the use of ligninases from microorganisms. For instance, white-rot fungi (*Basidiomycetes)* and closely related litter- decomposing fungi are lignin degraders. Also, soft rot fungi such as genera *Fusarium* and *Aspergillus* as well as some bacteria (*Nocardia* and *Streptomyces*) have been related with partial lignin degradation (Kirk et al., 1977-1980). Also, *Phanerochaete chrysosporium* is a white-rot fungus that has been reported to degrade

lignin with the addition of cellulose or sugar (Kirk et al, 1978). Biological treatment has disadvantages of being a slow process and the use of released sugars by the microorganism involved.

The Fenton Reaction has been proposed as a mimetic of the fungal delignification process since some wood-rotting fungi also utilizes OH^- radicals to degrade lignocellulose in nature (Hammel et al., 2002; Arantes et al., 2012). The Fenton reaction consists of the oxidation of organic compounds using H_2O_2 and a catalyst Fe^{+2} (Fenton, 1894).The reaction is represented by the following mechanism:

$$H_2O_2 + Fe_2+ \rightarrow Fe^{3+} + OH^- + H_2O^-$$

These OH^- free radicals act on polysaccharides and polymers such as lignin in the vegetable cell walls, and produce cleavages on the molecules, which allow easier penetration by lignocellulolytic enzymes (Mehdi Dashtban et al., 2010, Bentivenga et al., 2003.).

The next step is the saccharification process to hydrolyze complex polysaccharides such as cellulose and hemicellulose to monosaccharides (Demain et al., 2005). Cellulose degradation can be achieved by cellulolytic enzymes from different microorganisms. In nature, bacteria and fungi play a vital role to decompose plant biomass. In general, cellulases are classified in three categories: endoglucanases, cellobiohydrolases and β-glucosidases. Cellulose is a homopolymer of repeated units of cellobiose (two 1-4-β- glucoses linked), so, firstly, endoglucanases cleave the internal bonds of the long chains and provide cellooligosaccharides for cellobiohydrolases. The latter provides cellobiose (1-4-β- glucose disaccharide) as the major product. Lastly, cellobiose is hydrolyzed to glucose by the β-glucosidases (Lynd et al., 2002). Klyosov (1986) compared several cellulases from various sources such as *Trichoderma, Geotrichum, Myrothecium, Sporotrichum* and *Aspergillus* and found that there are different mechanisms of enzymatic hydrolysis of crystalline and amorphous cellulose. Moreover, cellulases from the fungus *Trichoderma ressei* and *Trichoderma viride* hydrolyze crystalline cellulose to glucose and cellobiose. *Aspergillus* cellulases also degrade crystalline cellulose; but, at a so much lower rate than *Trichoderma* (Klyosov, 1983). Also, anaerobic bacteria such as *C. thermocellum* produce a cellulase system as a cell-associated enzyme complex called cellulosome (Bayer et al, 2004*)*. In contrast,

aerobic bacteria typically produce enzymes in a "free" form, in which individuals work synergistically in the cellulose degradation. *Fibrobacter succinogenes* is an active rumen bacterium in the digestion of cellulose (Forsberg, 1990). A facultative bacterium *Paenibacillus curdlanolyticus B-6* was found to produce a multienzyme complex in aerobic conditions (Waeonukul et al., 2009). Bacterial cellulases are competitive in cellulose hydrolyzing efficiency, but modern commercial cellulases are still derived from fungal sources such as *T. reesei.* The latter is considered a great degrader of crystalline cellulose and it has high cellulase yields (van den Brink and de Vries, 2011).

Once the biomass is broken down into simple sugars, the fermentation process is the last step to convert monosaccharides to a biofuel. Both pentoses from hemicellulose and hexoses from cellulose are fermented for biofuels production (currently, ethanol). *Saccharomyces cerevisiae* is widely used to obtain ethanol from hexoses. Pentoses are fermented by a variety of microorganisms *(*Rosenberg, 1980*).* For instance, yeasts such as *Pichia stipitis, Candida sehatae,* and some filamentous fungi such as genera *Fusarium, Rhizopus* and *Pacilomyces* are able to ferment both five and six carbon sugars. Furthermore, *Clostridium thermohydrosulfuricum, C. thermosaccharolyticum and C. thermocellum* are able also to ferment both hexoses and pentoses. Genetically modified bacteria such as *Escherichia coli* or *Zymomonas mobilis* are able to ferment hexoses and pentoses and obtain high ethanol concentrations (40-58 g/l) (Mohagheghi et al., 2004; Zhou et al., 2008; Zhang et al., 1995).

Switchgrass as a dedicated energy crop

Switchgrass (*Panicum virgatum*) is a dedicated energy crop identified by the US Department of Energy (McLaughlin and Kszos, 2005). It has been proposed as lignocellulosic non-food biomass sources to produce biofuels (Parrish and Fike, 2005). Switchgrass is a warm-season C4 perennial grass native to North America. It is used for forage and hay, bedding, combustion and also as a way of conservation planting (Heaton et al., 2004; Moser et al., 1995). This grass is a perennial rhizomatous grass that begins to grow in late spring. It is a hardy plant composed of around 12-19% of lignin, 29-45% of cellulose and 30-37 % of hemicellulose (Sun and Cheng, 2002; Samson et al., 2008).

Some physical features are a height of 2.7 m and leaves 30–90 cm long. Switchgrass has the advantage to grow in conditions of drought and high temperature (Silzer et al., 2000). Switchgrass is not only a perennial but also a self-seeding crop. Once established, a switchgrass stand can survive for many years (Schmer et al., 2006). Additional features are high production yield (about 14 ton/acre), wide adaptation, and renewable root system (Yang et al., 2009). The grass has been used in several bioenergy conversion processes, including cellulosic ethanol production, biogas, and direct combustion for thermal energy production. According to a switchgrass model based on large scale field study (Proc Natl Acad Sci. USA 2008 105,464) switchgrass can provide cellulosic ethanol per acre to satisfy the energy demand in growing the plant and in converting it into biofuel. In terms of greenhouse gases, cellulosic ethanol from switchgrass produces 94% less than gasoline (Schmer et al., 2008).

Microbiology of biofuel production

Microbiologists and engineers are looking for novel ways to produce fuels using engineered microbes and new feedstocks. Industrial Microbiology has successfully accomplished the making of a variety of products by fermentation processes.

Fermentation is a process in which energy is extracted from the oxidation of organic compounds (Gottschalk 1986, Klein et al., 2004). In general, hexoses are utilized in the Embden-Meyerhoff pathway (Gottschalk 1986). One mol of glucose is converted into two mols of pyruvate during glycolysis, obtaining as a result energy stored as ATP and Nicotinamide Adenine Dinucleotide (NADH). During fermentation, pyruvate is metabolized to various compounds. According to these compounds, we denominate fermentation as homolatic fermentation which is the production of only lactic acid and carbon dioxide from pyruvate; alcoholic fermentation is the conversion of pyruvate into ethanol and carbon dioxide; and heterolactic fermentation is the production of lactic acid as well as other acids and alcohols (Dickinson, 1999). In addition, another well-known path is the acetone-butanol-ethanol (ABE) fermentation in which acetone, n-butanol, and ethanol are produced among acids such as acetate, butyric, lactate, and gases such as CO_2

and H_2. Carbon dioxide is a common factor in all fermentations, so then gas production can be used as a measure of fermentability (Gottschalk, 1986).

The gas production can be measured during the fermentation by the Ankom RF gas production measurement System (ANKOM RF2010). This system is being applied to record gas production, vacuum, or temperature in different areas such as ruminant nutrition, biogas/biomass production, yeast activity and dough rising, ethanol production, among others. The system consists in bottles (100, 250, 500, 1000 ml sizes) that are sealed with wireless digestion modules capable of measuring temperature and pressure. Then, temperature, the pressure or vacuum generated in each reaction bottle is recorded at certain intervals on computer software (Windows XP™, Windows 7™, or Windows Vista™ is required.) and real time graphing is shown. Data points are recorded on standard Microsoft Excel spreadsheets.

In the field of industrial microbiology, there are different fermentation approaches. The modes of bioreactor feeding are: batch fermentation processing, continuous fermentation processing or fed batch processing (Madigan and Martinko, 2006). The batch mode bioreactor is fed just once; when the process is done the entire medium is removed from the fermentation tank. The tank is then thoroughly washed, cleaned and the new batch is started only thereafter. In the continuous mode, the feeding is continuous and a part of the medium is removed at regular intervals, and a constant volume is kept or in other words, it is operated under steady state conditions. The most common type of continuous culture device is the chemostat. Fed batch is a mixed-model of bioreactor operation. The fed batch mode is characterized by predetermined or controlled addition of nutrients into the bioreactor at certain times (Demain and Solomon, 1981).

Furthermore, according to the physical phase, the process is defined as liquid or submerged fermentation (SmF) or solid substrate fermentation (also known as solid substrate cultivation-SSC). The former is the typical method for biofuel production because it has physiological and economic advantages when scaling up the process (Jorgensen et al., 2007). The solid substrate fermentation consists of the growth of the microorganisms on solid substrate. A high solid environment is characterized by having solids concentration of 10-15% w/w. This method presents advantages, such as, high

yields, low production costs due to availabilities of equipment, stability of the products and less effluent generation (Nigam and Singh, 1994). However, SSC has disadvantages as unsolved technological and scale up problems such as the oxygen transfer and heat elimination as well as higher concentration of inhibitors (Lekanda and Perez-Correa, 2004; Dharmagadda et al., 2010, Jorgensen et al., 2007).

The objectives of this study are to develop enhanced biomass conversion to value-added products using acetone-butanol-ethanol (ABE) fermentation. Cellulose hydrolysis will be followed by bacterial solventogenesis in solid substrate cultivation. Several fermentation parameters will be tested along the fermentation.

Chapter Two

ABE Fermentation in Batch System.

2.1 Introduction

Traditionally, the Acetone-butanol-ethanol (ABE) fermentation was used for acetone production during the World War I. Butanol was used as precursory of rubber. Also, butanol was used as aviation fuel in Japan during World War II (McCutchan W.N. and Hickey R.J., 1954). However, limitations in the fermentation process such as low conversion of products and technical difficulties such as avoiding contamination in the process, together with the competitors derived from petroleum and natural gas, lead to ABE fermentation declination since the 1950´s (Spivey, 1978). ABE fermentation consists of one stage batch process using *Clostridium* species (generally *C. beijerinckii or C. acetobutylicum*). It is an anaerobic process (done in the absence of oxygen) and generally produces these solvents in a ratio of 3:6:1, or 3 parts acetone, 6 parts butanol and 1 part ethanol. (Spivey, 1978) (Figure 2.1.1). The total solvent yield obtained in batch fermentation is about 20-30g/l (Qureshi and Blaschek, 2001). ABE fermentation is not economic viable due to the cost of carbohydrate substrates (approx. 60%) (Qureshi and Blaschek, 2000). Also, the cost effectiveness of the ABE fermentation is affected keeping the process under sterile conditions. However, in the case of using lignocellulosic biomass, some pretreatments are typically performed at 45 or 50 ºC, and then the no wished mesophilic anaerobic bacteria will not interfere in the fermentation (Wang and Chen, 2011).

The batch ABE fermentation generally involves two stages: acidogenic and solventogenic. Glucose enters in the glycolytic pathway for conversion to pyruvate (Gottschalk, 1986). Pyruvate is converted to acids in the acidogenic phase. During solventogenesis the acids are reduced to acetone, butanol and ethanol. The toxicity of the solvents limits the yields and it reduced the productivity of the ABE fermentation.

Currently, the necessity to find a sustainable and economically feasible process to produce biofuels have positioned ABE fermentation for butanol production as an

alternative; however, it should be optimized to make this process competitive with the current fuels production methods (Al-Shorgani et.al., 2011).

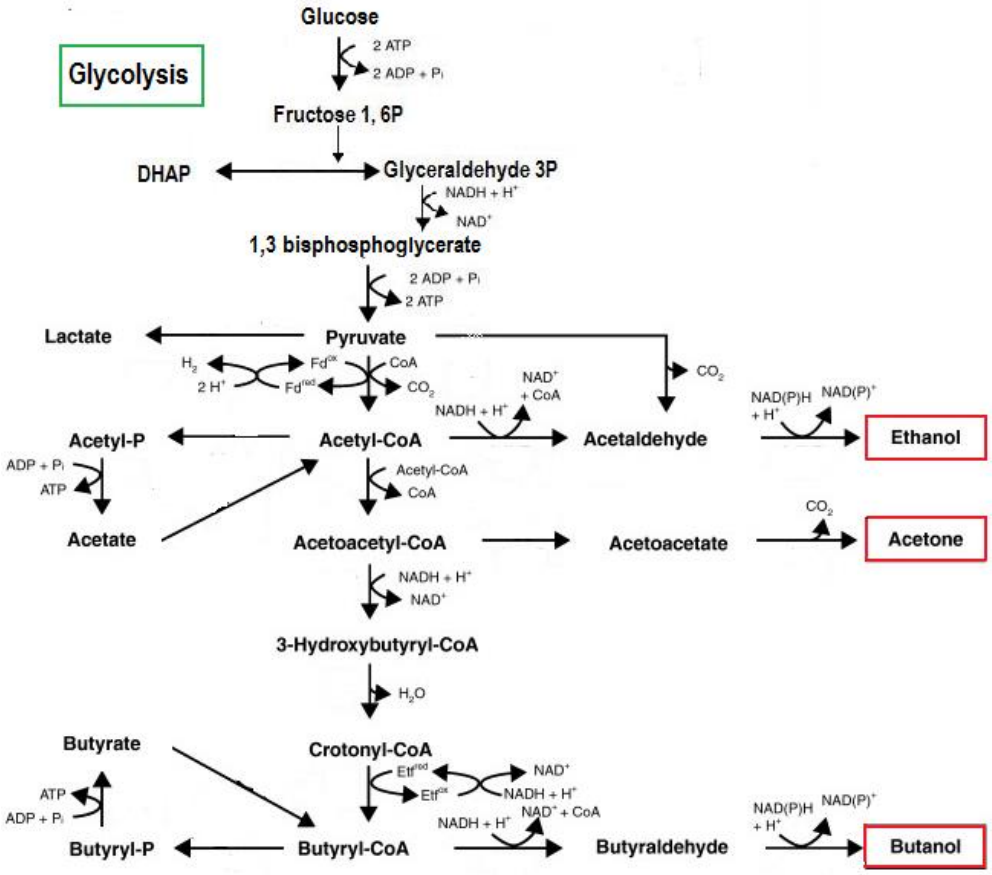


Figure 2.1.1.Simplified figure of glycolysis leading acetone, butanol and ethanol production from *Clostridium* species. Straight arrows indicate one or more enzymatic steps. Boxes highlight solvent products.

Microorganisms of interest

Cellulolytic clostridia

Clostridium thermocellum is a Gram-positive, cellulolytic, thermophilic anaerobe that catabolizes a wide range of polysaccharides and has the potential to be an ethanol producer using renewable lignocellulosic material (Lynd et al., 1989). *C. thermocellum* was described by McBee (1954), as an active cellulose digester. This thermophilic microorganism has been found on horse manure, soil, and marine mud in association with

methanogens. Their optimum temperature is between 50-65 °C; they are straight or slightly curved rods (0.6-07 by 2.5-3.5 µm). *C. thermocellum* can degrade cellulose and cellodextrins and produces carbon dioxide, hydrogen, acetic acid and ethanol as main fermentation products (Gaughran, 1947; Weimer et al., 1977; Collins et al., 1994, Rydzak et al., 2008) (Figure 2.1.2). The strain *Clostridium thermocellum* ATCC 27405 has the ability to degrade both crystalline and amorphous cellulose, converting it into acetate, ethanol and gases such as CO_2 and H_2 (Weimer et al., 1977). *C. thermocellum* has been reported to degrade substrates such as sugar cane baggase, paper pulp, and corn stover in high solid substrate cultivation (Chinn et al., 2006, Chinn et al., 2007). *C. thermocellum* is one of the clostridia able to produce their own cellulose complex. It is called the cellulosome and it has the ability to hydrolyze cellulose in lignin content materials. This bacterium has the highest rate of cellulose degradation among all known cellulolytic microorganisms (Lynd et al., 1989, Halliwell et. al., 1995, Bayer et.al., 1994, Demain et al., 2005).

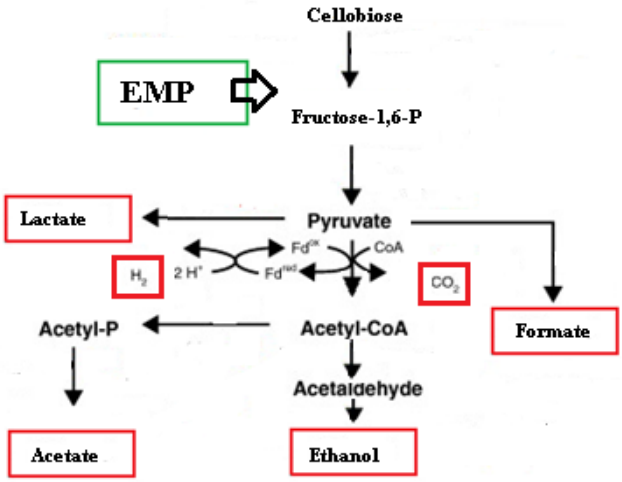


Figure 2.1.2.Simplified figure of central metabolic pathway of *C. thermocellum*. Straight arrows indicate one or more enzymatic steps. EMP: Embden-Meyerhof Pathway. Boxes highlight the characteristic end-products (Rydzak et al., 2008)

The cellulosome is a multicomponent cellulolytic complex that hydrolyzes cellulose into the disaccharide cellobiose and cellodextrins (Bayer et al., 1983). It was first described as a cellulose-binding factor located on the cell surface in a culture of *C.*

thermocellum (Ljungdahl and Eriksson, 1985). It hydrolyzes both amorphous and crystalline cellulose. The hydrolysis of cellulose to glucose is carried out by three types of enzymes: endo-1,4 beta glucanase, exo-1,4- beta glucanase or cellobiohydrolase and cellobiase or beta-glucosidase (Felix et al., 1993). Currently, it is known that the cellulosome is composed of three main components: 1) a cell surface-anchoring protein, 2) the CipA scaffoldin protein, and 3) enzyme subunits (Bayer et al., 1994, 2004) (Figure 2.1.3).

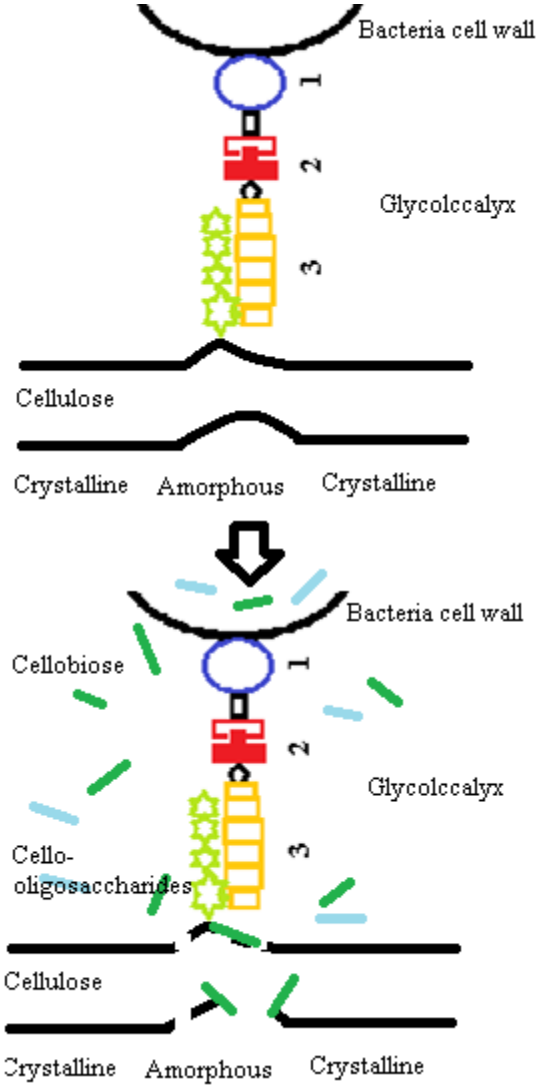


Figure 2.1.3. Schematic representation of the cellulosome from *C. thermocellum* adapted from Lynd et al. (2002) and Currie et al. (2010). The cellulosome is composed of three types of proteins: 1) a cell-surface-anchoring protein, 2) the CipA scaffoldin protein, and 3) enzyme subunits.

Furthermore, *C. thermocellum* has the ability to combine the cellulase production and the ethanol conversion using the cellulosic biomass (Lynd et al., 2002). Consolidated bioprocessing is an alternative to reduce the capital cost and increase the efficiency of the process since it combines the enzymatic production, hydrolysis and fermentation into one reactor (Lynd et al., 2005). Furthermore, preliminary results showed that *C. thermocellum* produced gas steadily for 20 h when switchgrass was the sole carbon source (Figure 2.1.4). These results not only indicate the suitability of the switchgrass as a growth substrate, but also that the endogenous gas production circumvent the concern of maintaining the fermentation under anaerobic conditions on large scale.

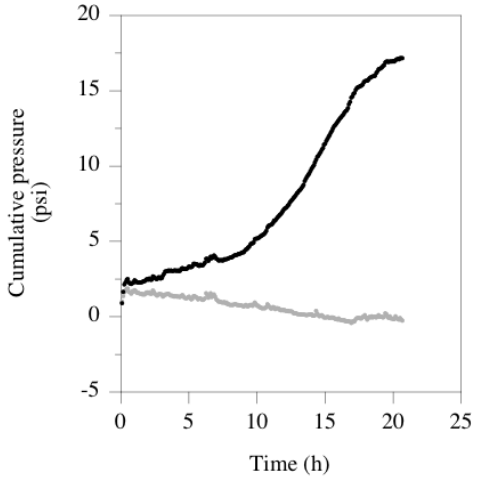


Figure 2.1.4. Gas production from switchgrass by *Clostridium thermocellum*. Cells were inoculated into the basal medium with switchgrass (2 mm, 2% w/v) as the sole carbon source (black symbols). An un-inoculated control is also shown (gray symbols). Gas pressure in the headspace was measured in 5 min intervals.

Overall, *C. thermocellum* features such as being anaerobic, thermophilic and cellulolytic are suitable for solid substrate cultivation. Also, these characteristics are attractive comparing with heat and mass transfer difficulties for aerobic, mesophilic organisms.

Solvent producer clostridia

Solventogenic clostridia ferment a variety of carbon sources such as glucose, galactose, cellobiose, mannose, xylose, among others (Gottschalk, 1986). Acetic and butyric acids and solvents such as acetone, ethanol and butanol (ABE) are obtained. In a batch mode, there are two phases: acidogenic and solventogenic periods. The limitation of the ABE production is the toxic effect of solvents which affects the profitability of ABE fermentation as a tool for a biofuel production.

Clostridium acetobutylicum is a mesophilic anaerobe that it has been one of the most studied during the first decades of the 20th century for the ability to ferment sugars into butanol and acetone (patent issued to Weizmann, 1919). It is able to ferment raffinose, saccharose and pectin (Cato et al., 1986). Growing in a batch culture, firstly, the acidogenesis phase takes place and fermentation products are a mixture of butyric, lactic and acetic acid (Bahl et al., 1982). Later, certain alterations (pH drop and butyric acid concentration) lead to the solventogenesis phase, in which butanol, acetone and ethanol are produced (Bahl and Gottschalk, 1988).

Clostridium beijerinckii shares phenotypic characteristics with *C. acetobutylicum.* It is also a mesophilic anaerobe (rod shape) that ferments sugars into acids, alcohols and solvents on similar conditions (Cato et al., 1986, Gottschalk 1986, George et al., 1983). It has the ability to ferment saccharose and utilizes alcohol sugars d- and larabitol, dulcitol and inositol as well as methyl-glucopyranoside, turanose, dextrin and pectin (Cato et al., 1986). It has been found in feces and soil. *Clostridium beijerinckii* is also endospore-forming and the solventogenesis phase has been related with sporulation (Paredes et al., 2005). The solvent production for these organisms is limited by the toxicity of the product accumulation. Butanol and ethanol causes damage on the cell membranes of these microorganisms and lead to cell growth inhibition (Herrero et al., 1985; Hutkins and Kashket, 1986). As a consequence, genetic engineered manipulations have been done to increase solvent resistance and increase solvent production (Ezeji et al., 2004). Indeed, the total solvents concentration produced in the fermentation is not higher than 20 g l^{-1} with butanol concentration of 13 g l^{-1} (Qureshi and Blaschek, 2001).

Another solventogenic bacterium that is being researched is *Clostridium saccharoperbutylacetonicum* ATCC 27021 (N1-4). Its name derived from saccharon sugar juice, per throughout, butylum butanol, and acetonicus acetonic; so,

saccharoperbutylacetonicum means the production of large amount of butanol and acetone from sugar first named and described by Hongo (1960, US patent no. 2945786). They are Gram positive straight, short or long rods with rounded ends occurring singly or occasionally in pairs. The cells are motile and in exponential phase use to accumulate as granulose which is associated with the metabolic change from acid production to solvent production. *Clostridium saccharoperbutylacetonicum* is an endospore forming bacterium (oval). Being a mesophilic, the optimum growth for solvent production is between 25-35 °C. They are able to ferment arabinose, xylose, glucose, mannose, cellobiose, lactose, maltose, saccharose, d-arabitol, l-arabitol, mannitol, melibiose, methyl-glucopyranoside, raffinose, salicin, trehalose, turanose, amygdalin, starch, glycogen, dextrin, pectin, melezitose and inulin (Hongo, 1960). This butanol hyperproducing bacterium has been used commercially in butanol fermentation (Jones and Keis, 1995). It has been shown to produce 13.61 g l^{-1} of acetone, butanol and ethanol, in which 8.69 g l^{-1} corresponds to butanol using a concentration of 50 g l^{-1} of glucose. Furthermore, this bacterium is able to utilize different carbon sources on the anaerobic fermentation, making it a potential inoculant for biobutanol production (Al-Shorgani Najeed et al., 2011). Thang et al. (2010) showed that *C. saccharoperbutylacetonicum* possesses hyper amylolytic activity; and, butanol from starch is produced without a hydrolysis step. Moreover, the lignocellulosic biomass such as rice and de oiled rice bran was efficiently fermented by *C. saccharoperbutylacetonicum* (Al-Shorgani et al., 2012).

In conclusion, the genus *Clostridium* has a diversity of Gram positive, spore forming anaerobic organisms. They have value for industrial production of acids and solvents. This project studies the sequential culture of the cellulolytic *Clostridium thermocellum* followed by different solvent producer clostridia in a simultaneous saccharification and fermentation (SSF). SSF is a method for fermenting sugars released from lignocellulose and obtains biofuels (Wyman, 1999). The role of the thermophilic culture (*C. thermocellum*) is freeing the sugars by depolymerization of the lignocellulosic substrate; and, the purpose of the mesophilic culture is to ferment the sugars and produce the biofuels of interest.

2.2 Objectives

Compare different clostridia strains and substrate particle size for the sequential culture of *Clostridium thermocellum* and solvent producer clostridia in a batch fermentation system.

Short term fermentation: Solvent producer strains on glucose and switchgrass.

Compare the end product formation by three different solvent producer clostridia in batch system. Short term fermentations were carried out with either: 1-soluble sugars (RCM media) or 2- switchgrass (basal media) as the growth substrate. The most efficient solvent maker on switchgrass was selected for further studies.

Short term fermentation: Effect of the particle size

The hypothesis is that there is an inverse relationship exists between particle size and gas production.

The ability of *C. thermocellum* and *C. beijerinckii* to catabolize cellulosic biomass was evaluated through gas production experiments on different particle sizes of switchgrass. Among the gasses produced, CO_2 is the most universal measure of substrate fermentability. Moreover, because not only carbon dioxide but also hydrogen is produced during the fermentation, the cumulative pressure obtained in order to maintain adequately anaerobic conditions without supplying gas continuously will be quantified. The optimum particle size was determined by the maximum gas production of sequential cultures using the Ankom RF system.

2.3 Materials and Methods

Strains and Culture Conditions

Solvent producer strains

C. beijerinckii ATCC 51743 was obtained from the American Type Culture Collection. Strains of *C. acetobutylicum* ATCC 824, *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) and *C. acetobutylicum* ATCC 824 NC were obtained from the culture collections of Herbet Strobel (University of Kentucky), the American Type Culture Collection (VA), and Department of Microbiology and Immunology, University of North Carolina (Bruno Barcena), respectively . All cultures were grown and routinely transferred anaerobically at 35 °C in Reinforced Clostridial Media (RCM; Difco Laboratories, Detroit, Mich.). The RCM media contain (per liter) 10.0 g peptone, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g dextrose, 5.0 g NaCl, 1.0 g soluble starch, 0.5 g cysteine, 3.0 g $C_2H_3NaO_2$, and 0.5 g agar. Media for batch liquid cultures was anaerobically dispensed into serum bottles with butyl rubber stoppers, and autoclaved.

Cellulolytic strain

The strain of *C. thermocellum* ATCC 27405 was obtained from the culture collection of Dr. Michael Flythe, University of Kentucky. *C. thermocellum* was grown anaerobically at 63 °C in the basal medium containing (per liter): 30.6 g Na_2HPO_4, 30.0 g KH_2PO_4, 10.0 g NH_4Cl, 10.0 g $(NH_4)_2SO_4$, 1.8 g $MgCl_2$ 6-H_2O, 0.6 g $CaCl_2$, 2.0 g yeast extract, 10 ml standard vitamin mixture (Cotta and Russell 1982), 5.0 ml modified mineral mixture (Pfennings Metals plus 10 mg Na_2WO_4.2 H_2O and 1 mg Na_2SeO_3 per liter) and 1 ml resazurin. The pH was adjusted to 6.7 using NaOH. The buffer, Na_2CO_3 (4 mg ml^{-1}) was added after sterilization (121 °C, 104 kPa, 20 min) at room temperature. Broth for batch liquid cultures was anaerobically distributed into serum bottles and sealed with butyl rubber stoppers, and autoclaved. *C. thermocellum* was grown in the basal medium with amorphous cellulose (Whatman #1 filter paper; 4.5 mg ml^{-1}) at 63 °C.

Biomass preparation

The switchgrass was obtained from small plots maintained at the University of Kentucky's North farm. The switchgrass is harvested once after the end of the season. Switchgrass is usually cut at least 6 inches high and stored in small square bales that are ground (passed through 2, 5 and15 mm screen) using a hammer mill in the lab. The chemical characterization of the switchgrass was done by Dairy One Forage Testing Laboratory, Ithaca, NY (Ration Balancer Plus) (Table 2.1).

Table 2.1. Analysis results of switchgrass (5 mm) by "DairyOne" Forage Testing Laboratory, Ithaca, New York (Sample N° 18655760).

Components	Total	DM
%Moisture	6.0	
% Dry Matter	94.1	
%Crude Protein	3.6	3.8
%Adjusted Crude Protein Soluble Protein %CP		34
%Acid Detergent Fiber	51.6	54.9
%Neutral Detergent Fiber	83.6	88.9
%Lignin	8.3	8.8
%NFC	9.4	10.0
%Crude Fat	1.2	1.2
%Ash	1.15	1.23
%TDN	48	51
NEM,Mcal/Lb	.35	.37
NEG, Mcal/Lb	.12	.12
Relative Feed Value		48
%Calcium	.32	.35
%Phosphorus	.06	.07
%Magnesium	.13	.14
%Potassium	.07	.08
%Sodium	.017	.018
PPM Iron	94	100
PPM zinc	16	17
PPM Copper	7	7
PPM Manganese	66	70
PPM Molybdenum	< 0.1	< 0.1
%Sulfur	.06	.06

Short term fermentation: solvent producer strains on glucose and switchgrass.

Inocula of *C. beijerinckii* ATCC 51743, *C. acetobutylicum* ATCC 824 and *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4*)* were grown anaerobically at 35 °C in RCM. Batch reactions were carried out in serum bottles with a working volume of 50 ml at 35 ºC. All media were sterilized at 121 °C for 15 minutes. Figure 2.2.1 shows the experimental design.

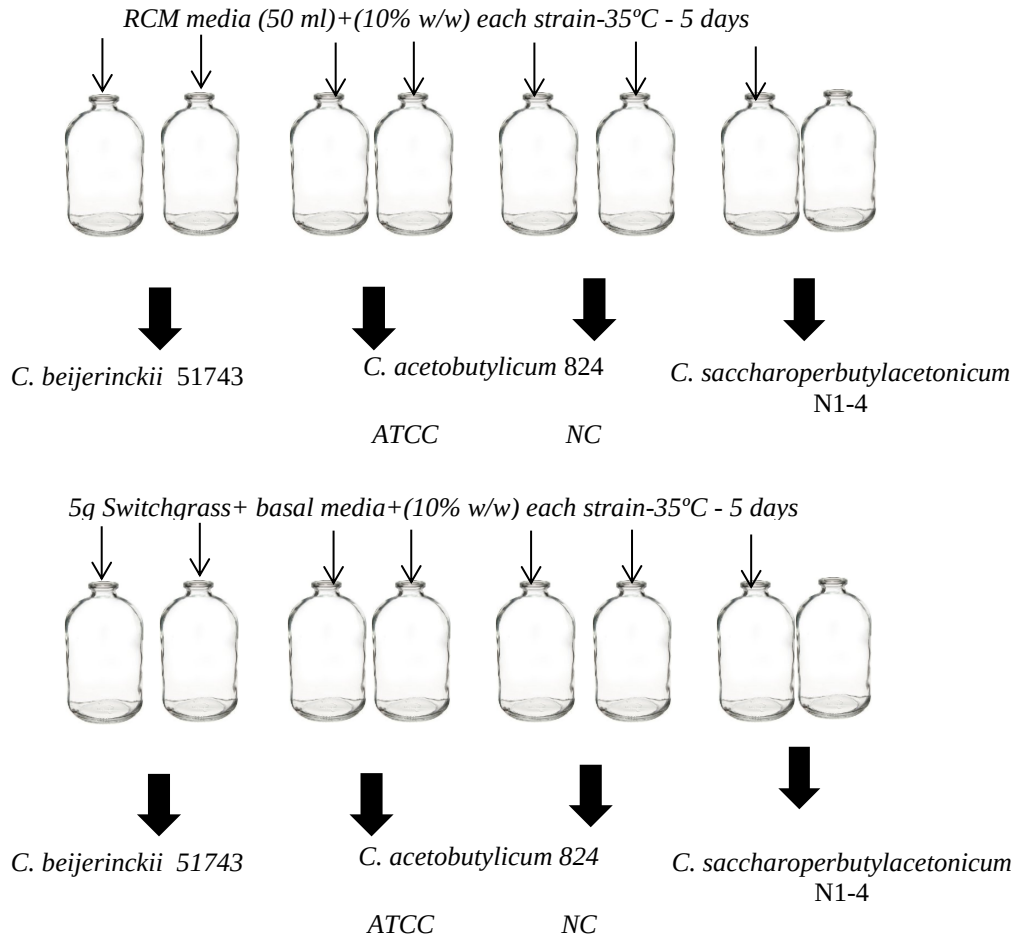


Figure 2.2.1. Experimental design to test different solvent producer on glucose and switchgrass as substrate.

On one side, 45 ml of RCM was inoculated with 5 ml (10% w/w) of each strain and incubated at 35 °C. On the other hand, 50 ml of basal media (K_2HPO_4 used as buffer) was added to serum bottles already contained 5 grams of sterilized switchgrass. Then, 5 ml

(10% w/w) of each strain were added and incubated at 35 °C. Daily samples were clarified by centrifugation (14.8 g 2 min) and frozen for further analysis. pH was also measured. The experiments were performed in duplicated.

Effect of the particle size: Gas production

Switchgrass (4.7 g of substrate that passes through a 2, 5 and 15 mm screen) was added to the fermentation vessels (Pyrex bottle with sidearm port, 1000 mL). The vessels were sealed and purged with O_2-free CO_2 through the sidearm septa. Basal medium (45 mL) was added through the septa of the sidearm ports. There were carried out two experiments: a sequential culture of *C. thermocellum* followed by *C. beijerinckii*, and a pure culture of *C. beijerinckii*. For the former, the vessels were warmed in a water bath (63 °C) before inoculation. The vessels were inoculated with 10^8 cells/ml broth (10% w/w) through the septa with *C. thermocellum* (48 h cultures), and incubated (63 °C; 48 h). After the *C. thermocellum* incubation, the vessels were cooled down, and inoculated with *C. beijerinckii* (10% w/w, 24 h cultures). The culture was incubated at 35 °C for 48 h. In the pure culture experiment, the previous inoculation with *C. thermocellum* was omitted. Gas production was monitored using the Ankom RF Gas Pressure System (Ankom, Macedon, NY, USA). Gas pressure was tested in 1 min intervals, and cumulative pressure was recorded every 5 min. The global pressure release was set at 104 kPa. Control vessels were uninoculated media. They were tested in each replication. The pressure values of the uninoculated vessels were subtracted from each treatment to control for the effects of temperature. Each gas production treatment was performed in triplicate on separate days. The data were analyzed in SAS v. 9.3 (SAS Inst. Inc., Cary, NY, USA) using an ANOVA with a Tukey's test *post hoc* ($P < 0.05$ considered significant).

Effect of the particle size: Product quantification

Likewise, the product quantification was done on the sequential culture of *C. thermocellum 2*7405 and *C. beijerinckii* 51743 on different particle sizes (ground through

a 2, 5 and 15 mm screen). Serum bottles (100 ml size) containing 4.7 g (10%) of biomass were autoclaved. 45 ml of basal medium was added and the serum bottles were warmed in a water bath (63 °C) prior inoculation with *C. thermocellum* (5 ml or 10% w/w). After 48 h, the bottles' temperature was decreased to 35 °C, and they were inoculated with *C. beijerinckii* (5 ml or 10% w/w). Also, the pure culture of *C. beijerinckii* was tested in similar manner where the prior inoculation with *C. thermocellum* was omitted.

Daily samples (1 ml) were clarified by centrifugation (14800 × g, 2 min), and they were frozen for later analyses. Acetate, ethanol, butanol, lactate and formate were quantified by HPLC (Dionex, Sunnyvale, CA). The anion exchange column (Aminex 87H; BioRad) was operated at 50 °C, flow rate 0.4 ml min^{-1}. Eluting compounds were detected by refractive index (Shodex/Showa). The experiment was performed in duplicate.

2.4 Results

Short term fermentation: solvent producer strains on glucose and switchgrass

To analyze the performance of different solvent producers, short term fermentations on glucose and switchgrass were carried out by *C. beijerinckii*, *C. acetobutylicum* and *C. saccharoperbutylacetonicum* (Table 2.4.1 and 2.4.2). First, looking at the results on RCM as substrate, the conversion on glucose into metabolites was 100% and it was carried out in the first 24 hours for all the strains. Acetic and butyric acids were the principal acids produced. Figure 2.4.1 (a) shows that *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) total product formation was 40-45% higher than other strains when glucose was the substrate. Moreover, *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) transformed 26.5 % (32 mM) of substrate into butanol which was more than the other strains ($P<0.05$). Solvent production occurred between the 48 to 72 hours (Figure 2.4.2). The growth on switchgrass was poor for all the strains (Table 2.4.2). Xylose was found in small amounts between 0.80 and 1.25 mM and 100% was consumed in the first 24 hours. Acids and solvents were poorly produced. Total product formation was around 100 mM (Figure 2.4.1 (b)), around 25% of total yield reached on RCM. There was no difference between the conversions of

switchgrass by the different strains. The pH values decreased along with the acid formation reaching a minimum of 5 (data not shown).

Table 2.4.1. Product formation by *C. beijerinckii* ATCC 51743, *C. acetobutylicum* ATCC 824, *C. acetobutylicum* 824 NC and *C. saccharoperbutylacetonicum* ATCC 27021 N1-4 on RCM. Product formation on day 0 corresponds to the inoculum.

Day	glucose	lactate	formate	acetate	butyrate	butanol	acetone
C. beijerinckii ATCC 51743				(mM)			
0	124.5±12.0	0.115±0	-	33±2.8	-	-	-
1	-	-	1.55±0.07	28±1.4	28±2.8	-	-
2	-	-	1.7±0.28	32.5±0.7	31.5±0.7	-	-
3	-	-	1.85±0.3	32.5±0.7	33±0.01	-	-
4	-	-	1.5	29.5±2.12	31±0.01	-	-
5	-	-	1.45±0.07	27.5±2.1	28±1.4	-	-
C. acetobutylicum ATCC 824							
0	123.5±9.1	0.235±0.15	-	34±1.4	-	-	-
1	-	3.1±0.4	-	39.5±3.5	15.5±0.7	0.95±0	-
2	-	3.15±0.3	-	41±2.8	15.5±0.7	1.85±0.3	-
3	-	3±0.3	-	40±2.8	17±1.4	1.85±0.2	-
4	-	3.3±0.3	-	42.5±3.5	17.5±0.7	1.9±0.4	-
5	-	3±0.2	-	41.5±3.5	17.5±0.7	1.9±0.1	-
C. acetobutylicum 824 NC							
0	119.5±6.3	0.09	-	34±2.8	-	9±0	-
1	-	-	-	27.5±4.0	17.5±2.1	8.55±1.6	-
2	-	-	-	28±1.4	23.5±-.7	10.4±0.8	-
3	-	-	-	28±1.4	25±1.4	11±0	-
4	-	-	-	26±0	24±0	10.2±1.1	-
5	-	-	-	27±1.4	25±2.8	10.4±0.8	-
C. saccharoperbutylacetonicum ATCC 27021							
0	120.5±3.5	0.18±0.07	1.1±0	34.5±0.7	-	-	-
1	-	1.05±0.3	-	32.5±6.3	-	28±4.2	17.5±2.1
2	-	0.8±0	-	36±0	-	30.5±0.7	23±1.4
3	-	0.45±0.2	-	34.5±4.0	-	32±4.2	23±1.4
4	-	0.35±0.2	-	35.5±2.1	-	31.5±2.1	24.5±3.5
5	-	0.05±0	-	31.5±0.7	-	29±0	23±1.4

Table 2.4.2. Product formation by *C. beijerinckii* ATCC 51743, *C. acetobutylicum* ATCC 824, *C. acetobutylicum* 824 NC and *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) on switchgrass.

Day	xylose	lactate	formate	acetate	butyrate	butanol	EtOH
C. beijerinckii ATCC 51743				mM			
0	0.8	-	-	5.0±0	3.0±0	-	-
1	-	-	0.9±0.25	9.0±1.41	5.5±0.7	0.3±0.01	-
2	-	-	1.2±0.37	12.5±3.5	6.0±1.4	0.3±0.03	-
3	-	-	1.6±0.56	18.0±1.4	7.5±0.7	0.4±0.03	-
5	-	-	1.6±0.14	18.0±1.4	7.0±1.41	0.4±0.01	2.8±0
C. saccharoperbutylacetonicum ATCC 27021							
0	1.1±0.07	0.3	-	6.0±0	2.0±0	0.7±0	-
1	0.7±0	0.3	0.9±0.18	9.5±0.7	5.0±1.4	0.4±0.3	-
2	-	0.2	1.3±0	19.0±2.8	6.0±1.4	0.5±0.5	-
3	-	-	1.2±0.74	21.0±0	7.5±0.7	0.6±0.1	-
5	-	-	1.2±0	21.5±0.7	5.5±0.7	0.7±0.4	-
C. acetobutylicum 824 NC							
0	1.3±0.07	-	-	4.5±0.7	3.0±0	0.2±0	-
1	-	0.4	0.5±0	10.0±4.2	4.0±1.4	0.1±0	-
2	-	-	1.2±0	18.5±0.7	6.5±0.7	0.2±0.04	-
3	-	-	-	33.5±2.1	6.5±2.1	0.1±0.01	-
5	-	-	-	34.5±3.5	5.5±0.7	-	-
C. acetobutylicum ATCC 824							
0	1.2±0.14	0.5±0.01	-	6.0±0.1	2.0±0	0.1±0	-
1	1.4±0.2	0.4±0.07	0.7±0.06	12.0±0.2	3.0±1.4	0.8±0	-
2	-	0.4±0	0.9±0.1	18.5±2.1	4.5±0.7	0.5±0.3	-
3	-	0.4±	2.0±0	25.0±8.4	5.0±1.4	0.4±0.3	2.2±0
5	-	0.4±0	2.4±0	26.0±8.4	4.5±2.1	0.3±0.1	3.5±0

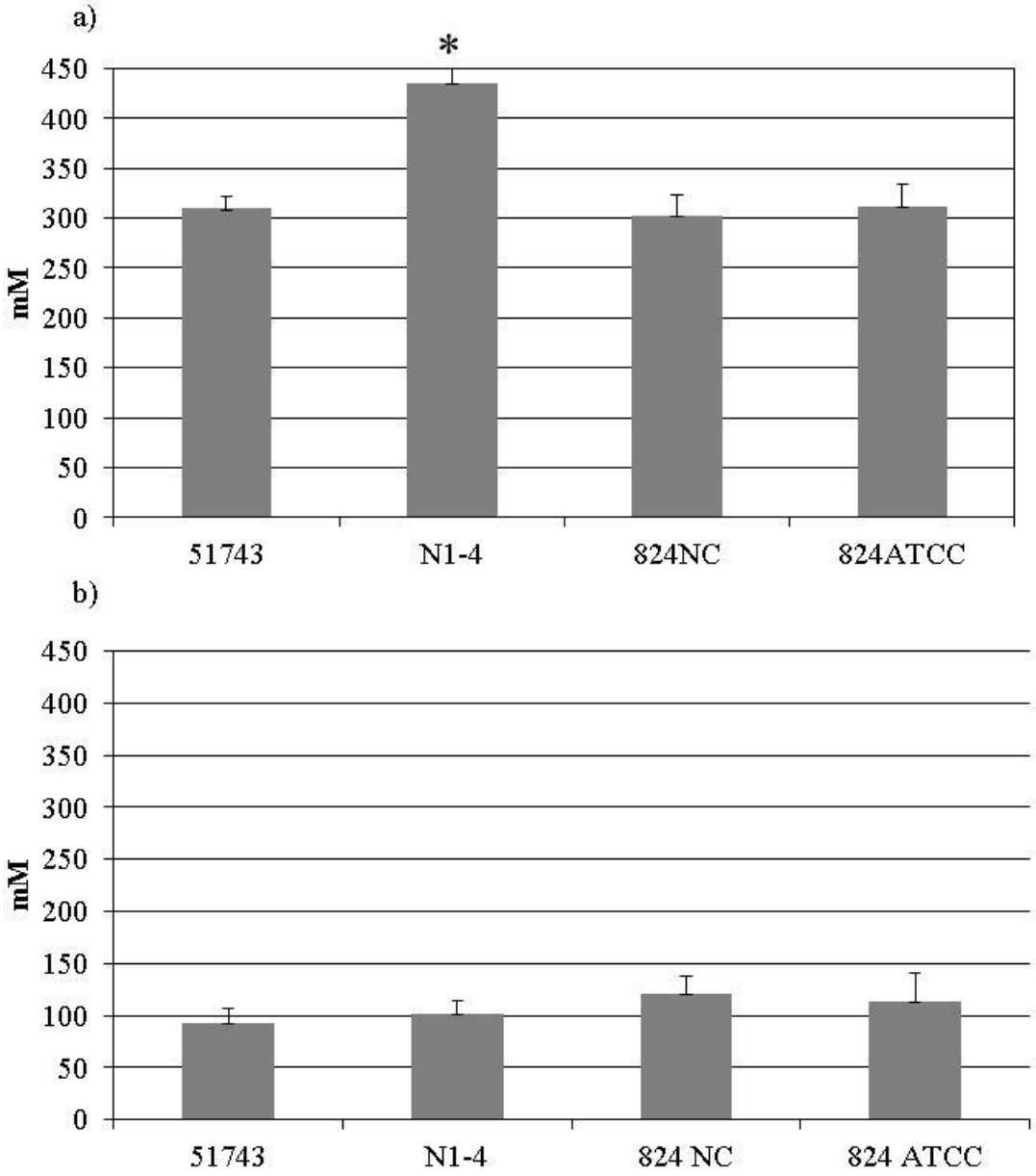


Figure 2.4.1. Total product formation by *C. beijerinckii* ATCC 51743, *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4), *C. acetobutylicum* 824 NC and *C. acetobutylicum* ATCC 824 on RCM (a) and switchgrass (b). * indicates significance difference ($P<0.05$). Error bars indicate ± one standard deviation.

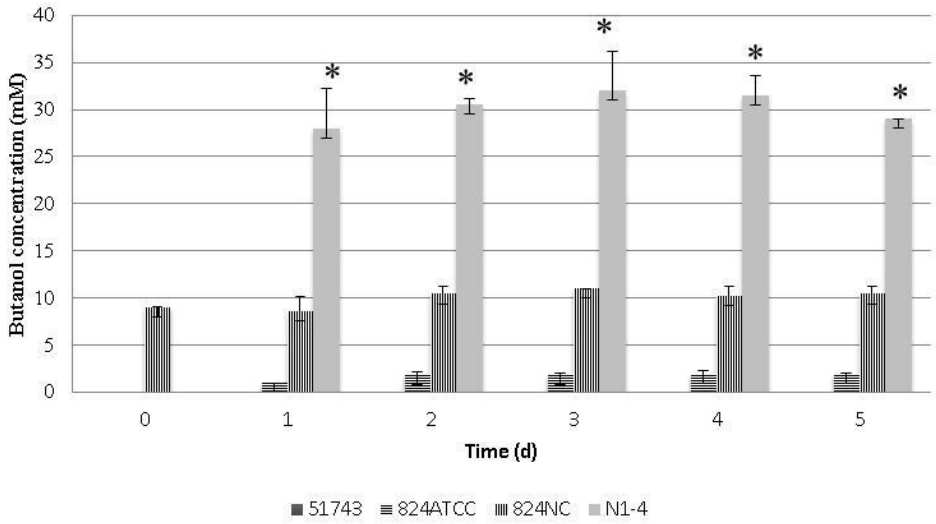


Figure 2.4.2. Butanol production over the time by *C. beijerinckii* ATCC 51743, *C. saccharoperbutylacetonicum* ATCC 27021 *N1-4*, *C. acetobutylicum* 824 NC and *C. acetobutylicum* ATCC 824 on RCM (35 ºC) during 5 days. Plot from Table 2.4.1. * Indicates significance difference ($P<0.05$). Error bars indicate ± one standard deviation.

Effect of the particle size: Gas production

The cumulative pressure was recorded initially in psi unit (See Appendix Chapter 2). The cumulative pressure was converted into moles of gas, which are characterized as moles CO_2, since it is the major gas component. *C. beijerinckii* grown on switchgrass pushed through 2 mm and 5 mm screen produced approximately 1000 µmol CO_2 in 24 h (Figure 2.4.3). The longest particle size (15 mm) gas production was 30% less. Sequential culture promoted gas production. Specifically, when *C. thermocellum* was grown on the substrate (2 or 5 mm) prior to inoculation with *C. beijerinckii*, CO_2 produced by *C. beijerinckii* increased 500%. Also, gas production from 15 mm particle switchgrass was more than doubled by sequential culture (Figure 2.4.4).

Effect of the particle size: product formation

Acetate was the major product in pure cultures. Also, butyric and formic acid were produced. In pure cultures, there was no significant difference between particle

sizes product formation (Table 2.4.3). In sequential cultures on switchgrass, acids (acetate, formate, lactate) and ethanol were produced *by C. thermocellum* (Table 2.4.4). After inoculation with *C. beijerinckii*, butyric acid and butanol were also produced. There was no effect of particle size ($P< 0.05$).

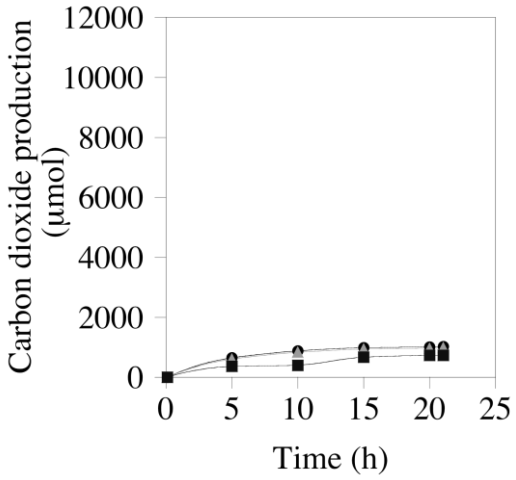


Figure 2.4.3. Gas production by *C. beijerinckii* on switchgrass. 2 mm (black circles), 5 mm (grey triangles) and 15 mm (black squares) are shown.

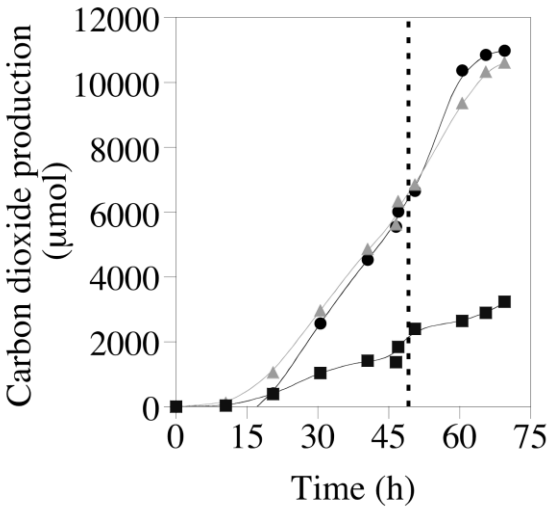


Figure 2.4.4. Gas production by sequential culture on switchgrass. 2 mm (black circles), 5 mm (grey triangles) and 15 mm (black squares) are shown. *C. beijerinckii* was added after 48 h (dashed line) and temperature was dropped to 35 ºC. After 24 hour of *C. beijerinckii* incubation, the cumulative pressure leveled out. Standard deviation is shown in the Appendix Chapter 2.

Table 2.4.3. Product formation by *C. beijerinckii* on different particle size of switchgrass during 10 days.

Day	glucose	lactate	formate	acetate	butyrate	butanol
2 mm			(mM)			
0	2.97±0.86	-	-	4.33±0.58	2.67±0.58	0.25±0.13
1	-	-	1.93±0.153	9.00±1.7	7.33±0.57	0.19±0.11
2	-	-	1.40±0.61	19.00±0	9.33±0.58	0.16±0.05
3	-	-	-	28.33±2.52	9.00±1.0	0.28±0.11
5	-	-	-	38.67±3.79	9.67±1.15	0.11±0.02
10	-	-	-	46.00±3.46	10.33±0.58	0.14±0.01
5 mm						
0	0.77±0.15	0.08±0	-	4.00±2	2.33±2.12	0.21±.12
1	-	0.08±0	0.10±0	6.67±2.08	5.67±3.51	0.27±.17
2	-	-	0.70±0.49	13.00±3.6	8.33±4.04	0.20±0.09
3	-	-	0.57±0.21	15.67±3.5	7.67±3.51	0.19±0.08
5	-	-	0.73±0.14	2-±4.0	8.33±4.04	0.23±.152
10	-	-	0.93±0.14	23.00±5.56	8.67±3.51	0.20±0.14
15 mm						
0	2.63±0.68	-	-	4.00±0	3.33±1.15	0.19±0.028
1	-	-	0.73±0.21	8.33±0.57	5.67±0.58	0.22±0.08
2	-	-	0.43±.35	17.33±1.52	7.33±0.58	0.19±0.02
3	-	-	0.67±0.06	21.33±4.51	9.67±2.88	0.18±0.03
5	-	-	0.77±0.4	29.00±4.58	9.00±0	0.25±0.01
10	-	-	0.30±0	36.33±1.16	9.00±0	0.26±0.04

Table 2.4.4. Product formation by the sequential culture of *C. thermocellum* followed by *C. beijerinckii* on different particle sizes of switchgrass during 10 days. * indicates *C. thermocellum* cultivation.

Day	glucose	lactate	formate	acetate	EtOH	butyrate	butanol
2 mm				(mM)			
0*	0.13±0.05	-	-	1.67±0.6	4.00±0	-	-
1*	-	0.93±0.2	8.00±0.7	8.67±1.1	8.67±1.1	-	-
2*	-	1.50±0.45	9.33±1.5	22.67±2.8	18.33±2.5	-	-
3	-	1.37±0.35	11.0±1	20.67±3.5	-	3.33±2.5	-
4	-	-	8.67±1.52	22.33±3.2	-	10.00±0	0.70±0
5	-	-	10.33±0.57	24.33±0.5	-	11.67±1.2	0.80±0.01
6	-	-	6.33±2.1	31.67±7.0	-	12.00±1.0	0.97±0.01
7	-	-	5.00±2.1	36.67±9.0	-	12.33±2.5	1.17±0.1
8	-	-	3.67±2.1	42.0±11.1	-	13.00±2	1.37±0.4

Table 2.4.4 (Continued). Product formation by the sequential culture of *C. thermocellum* followed by *C. beijerinckii* on different particle sizes of switchgrass during 10 days. * indicates *C. thermocellum* cultivation.

Day	glucose	lactate	formate	acetate	EtOH	butyrate	butanol
5 mm							
0*	0.10	-	-	2.00±0	4.00±0	-	-
1*	-	0.93±0.15	-	9.00±2.0	8.33±0.58	-	-
2*	-	1.53±0.38	5.33±2.83	22.33±4.9	19.3±2.3	-	-
3	-	1.17±0.4	4.00±0	20.00±5.2	5.00±0	1.00±0	-
4	-	-	7.33±0.58	23.67±3.1	-	11.67±0.58	0.70±0
5	-	-	7.33±1.53	23.33±4.0	-	13.00±0	0.77±0.1
6	-	-	7.67±1.15	23.67±4.5	-	14.00±1.0	0.77±0.06
7	-	-	4.67±1.0	23.67±4.7	-	16.67±0.5	1.03±0.15
8	-	-	4.67±1.41	14.00±2.8	-	12.67±0	0.73±0.12
10	-	-	4.67±0	13.33±2.8	-	14.00±1.41	0.80±0.2
15 mm							
0*	0.08±0.03	0.93±0.06	-	2.00±0	4.33±0.5	-	-
1*	-	0.93±0.06	3.00±0	8.33±0.5	6.67±0.5	-	-
2*	-	0.70±0.10	5.00±2.0	18.33±2.0	11.33±2.3	-	-
3	-	-	4.33±2.5	17.00±1.0	6.33±0.71	0.67±1.1	-
4	-	-	7.00±1.5	16.50±3.5	6.00±0.7	6.00±2.0	0.70±0.06
5	-	-	6.33±2.0	22.00±3.0	4.00±0	7.33±1.5	0.80±0
6	-	-	5.33±1.4	26.33±7.5	-	8.00±1.7	1.00±0.06
7	-	-	-	41.33±2.5	-	8.67±1.5	0.67±0.06
8	-	-	-	44.33±2.5	-	8.67±1.0	0.70±0.3
10	-	-	0.33±0	47.33±2.6	-	9.33±0	0.80±0.3

2.5 Discussion

Short term fermentation: solvent producer strains on glucose and switchgrass

Acetone-butanol-ethanol (ABE) fermentation common products are acetone, n-butanol, and ethanol together with acids such as acetate, butyric, lactate; and gases such as CO_2 and H_2 (Gottschalk, 1986). Currently, ABE fermentation is investigated for a sustainable way to produce advanced biofuels (Al-Shorgani et al., 2011). Different clostridia have been involved with the commercial and traditional ABE fermentation a

century ago (Bahl et al., 1982). The performance of different solventogenic clostridia was researched on glucose as substrate. Batch fermentations were carried out by *C. beijerinckii*, *C. acetobutylicum* and *C. saccharoperbutylacetonicum.* Acetic and butyric acids were the principal acids produced as it had been described in ABE fermentation (Gottschalk, 1986).

Clostridium saccharoperbutylacetonicum ATCC 27021 is a butanol hyperproducing bacterium (Jones and Keis, 1995). *C. saccharoperbutylacetonicum* ATCC 27021 had total product formation 40-45% higher than other strains, and more than three times the butanol concentration of *C. acetobutylicum*, traditionally used in marketable ABE fermentation (Bahl et al., 1982). It has been described a production of 8.69 g l^{-1} butanol *C. saccharoperbutylacetonicum* using a concentration of 50 g l^{-1} of glucose (Jones and Keis, 1995); however, almost two times more butanol (2.4 g l^{-1}) was observed considering that a the substrate was 10% w/w of 50 g l^{-1} (RCM glucose concentration of 5 g l^{-1}). Solvent production occurred between 24-48 hours and then stopped. Product inhibition in batch mode is one of the limitations of ABE fermentation (Spivey, 1978).

Lignocellulosic material is an abundant resource that has the potential to serve as substrate for biofuels production. It is also known that the sugars are not readily available because of the recalcitrant nature of lignocellulosic biomass (Himmel et al. 2007). The growth of the solventogenic clostridia on switchgrass was at most 25% of total yield reached on glucose. Switchgrass was poorly fermentable (xylose was found in small amounts). The fact that solvent producer bacteria do not effectively use lignocellulosic material, as switchgrass, makes it clear the necessity of a previous step of saccharification (Zhang and Lynd, 2004). Simultaneous enzyme production, saccharification and fermentation is known as Consolidated bioprocessing (CBP). As clostridia that produce butanol are not able to grow on crystalline cellulose, *C. thermocellum* is a cellulolytic microorganism that could satisfy this action (Bayer et al, 2007).

This study was useful to determine the solvent producer model, *C. saccharoperbutylacetonicum* ATCC 27021, to run future experiments such as the sequential culture with *C. thermocellum* in high solid cultivation with continuous product removal using switchgrass as substrate.

Effect of the particle size:

It is known that physical pretreatment of crop biomass can reduce particle size which increases available surface area of intracellular components (Palmowski and Müller 1999; Vidal et al., 2011). In this experiment, the hypothesis that the effect of particle size on gas production was surface area-dependent was supported by the results. *C. beijerinckii* and *C. thermocellum* were studied in this case. Between switchgrass passed through 2, 5 and 15 mm screen, the former was the best fermentable size according to gas production experiments. However, particle size reduction as pretreatment is important in terms of energy consumption, the smaller the particle size the bigger the energy consumption (Sun and Cheng, 2002). But it has been reported that there is a maximum volume reduction for chopped switchgrass (Chevanan et al., 2010). Bulk density is a concern in handling and storing lignocellulosic biomass (Lam et al., 2007). Even though reducing particle size has a negative aspect in terms of energy, it has a positive side in handling and fermentability. However, it is important to mention that product formation was independent of the particle size.

Sequential culture experiment showed that *C. thermocellum* improves fermentation by *C. beijerinckii* on gas production experiments. However, *C. thermocellum* cellulase system retains some activity at 35 ºC (Ng et al., 1977); so, gas produced during the mesophilic range cannot be just attributed to *C. beijerinckii*. So, additional experiments have to be done to determine if *C. thermocellum* is still producing gas in the mesophilic range. *C. thermocellum* enzyme complex (cellulosome) degrades cellulose freeing sugars for other microorganism to ferment (Bayer et al, 2007*)*. Pretreatment options are crucial to reduce cost and energy consumed in biomass conversion. Thus, *C. thermocellum* could be used as a cheap biological pretreatment on lignocellulosic biomass. Moreover, the study indicated that gas production reduced the concern of maintaining fermentation under anaerobic conditions but also the suitability of the switchgrass as a substrate.

Chapter Three

Fenton reaction as a pretreatment on Switchgrass

3.1 Introduction

Lignin provides the defense against being degraded by microbial enzymes as well as strengthening of the plant (Himmel et. al., 2007). However, lignin content is not desirable in the conversion of lignocellulosic biomass into biofuels (Zhang and Lynd, 2004). Lignin encloses cellulose and hemicellulose and does not give fermentable sugar and interferes with the enzymatic hydrolysis of cellulose and hemicellulose.

The brown and white rot fungi are the most effective decomposers of lignocellulosic biomass in nature (Daniel, 2003). The biomass decomposition is due to the presence of lignin degrader enzymes together with low molecular weight chemicals such us H_2O_2, iron and chelators (Hammel et al., 2002; Arantes et al., 2012). Different pretreatments have been studied in the conversion of lignocellulosic biomass into biofuels. Among the non-enzymatic treatments, Fenton reaction is accepted as a mimetic of fungi decomposition (Fenton, 1894). Free radicals (OH^-) are generated due to the following reaction:

$$Fe^{+2} + H_2O_2 \rightarrow \quad Fe^{+3} + H_2O + {}^-OH$$

The lignin is attacked by these OH^- free radicals which produce cleavages on the polymers. Then, lignocellulolytic enzymes action is more efficient so that the conversion of the lignocellulosic biomass into biofuels (Mehdi Dashtban et al., 2010; Bentivenga et al., 2003.). Fenton reaction is a potential pretreatment for biofuel production (Arantes et.al, 2012).

In this chapter, switchgrass was pretreated with soluble phase Fenton solution. *C. thermocellum* in sequential culture with *C. beijerinckii* were studied during short and long term fermentations.

3.2 Objectives

Fenton reaction vs untreated switchgrass

The hypothesis is that the Fenton reaction improves not only the fermentability but also the survival of the bacteria because the bacteria have easier access to sugars. The purpose of this pretreatment is to achieve efficient separation of lignin from carbohydrates. The effectiveness of this pretreatment on switchgrass was evaluated by quantifying the substrate's subsequent conversion by simultaneous saccharification and fermentation using a sequential culture of *Clostridium thermocellum* and *Clostridium beijerinckii*. Gas production, viable cell number and product formation were evaluated.

Survival experiment

The ability of the inocula to survive in the substrate was determined by viable cell number. Viable cell number was determined by serial dilution into enriching media. The survival of each strain was evaluated in pure and co-culture on switchgrass as a substrate.

3.3 Materials and Methods

Strains and Culture conditions

See Chapter One section Materials and Methods.

Short term fermentation

Fenton-treated vs untreated switchgrass: Gas production

The Fenton-treated switchgrass (passed through a 2 mm screen) was provided by the laboratory of Bent C. Lynn, University of Kentucky, Department of Chemistry. The biomass was treated under the Fenton Reaction Protocol. 10 g of switchgrass were put

into an appropriate Erlenmeyer flask (i.e. 1 L). Then, 200 mL of H_2O_2 solution (6%) and 200 mL of the iron solution (500 mg $FeCl_2$ * $4H_2O$ (s) dissolved in 200 mL deionized water) were added to the biomass and stirred for about 120 h. The biomass reaction mixture was filtered with a filter paper. The aqueous portion was discarded and the Fenton-treated biomass was washed until neutral pH. The Fenton-treated switchgrass was dried at 105 ºC overnight.

Switchgrass (5 g of untreated or Fenton-treated, 6 % w-t) was added to the fermentation vessels (Pyrex bottle with sidearm port, 1000 mL). The vessels were sealed and purged with O_2-free CO_2 through the sidearm septa. Basal medium (45 mL) was added through the septa of the sidearm ports. There were carried out two experiments: a sequential culture of *C. thermocellum* followed by *C. beijerinckii*, and a pure culture of *C. beijerinckii*. For the former, the vessels were warmed in a water bath (63 °C) before inoculation. The vessels were inoculated (10% w/w) through the septa with *C. thermocellum* (48 h cultures), and incubated (63° C; 48 h). After the *C. thermocellum* incubation, the vessels were cooled down, and inoculated with *C. beijerinckii* (10% w/w, 24 h cultures). The culture was incubated at 35 °C during 48 h. In the pure culture experiment, the previous inoculation with *C. thermocellum* was omitted. Gas production was monitored using the Ankom RF Gas Pressure System (Ankom, Macedon, NY, USA). Gas pressure was tested in 1 min intervals, and cumulative pressure was recorded every 5 min. The global pressure release was set at 104 kPa. Control vessels were just basal media. They were tested in each replication. The pressure values of the uninoculated vessels were subtracted from each treatment to control for the effects of temperature. Each gas production treatment was performed in triplicate on separate days. The data were analyzed in SAS v. 9.3 (SAS Inst. Inc., Cary, NY, USA) using an ANOVA with a Tukey's test ($P < 0.05$ considered significant).

Fenton-treated vs untreated switchgrass: Survival experiment (Viable cell number)

The ability of the inoculum to survive on the substrate was determined by viable cell number by serial dilution into enriching media. The substrate (switchgrass, 0.05 g, 6 % wt) was autoclaved and inoculated in two ways: pure culture of *C. beijerinckii* (10%

w/w) or sequential culture of *C. thermocellum* (10% w/w) followed by *C. beijerinckii* (10% w/w). For the former, *C. beijerinckii* cells were grown anaerobically at 35 °C in Reinforced Clostridial Media and 0.5 ml was used to inoculate the set of Hungate tubes with 0.05 g of treated and untreated switchgrass. These tubes were incubated at 35 °C. Viable cell number was determined on day 0, 1, 2, 3, 4, 5, 6, 7, 14, 21. Similarly, *C. thermocellum* cells were grown anaerobically at 65 °C in basal media and 0.5 ml was used to inoculate the set of Hungate tubes with 0.05 g of treated and untreated switchgrass. These tubes were incubated at 65 °C during 24 hours. *C. beijerinckii* cells were added to these tubes and they were incubated at 35 °C. Viable cell number was determined on day 0, 1, 2, 3, 4, 5, 6, 7, 14, 21. Experiments were performed in triplicate (Figure 3.3.1).

Fenton-treated vs untreated switchgrass: Product quantification of C. thermocellum

End product quantification of *C. thermocellum 2*7405 on Fenton-treated and untreated switchgrass was studied. Serum bottles (100 ml size) containing 5 g (6% wt) of both types of biomass were autoclaved. 50 ml of basal medium was added and the serum bottles were warmed in a water bath (63 °C) prior to inoculation with *C. thermocellum* (5 ml or 10% w/w).

Daily samples (1 ml) were clarified by centrifugation (14800 × g, 2 min), and they were frozen for later analyses. Acetate, ethanol, butanol, lactate and formate were quantified by HPLC (Dionex, Sunnyvale, CA). The anion exchange column (Aminex 87H; BioRad) was operated at 50 °C, flow rate 0.4 ml min^{-1}. Eluting compounds were detected by refractive index (Shodex/Showa). Experiments were performed in duplicate.

Toxicity of C. thermocellum inoculum.

To evaluate the toxicity of the inoculum of *C. thermocellum* to *C. beijerinckii* cultures the following experiment was set up (Figure 3.3.2). *C. thermocellum* cells were grown in the basal medium with amorphous cellulose (Whatman #1 filter paper; 4.5 mg ml^{-1}) or in the basal medium with switchgrass (untreated, 10% w/w) at 63 °C. Different

volumes of *C. thermocellum* (10, 25, 50%) were added to serum bottles containing RCM media. Cells of *C. beijerinckii* (10 % w/w) were added all the serum bottles. They were incubated at 35 °C. Samples were taken at 0, 4, 7, 12, 24, 48 hours to measure the pH and Optical Density (OD_{600nm}).

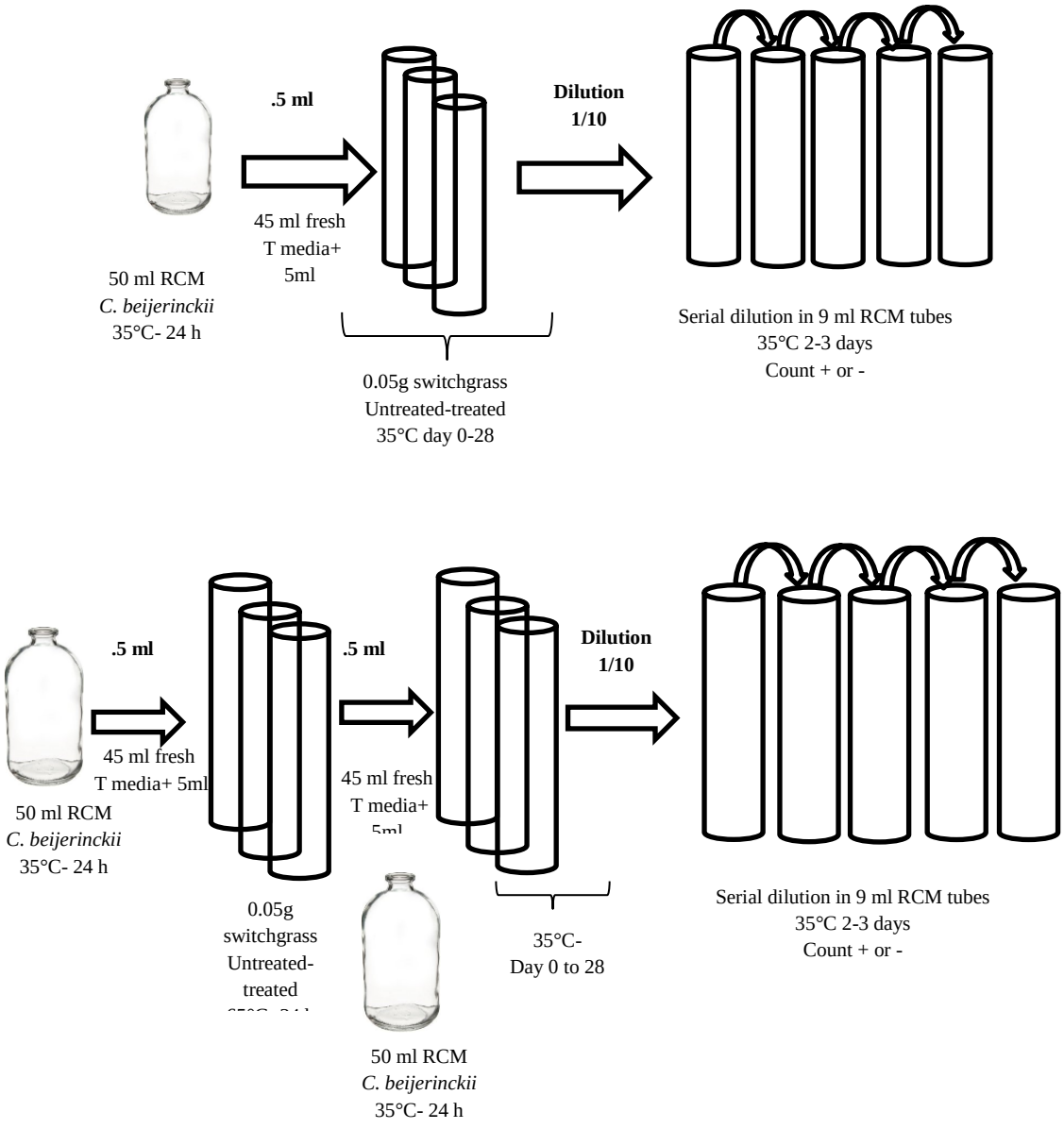


Figure 3.3.1. Experimental design to count the viable cell number of pure cultures and sequential cultures of *C. beijerinckii* and *C.thermocellum* on Fenton-treated and untreated switchgrass.

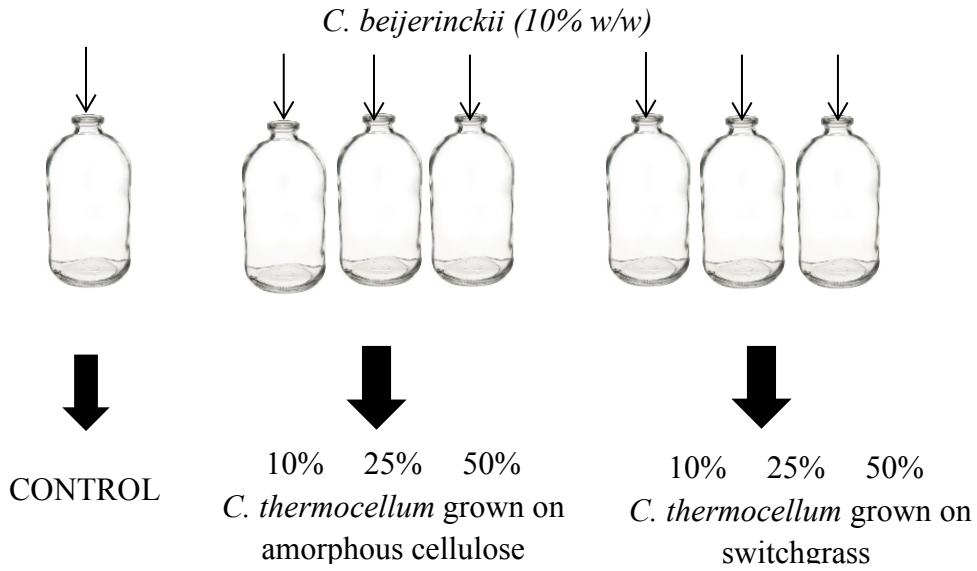


Figure 3.3.2. Experimental design to evaluate the toxicity of the inoculum of *C. thermocellum* on *C. beijerinckii*.

Product quantification

The cultures were sampled as indicated. Samples (1 ml) were clarified by centrifugation (14800 × *g*, 2 min), and frozen for later analyses. The pH and acetate were measured daily. Acetate was quantified by an enzymatic method assay (Roche, Nutley, NJ). Acetate, ethanol, butanol, lactate and formate were quantified by HPLC (Dionex, Sunnyvale, CA). The anion exchange column (Aminex 87H; BioRad) was operated at 50° C, flow rate 0.4 ml min^{-1}. Eluting compounds were detected by refractive index (Shodex/Showa).

3.4 Results

Short term fermentation

Fenton-treated vs untreated switchgrass: Gas production

The sequential culture of *C. thermocellum* followed by *C. beijerinckii* as well as the

pure culture of *C. beijerinckii* were carried out on Fenton-treated and untreated switchgrass. Gas production was monitored and cumulative pressure was measured. In the first 48 h, *C. thermocellum* gas production was higher on untreated switchgrass than the Fenton-treated one (Figure 3.4.1). The sequential addition of *C. beijerinckii* showed an increase in gas production on Fenton-treated substrate reaching a cumulative pressure of 109.24 kPa (Figure 3.4.1). When *C. beijerinckii* was studied without prior inoculation of *C. thermocellum*, the cumulative pressure was no more than 23.58 kPa (Figure 3.4.2). There was no significance difference between both substrates. To be able to determine just the gas production by *C. beijerinckii*, the cumulative pressure from the culture of *C. thermocellum* was subtracted (Figure 3.4.3, to see numerical values Appendix).

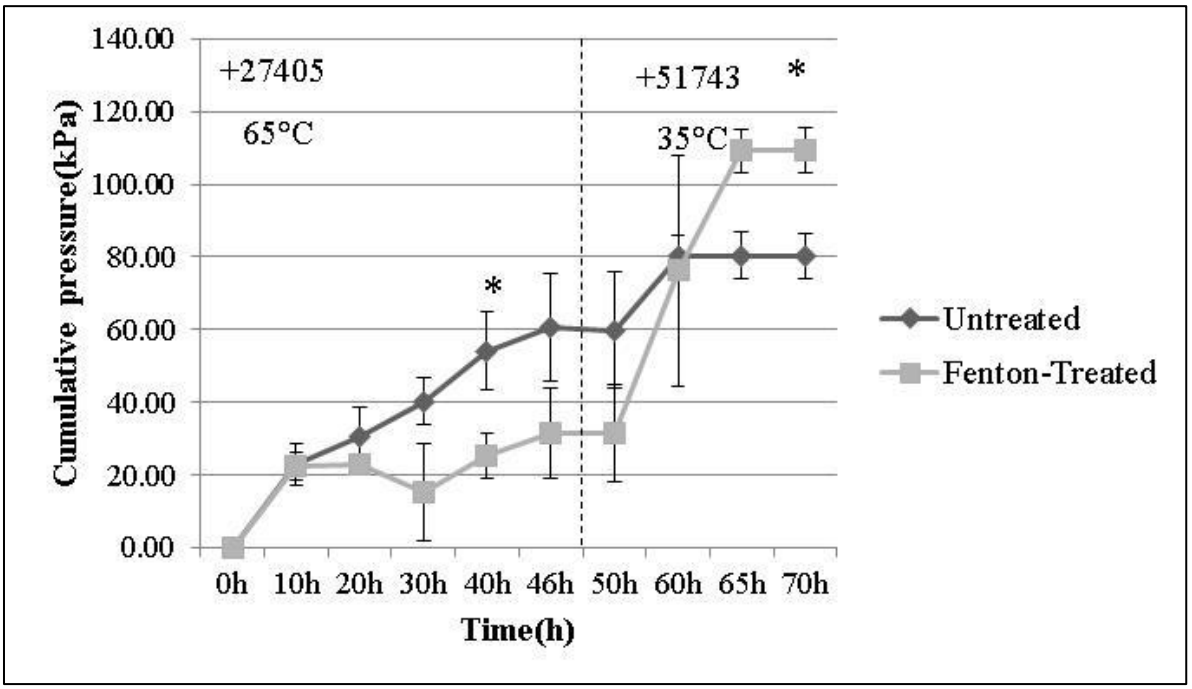


Figure 3.4.1. Gas production by the sequential culture of *C. thermocellum* followed by *C. beijerinckii* on untreated and Fenton-treated switchgrass. The dashed line indicates the inoculation with the mesophile and the associated switch in temperatures. * indicates significance difference. Error bars indicate ± one standard deviation. After 70 hours, the cumulative pressure levels out.

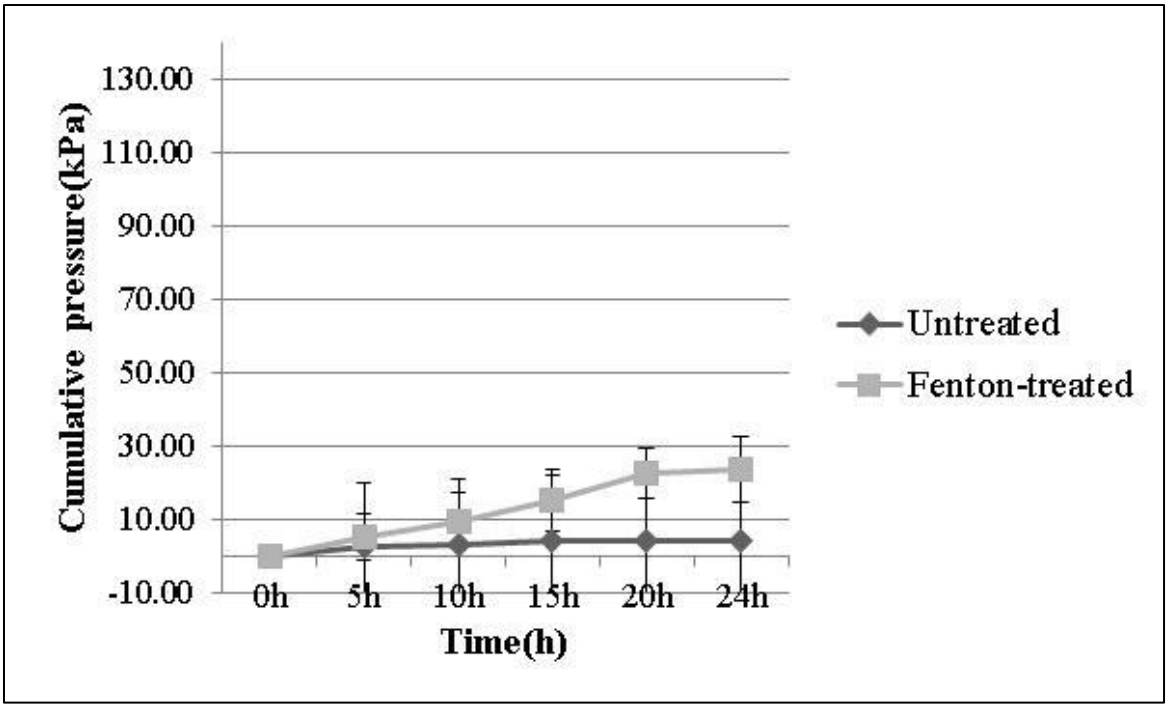


Figure 3.4.2. Gas production by *C. beijerinckii* on untreated and Fenton-treated switchgrass. There was no significance difference between the values at any time. Error bars indicate ± one standard deviation. After 24 hours, the cumulative pressure levels out.

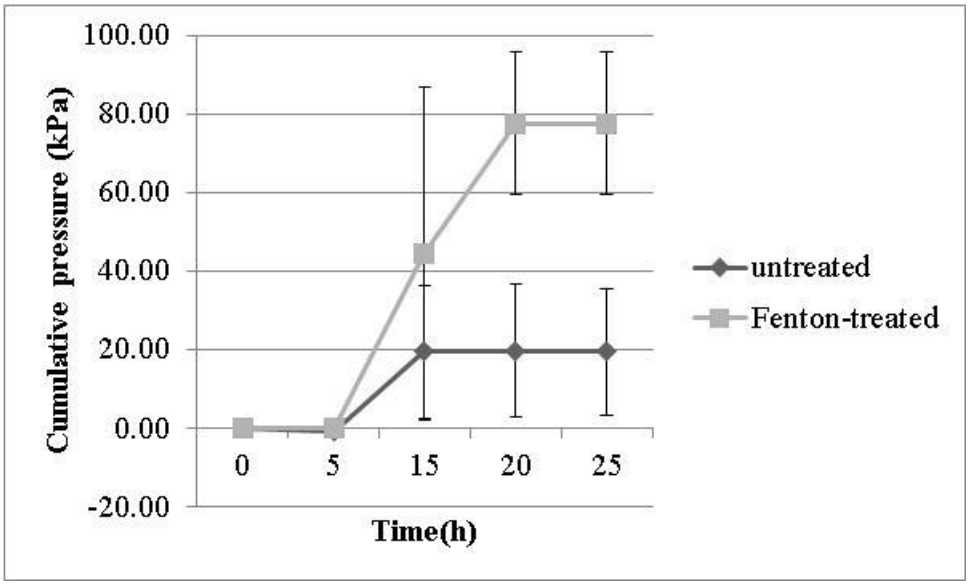


Figure 3.4.3. Comparison of gas produced by *C. beijerinckii* on Fenton-treated (light grey squares) and untreated switchgrass (grey rhomboid). Error bars indicates ± one standard deviation.

On untreated switchgrass, gas was produced for the first 15 h of incubation, and the final cumulative gas pressure was 19.5 kPa. However, the fermentation lasted a few more hours (20h) on Fenton-treated switchgrass and *C. beijerinckii* produced more than three times as much gas (77.7 kPa) (Figure 3.4.3). See numerical values in Appendix Chapter three.

Fenton-treated vs untreated switchgrass: Survival experiment (Viable cell number)

Survival experiments were carried out to study the ability of *C. thermocellum* and *C. beijerinckii* to survive in the substrate. The viable cell number of *C. beijerinckii* on Fenton-treated and untreated switchgrass was determined in both pure and sequential cultures. In order to know how long *C. thermocellum* had to be cultured, preliminary experimental design was carried out using basal media containing cellobiose (0.2 g ml^{-1}) at 63 ºC for serial dilution counting instead of RCM media. *C. thermocellum* viable cell number was 10^8 cells ml^{-1} during the first 48 hours (data not shown, see Appendix Chapter three). After that period, no visible growth was observed.

As follows, viable cell number of *C. beijerinckii* on untreated switchgrass is shown in Figure 3.4.4. The pure culture showed higher viable cell numbers than the sequential culture on untreated switchgrass. The bacteria survived on the untreated switchgrass for 14 days. The sequential cultures showed a dramatical decreased in *C. beijerinckii* viable cell number (Figure 3.4.4).

When *C. beijerinckii* was grown on Fenton-treated switchgrass there was no significance difference between sequential and pure cultures. The viable cell number reached 10^8 cells ml^{-1} and about 10^7 cells ml^{-1} number was kept along the time (Figure 3.4.5).

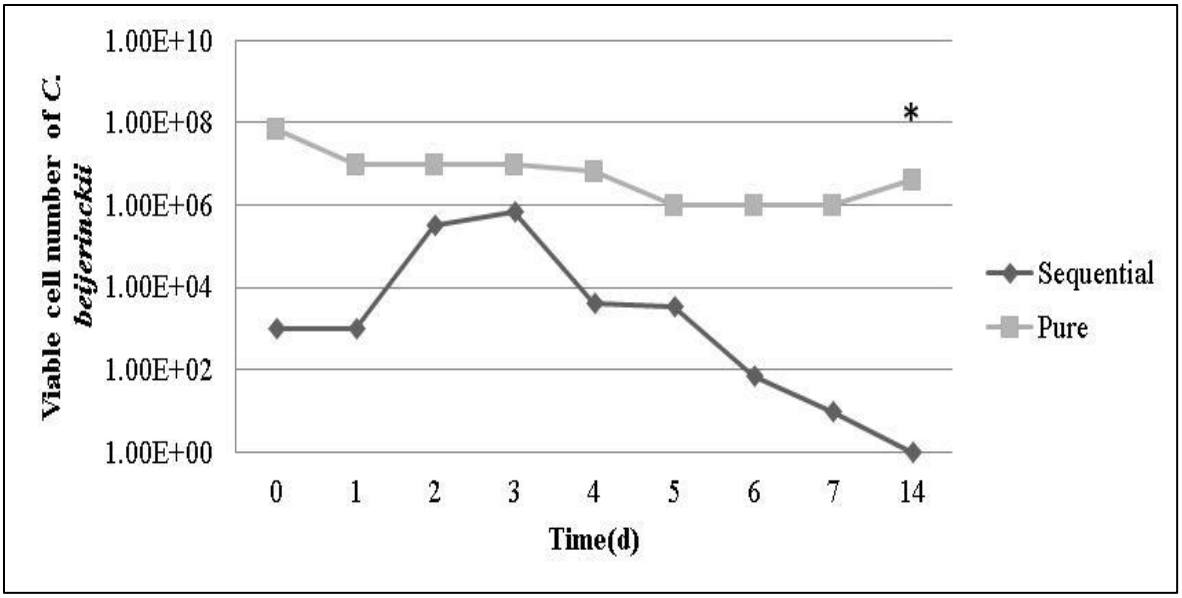


Figure 3.4.4. Viable cell number of *C. beijerinckii* grown on untreated switchgrass during 14 days measured by serial dilution. Sequential culture with *C. thermocellum* is shown by rhomboid symbol and pure culture of *C. beijerinckii* is shown by squared symbol. See data and standard error in Appendix Chapter three.

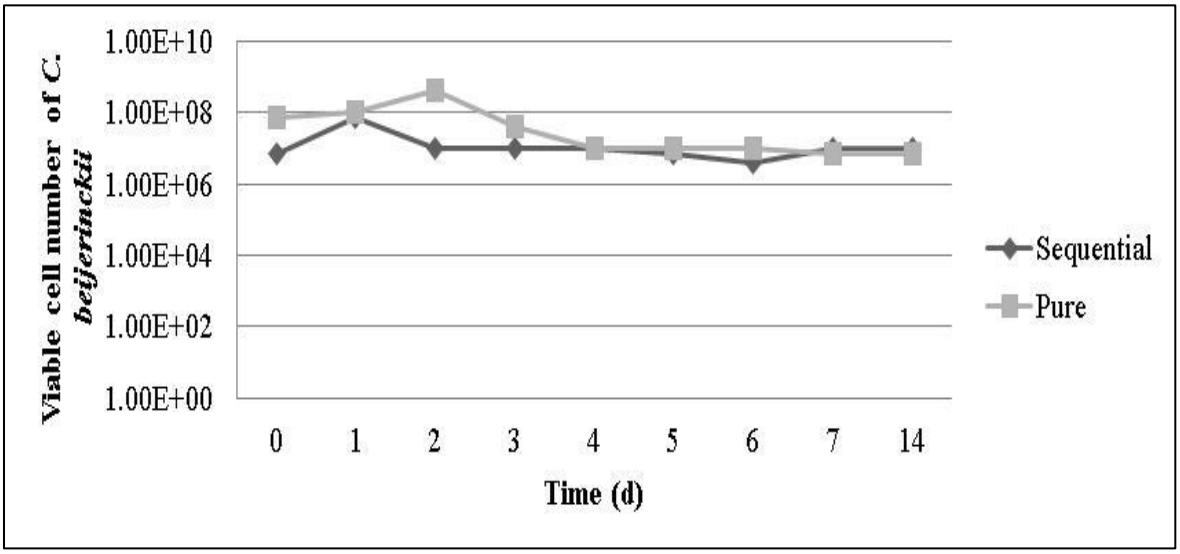


Figure 3.4.5. Viable cell number of *C. beijerinckii* grown on Fenton-treated switchgrass during 14 days measured by serial dilution. Sequential culture with *C. thermocellum* is shown by rhomboid symbol and pure culture of *C. beijerinckii* is shown by squared symbol. See data and standard error in Appendix Chapter three.

Fenton-treated vs untreated switchgrass: Product quantification of C. thermocellum

When gas production experiments determined that *C. thermocellum* ATCC *2*7405 gas production was poor on Fenton-treated switchgrass compared with untreated, the study of product formation was planned to observe the behavior of this strain. So, *C. thermocellum* was grown on Fenton-treated and untreated switchgrass during 12 days and pH plus end products were studied (Figure 3.4.6, 3.4.7 and 3.4.8). The results indicated that *C. thermocellum* grew better on untreated switchgrass when total products are quantified. Acetate production was observed earlier in untreated switchgrass, and it paralleled the pH drop on day 3 observed in Figure 3.4.6. Even though acetate formation reached similar amounts on treated switchgrass, it took more time to be produced. Ethanol formation was significantly higher on untreated switchgrass, but it was poorly produced on Fenton-treated switchgrass.

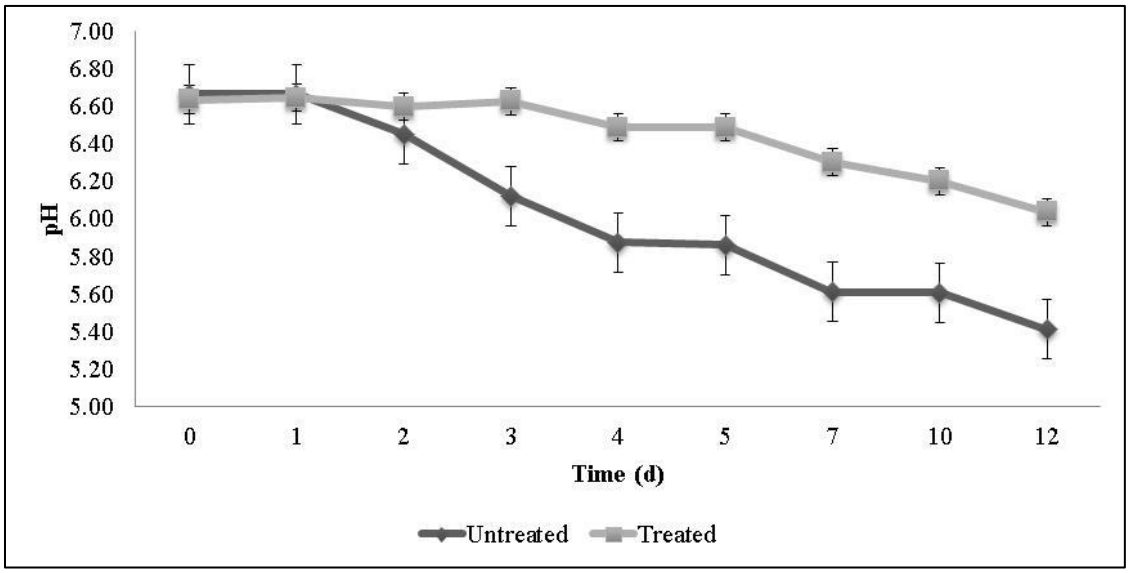


Figure 3.4.6. pH variation along the growth of *C. thermocellum* on Fenton-treated(square symbol) and untreated (rhomboid symbol) switchgrass.

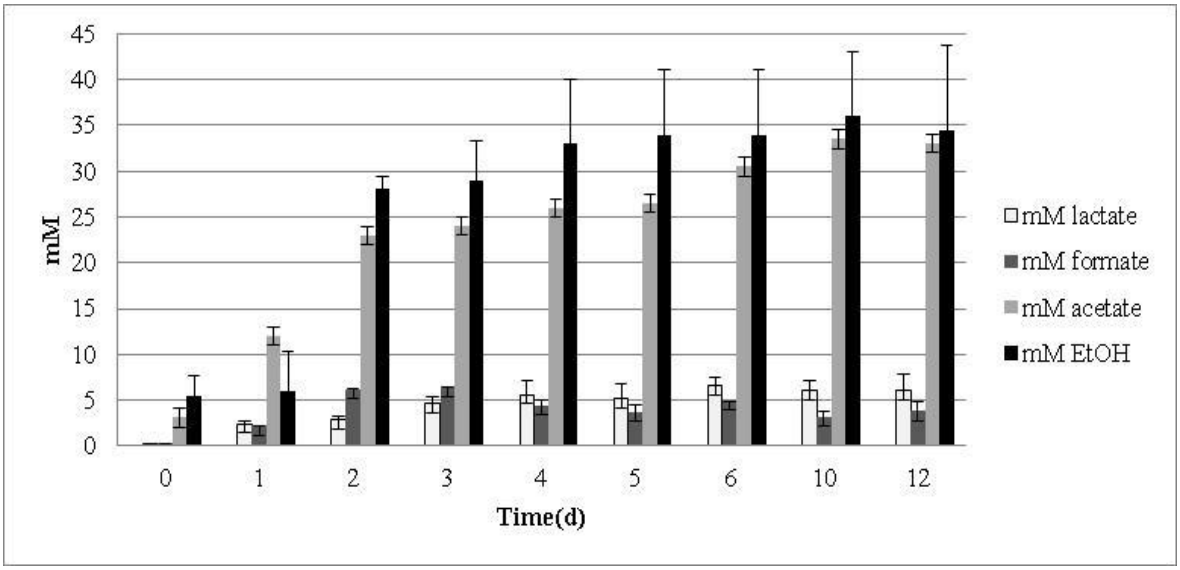


Figure 3.4.7. Product formation by *C. thermocellum* ATCC 27405 on untreated switchgrass (passed through a 2 mm screen) during 12 days at 65 ºC. Error bars indicates ± one standard error. See numerical values in the Appendix Chapter three.

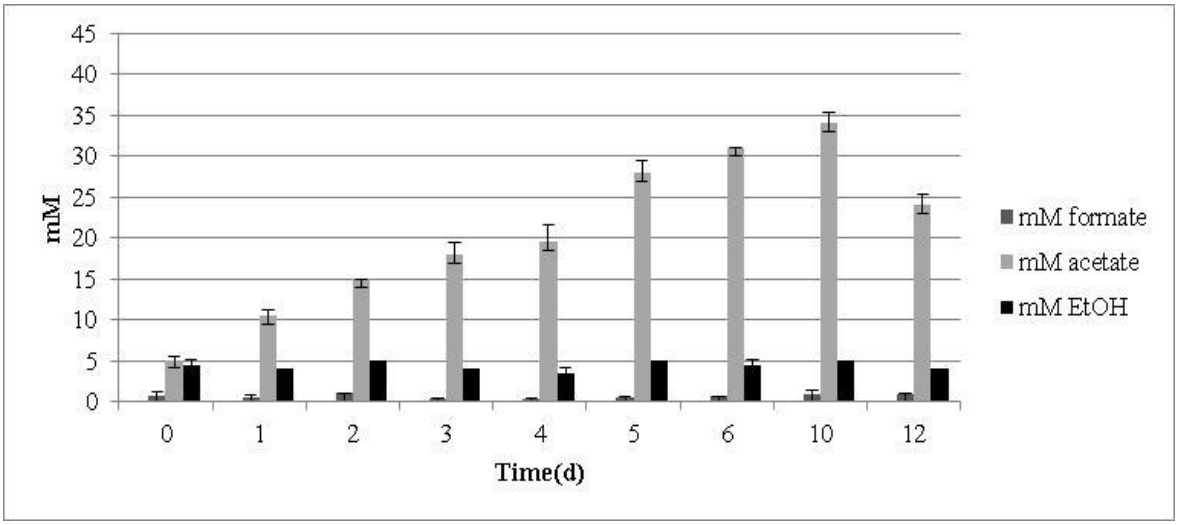


Figure 3.4.8. Product formation by *C. thermocellum* ATCC 27405 on Fenton-treated switchgrass (passed through a 2 mm screen)) during 12 days at 65 ºC. Error bars indicates ± one standard error. See numerical values in the Appendix Chapter three.

Toxicity of C. thermocellum inoculum

To analyze if the inoculum of *C. thermocellum* was toxic to *C. beijerinckii* according to the observed data, different concentrations of *C. thermocellum* were added

to the *C. beijerinckii* cultures in RCM media. Optical density and pH were measured along time. Optical density from initial cultures was subtracted from the data (Figure 3.4.9 and 3.4.10). 10% and 25% *C. thermocellum* inoculum were not toxic for *C. beijerinckii*. Optical density was significantly decreased when 50% of inoculum, grown either filter paper or switchgrass, was added on *C. beijerinckii* culture. Table 3.4.1 shows the pH variation on both treatments.

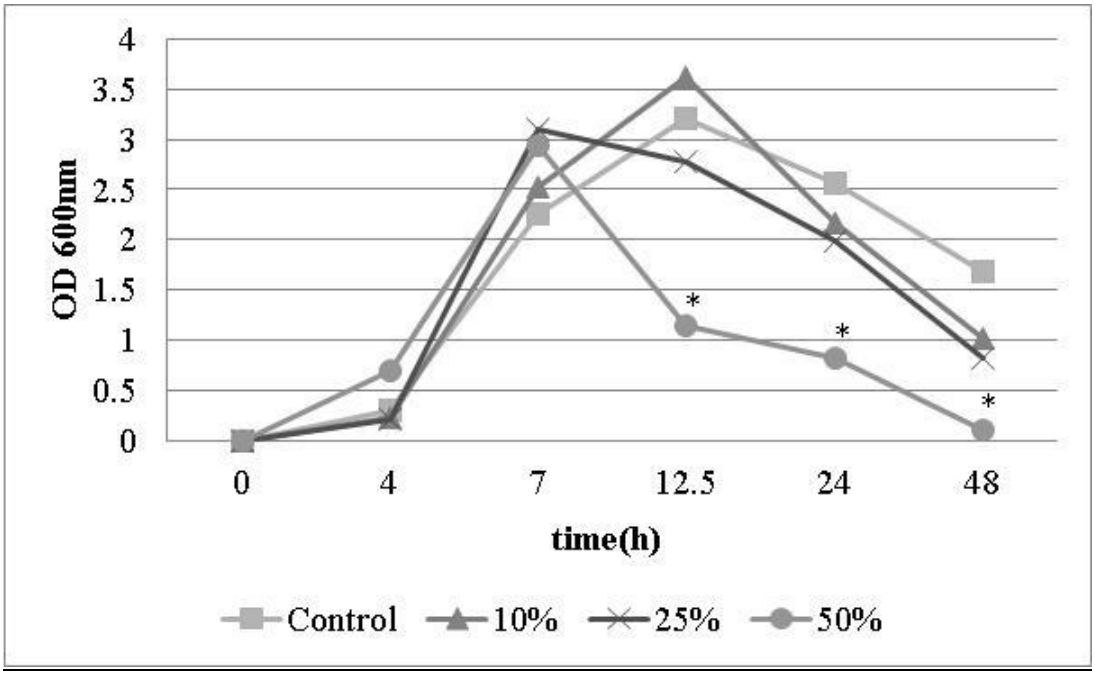


Figure 3.4.9. ΔOptical density (600nm) of cultures of *C. beijerinckii* grown on different concentrations (10 %, 25 % and 50 %) of *C. thermocellum* cultured on switchgrass. Δ indicates that the optical density of time zero was subtracted from the other values. * indicates significant difference($P<0.05$). See numerical values and standard error in Appendix Chapter three.

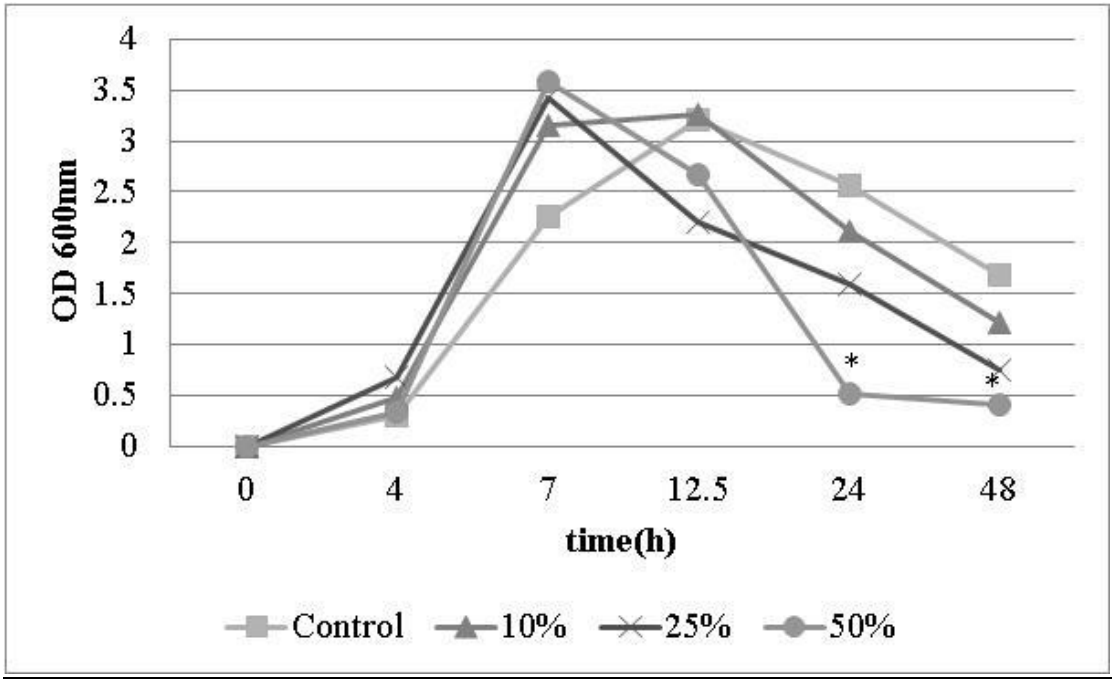


Figure 3.4.10. ΔOptical density (600nm) of cultures of *C. beijerinckii* grown on different concentrations of *C. thermocellum* cultured on filter paper. Δ indicates that the optical density of time zero was subtracted from the other values. * indicates significant difference($P<0.05$). See numerical values and standard error in Appendix Chapter three.

Table 3.4.1.pH of cultures of *C. beijerinckii* grown on different concentrations of *C. thermocellum* cultured on either filter paper or switchgrass. Control represents pure culture of *C. beijerinckii*.

		pH: *C.thermocellum* grown on					
	Control	switchgrass			filter paper		
Time (h)		10%	25%	50%	10%	25%	50%
0	5.97 ±0.07	6.16 ±0.02	6.36 ±0.01	6.79 ±0.01	6.16 ±0.06	6.6 ±0.14	6.8 ±0.06
4	5.73 ±0.01	5.88 ±0.09	6.10 ±0.02	6.49 ±0.02	5.93 ±0.01	6.31±0.25	6.4 ±0.01
7	6.84 ±0.01	7.00 ±0.01	6.85 ±0.07	7.14 ±0.01	6.92 ±0.02	6.88±0.07	7.3 ±0.08
12.5	5.33 ±0.08	5.27 ±0.01	5.44 ±0.01	6.12 ±0.02	5.31 ±0.02	5.53±0.01	5.91 ±0.09
24	5.24 ±0.07	5.32 ±0.01	5.46 ±0.02	6.23 ±0.01	5.36 ±0.03	5.61±0.01	6.01 ±0.13
48	5.23 ±0.04	5.33 ±0.04	5.61 ±0.01	6.34 ±0.02	5.45 ±0.03	5.65±0.03	5.99 ±0.01

3.5 Discussion

Fenton treatment was used for hydroxyl radical generation to make cellulose available for microbial fermentation (Flournoy, 1994). Microbial fermentation was carried out on Fenton treated switchgrass to evaluate the Fenton treatment. Untreated switchgrass was inoculated with *C. thermocellum*, followed by *C. beijerinckii*. Since *C. thermocellum* is a cellulolytic, the incubation step with this bacterium can be defined as a biological pretreatment for completeness of the cellulose degradation and allowed *C. beijerinckii* to utilize sugar liberated from cellulose.

CO_2 is the most common fermentation product (Gottschalk 1986). Thus, CO_2 can be measured to determine the fermentability of the biomass. In the sequential cultures, *C. beijerinckii* produced about 300% more gas when Fenton-treated switchgrass was the substrate (Figure 3.4.3). *C. thermocellum* seems to improve fermentation by *C. beijerinckii* on gas production experiments. However, *C. thermocellum* cellulase system retains some activity at 35 ºC (Ng et al., 1977); so, gas produced during the mesophilic range cannot be just attributed to *C. beijerinckii*. But, C. *thermocellum* also should be growing better on Fenton-treated material, it did not. According to these deductions, gas production experiments were carried out by incubating *C. thermocellum* at room temperature (~22 ºC) during 24 hours prior inoculation with *C. beijerinckii* on untreated and Fenton-treated switchgrass (2 mm) (data not shown, one replication). During the first 24 hours, the cumulative pressure was the same as control (just basal medium), indicating that *C. thermocellum* was not producing gas on both substrates. Moreover, *C. thermocellum* was inoculated together with *C. beijerinckii* on untreated and Fenton-treated switchgrass and incubated at 35 ºC (data not shown, one replication). The results indicated that cumulative pressure on both substrates was the same as those experiments carried out with pure cultures of *C. beijerinckii* (Figure 3.4.2). However, extra gas experiments should be done to clarify this issue.

Because gas experiments showed poor gas production by *C. thermocellum* on Fenton-treated material, supplementary analysis were done. Product formation by *C. thermocellum* ATCC 27405 on untreated and Fenton-treated switchgrass was studied (Figure 3.4.7 and 3.4.8). Even tough, Fenton-treated switchgrass was fermentable; this bacterium grew better on untreated material. It is known that *C. thermocellum*

cellulosome is sensitive to glucose; about 60 g l^{-1} produced 35% enzymatic inhibition. But, glucose concentrations were very low and not close to inhibitory levels. So, an experiment was carried out to study why *C. thermocellum* did not grow well on Fenton treated switchgrass (data not shown). It consisted of incubating *C. thermocellum* with different aliquots of different stages of Fenton-treated material. The results showed that one of the solutions used in the Fenton protocol inhibited the bacterium growth. So, why did *C. beijerinckii* grow better on Fenton-treated material, because *C. thermocellum* was not growing there? So, incubating *C. beijerinckii* alone on Fenton treated and untreated material may answer that question. However, gas production on both substrates was poor. Then, C. *beijerinckii* is poorly fermenting switchgrass without a previous saccharification step. Thus, was *C.thermocellum* inoculum toxic to C. *beijerinckii* cells?. After doing an assay measuring optical density of C. *beijerinckii* cultures inoculated with different concentrations of *C. thermocellum* showed that 10% and 25% *C. thermocellum* inoculum were not toxic for *C. beijerinckii*. However, 50% of inoculum grown either on filter paper or switchgrass affected *C. beijerinckii* growth. Subsequently, it can be hypothesized that the cellulosome is still active and it is why *C. beijerinckii* grew better on Fenton treated biomass and *C. thermocellum* enzymes may be working. So, it is known that Fenton chemistry increases enzymatic saccharification by an average of 312% relative to untreated biomass in switchgrass (Dawn Kato, unpublished communication). Because Fenton pretreatment showed an increase in gas production, the Fenton reaction could be an option in lignocellulosic biomass pretreatment methods.

Chapter four

ABE Fermentation in a Solid Substrate Cultivation with Continuous Product Removal.

4. 1 Introduction

In the early 1900, ABE fermentation was carried out by *Clostridium acetobutylicum* on different substrates (Weizmann and Rosenfeld, 1937). ABE fermentation was valuable for butanol production and during World War II, this fermentation process provided acetone used for the manufacture of explosives. Later on, ABE plants were constructed in the United States due the high availability of agricultural substrates. For example, butanol production was carried out in Teere Haute, IN and Peoria, IL on an industrial scale using corn mash. But, butanol toxicity at 16 g l^{-1} concentration led to cell growth inhibition and low yields. So, this fact together with the petroleum industry brought down this ABE development in 1960's (Spivey, 1978). Currently, there is a new interest for ABE fermentation not only because butanol has many advantages as fuel but also due to the potential of using different substrates such as corn, whey permeate, molasses and many other agricultural biomass such us corn stover, wheat straw and switchgrass (Quereshi and Ezeji, 2008). Lignocellulosic crops are searched for biofuel production because their cheap cost, their high biomass yields and availability. Switchgrass (*Panicum vergatum*) is a perennial grass with high yields (12-18 tonnes of dry solids per hectare) and it has been chosen as a model for energy production (Parrish and Fike, 2005). However, several challenges have to encounter to be able to ferment this lignocellulosic substrate as well as to overcome challenges related with the traditional batch fermentation process such as long lag phase, product inhibition and low yields.

In the batch ABE fermentation, the growth-associated phase is known as acidogenesis. The non-growth associated phase, solventogenesis, is the uptake of acids where solvents are produced. When acid concentration increases, acetic and butyric acids, and some proteins on cell membrane start to move and it facilitates the movement of

molecules through the cell membrane. Acids staying in the cell end up producing some inhibitory interactions leading to inhibition of cell growth (Flythe and Russel, 2006). Generally, the inhibition is because a negative enzymatic feedback. The acids are used to produce solvents to decrease the toxic effect on the cell environment. However, an excessive accumulation of solvents can be detrimental to the cells. Butanol not only affects the cell membrane permeability but also inhibits the membrane protein ATPase which is important to maintain the pH and electrochemical gradient (Gutierrez and Maddox, 1992). Membrane changes are the principal cell adaptations to the high alcohol concentration. However, there is a limitation in the butanol yields (Ezeji et al., 2004). Usually, the ABE fermentation in a reactor does not exceed 20 g l^{-1} of total solvents, and butanol yields are rarely over 13 g l^{-1} (Qureshi and Blaschek, 2001).

Solid substrate fermentation (SSC) consists in the growth of the microorganisms on solid substrate. This has certain advantages, such as, high yields, low production costs due to availabilities of equipment, stability of the products and less effluent generation (Nigam and Singh, 1994). SSC has disadvantages such as the oxygen transfer and heat elimination as well as higher concentration of inhibitors (Lekanda and Perez-Correa, 2004; Dharmagadda et al., 2010, Jorgensen et al., 2007). However, the comparison of different anaerobic bacteria on different lignocellulosic substrates resulted in double the concentration of products in SSC compared with SmF (Submerged fermentation) which is the typical method for biofuel production (Chinn et al., 2006; Jorgensen et al., 2007).

C. thermocellum has the potential to overcome the challenges related with solid substrate cultivation such as heat transfer (thermophilic bacterium) and limited oxygen diffusion (anaerobic bacterium) (Lynd et al., 1989). This bacterium has been found in low moisture environments. There are different studies of *C. thermocellum* growing on Solid Substrate Cultivation (SSC) by Chinn et al (2008). In another investigation, *C. thermocellum* grown on solid substrates had ethanol yields 4 times greater than liquid media (Dharmagadda et al., 2010). However, in solid substrate cultivation, cessation of end products occurs even though sufficient substrate remained. Dharmagadda et al. investigated the metabolic inhibition on SSC of *C. thermocellum* on cellulose. At the beginning, higher initial production rates for all fermentation products were observed; however, the rates were not maintainable. Acids produced by the fermentation were

related with unfavorable osmotic conditions which resulted in metabolic inhibition. Solid substrate cultivation with media replacement by periodic flushing (FSSC), on the other hand, maintained favorable growth conditions for *C. thermocellum* with constant amounts of ethanol, acetate and lactate yields (Dharmagadda et al., 2010).

Clostridium saccharoperbutylacetonicum ATCC 27021 (N1-4) is an anaerobic, mesophilic, butanol hyperproducing bacterium (Jones and Keis, 1995). This bacterium is able to utilize different carbon sources in anaerobic fermentation including lignocellulosic biomass such as rice and deoiled rice bran (Thang et al., 2010; Al-Shorgani et al., 2012).

To put it briefly, ABE fermentation is an alternative for biofuels production. This process involves the genus *Clostridium* which has the ability for acids and solvents production. When lignocellulose is the substrate, it is needed a community of organisms that work together in depolymerizing the lignocellulose and freeing the sugars for the production of acids and solvents. For that, it will be tested the sequential culture of the cellulolytic *Clostridium thermocellum* ATCC 27405 followed by *Clostridium saccharoperbutylacetonicum* ATCC 27021 (N1-4) using switchgrass, a lignocellulosic non-food feedstock. As optimum growth temperature of these microorganisms are different and in order to avoid accumulation of end products and its associated toxicity, a solid substrate cultivation cycling temperatures and continuous removal of products will be carried out.

4.2 Objectives

Solid-substrate cultivation (SSC) is an alternative to tradition liquid and submerged-substrate fermentations. Continuous flow solid-substrate cultivation comprises of temperature cycling between 65 °C and 35 °C with re-inoculation of the solvent producer strain. The parameter of Flow rate (F) will be tested at a high rate (2 l d^{-1} or 83.33 ml h^{-1}), medium rate (1 l d^{-1} or 41.66 ml h^{-1}) and a lower rate (0.5 l d^{-1} or 20.83 ml h^{-1}) having switchgrass as substrate.

4.3 Materials and Methods

Strains and Culture Conditions

Solvent producer strains

C. saccharoperbutylacetonicum ATCC 27021 N1-4 was obtained from the American Type Culture Collection. The bacteria were grown anaerobically at 35 °C in basal medium containing (per liter): 30.6 g Na_2HPO_4, 30.0 g KH_2PO_4, 10.0 g NH_4Cl, 10.0 g $(NH_4)_2SO_4$, 1.8 g $MgCl_2$ 6-H_2O, 0.6 g $CaCl_2$, 2.0 g yeast extract, 10 ml standard vitamin mixture (Cotta and Russell 1982), 5.0 ml modified mineral mixture (Pfennings Metals plus 10 mg Na_2WO_4.2 H_2O and 1 mg Na_2SeO_3 per liter) and 1 ml resazurin. The pH was adjusted to 6.7 using NaOH. The buffer was Na_2CO_3 (4 mg ml^{-1}) in case of "T media" and K_2HPO_4 (13.6 mg ml^{-1}) in case of "TP media" (which was used in preliminary assays). The buffer was added after sterilization (121 °C, 104 kPa, 20 min) at room temperature. Broth for batch liquid cultures was anaerobically dispensed into serum bottles with butyl rubber stoppers, and autoclaved. The sugar source (sterilized glucose, 4 g l^{-1}) was aseptically added to the basal medium prior to inoculation.

Cellulolytic strain

The strain of *C. thermocellum* ATCC 27405 was obtained from the culture collection of Dr. Michael Flythe, University of Kentucky. *C. thermocellum* was grown anaerobically at 63°C in the basal medium described above. Media for batch liquid cultures was anaerobically dispensed into serum bottles with butyl rubber stoppers, and autoclaved. *C. thermocellum* was grown in the basal medium with amorphous cellulose (Whatman #1 filter paper; 4.5 mg ml^{-1}) at 63 °C.

Preliminary Batch Fermentation

Sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* on switchgrass (4.7 g, passed through a 2 mm screen) was carried out in serum bottles (100 ml size). To test which medium was optimum, basal medium with K_2HPO_4 (13.6 mg ml^{-}

^{1}) buffer (called TP) and media with Na_2CO_3 (4 mg ml^{-1}) buffer (called T) were tested. 45 ml of each of the basal media was added to the serum bottles and they were warmed in a water bath (63 °C) prior to inoculation with *C. thermocellum* (5 ml or 10% w/w). After 48 h, the bottles' temperature was decreased to 35 °C, and they were inoculated with *C. saccharoperbutylacetonicum* (5 ml, 10% w/w). Daily samples (1 ml) were clarified by centrifugation (14800 × g, 2 min), and they were frozen for later analyses. Acetate, ethanol, butanol, lactate and formate were quantified by HPLC (Dionex, Sunnyvale, CA). The anion exchange column (Aminex 87H; BioRad) was operated at 50 °C, flow rate 0.4 ml min^{-1}. Eluting compounds were detected by refractive index (Shodex/Showa) (data not shown, one replication. See Appendix Chapter four).

Also, the ability of the inoculum to survive on the substrate was determined by viable cell number by serial dilution into enriching media. The substrate (switchgrass, 0.05 g) was autoclaved and inoculated in two ways: pure culture of *C. saccharoperbutylacetonicum* (10% w/w) or sequential culture of *C. thermocellum* (10% w/w) followed by *C. saccharoperbutylacetonicum* (10% w/w). For the former, *C. saccharoperbutylacetonicum* cells were grown anaerobically at 35 °C in basal media containing glucose (4 g l^{-1}) and 0.5 ml was used to inoculate the set of Hungate tubes with 0.05 g of untreated switchgrass. These tubes were incubated at 35 °C. Viable cell number was determined on day 0, 1, 2, 3, 4, 5, 6, 7, 14, 21. Similarly, *C. thermocellum* cells were grown anaerobically at 65 °C in basal media and 0.5 ml was used to inoculate the set of Hungate tubes with 0.05 g of untreated switchgrass. These tubes were incubated at 65 °C during 24 hours. *C. saccharoperbutylacetonicum* cells were added to these tubes and they were incubated at 35 °C. Viable cell number was determined on day 0, 1, 2, 3, 4, 5, 6, 7, 14, 21. Experiments were performed in triplicate (Figure 4.3.1).

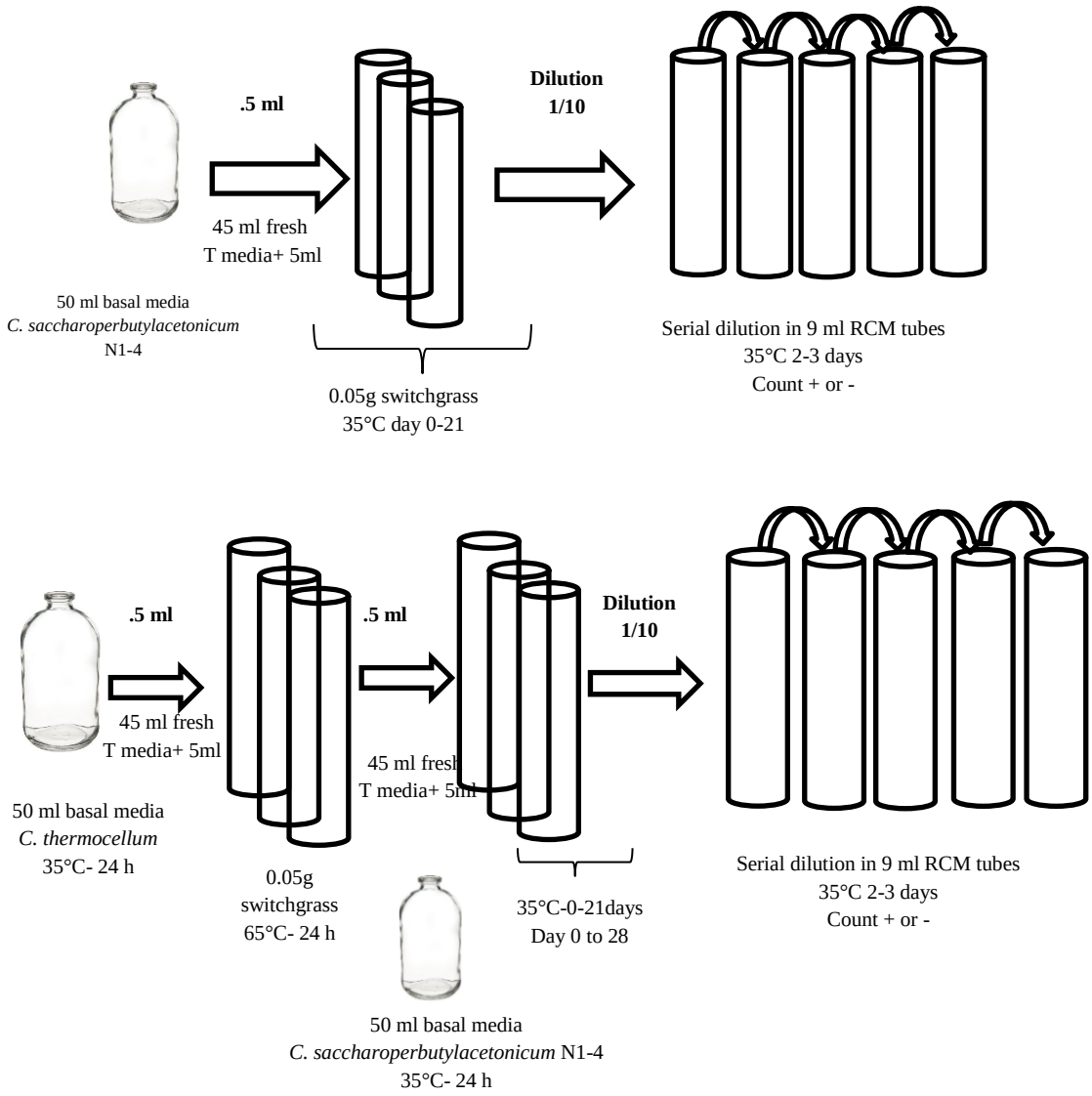


Figure 4.3.1. Experimental design to count the viable cell number of pure cultures and sequential cultures of *C. saccharoperbutylacetonicum* and *C. thermocellum* on switchgrass.

Continuous Fermentation

Continuous Flow Solid-Substrate cultivation was conducted in a water-jacketed Buchner funnel (See Figure 4.3.2). The vessels (500 ml working volume) containing 25 grams of switchgrass (6.15 % moisture content, passed through a 2 mm screen) were autoclaved (121 °C, 104 kPa, 20 min). The vessels were sealed with rubber stoppers and continually sparged with O_2 -free CO_2 to keep anaerobic conditions. The temperature of

the vessels was maintained using an immersion circulator system, which distributed water among the water vessel chambers. Once the temperature was 63 °C, 100 ml of sterilized basal media (pH 6.7) was added to the vessels to increase the water activity of the biomass. The fermentation was initiated by the inoculation of *C. thermocellum* ATCC 27405 (100 ml, 10^8 cells ml^{-1}). The inoculum was prepared in basal media containing amorphous cellulose (Whatman #1 filter paper; 4.5 mg ml^{-1}) with starting pH of 6.7. After 24 hours, the vessel temperature was dropped to 35 °C. Cells of *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) (100ml, 10^8 cells ml^{-1}) were added to the vessels. They were previously grown on basal media containing glucose (4 g l^{-1}).

For the continuous mode, 60 liters of basal media were prepared and autoclaved in 10 l batches. After the inoculation with *C. saccharoperbutylacetonicum*, the flow of media was initiated at different flow rates (high, medium and low flow rate; 83.33 ml h^{-1}, 41.66 ml h^{-1} and 20.833 ml h^{-1}) through PHARMED BPT NSF-51 tubing using a Watson Marlow Pump (205/CA, Watson Marlow, Inc, Wilmington, Wilmington, MA). For the high flow rate the pump was set to obtain 2 liters per day, 1 liter per day in case of medium flow rate; and, 0.5 liter per day for the low flow rate. Fermentation products were collected in an Erlenmeyer flask (previously autoclaved) and samples (4 ml) were daily taken. The Erlenmeyer flask (4 l) was replaced with another sterilized Erlenmeyer flask every time that the sample was taken. The samples were frozen for later analysis. The pH and acetate (Enzymatic method, Roche, Nutley, NJ) were also measured daily. When the acetate formation decreased, the temperature was increased to 65 °C for 24 h to permit cellulosome production by *C. thermocellum*. Lastly, the temperature of the vessels was returned to 35 °C. Re-inoculation with *C. saccharoperbutylacetonicum* (100 ml) was carried out and daily samples were taken as well. The experiments were performed in duplicate. The switchgrass was dried at 80 °C until the weight was constant. Once desiccated, the samples of switchgrass were analyzed by Dairy One, Forage Testing Laboratory, Ithaca, New York (Ration Balancer Plus).

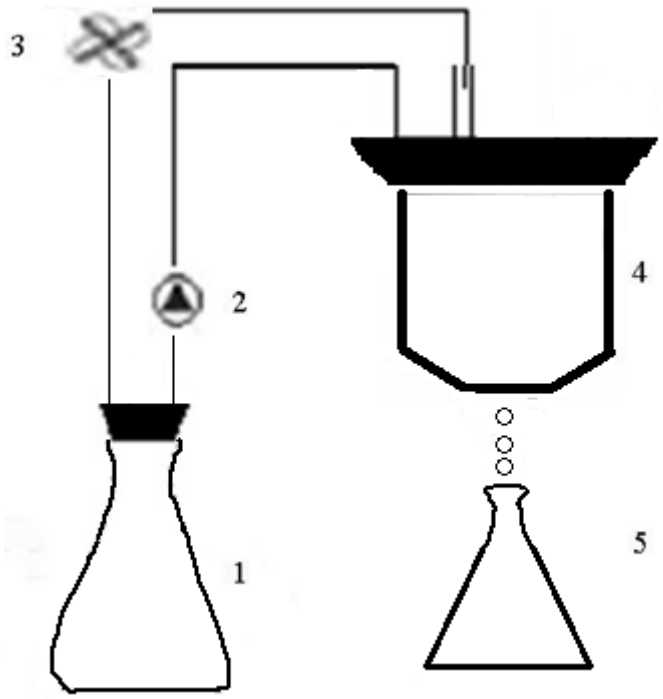


Figure 4.3.2 . Scheme of the Solid-substrate cultivation system with continuous product removal. 1- Medium, 2- Pump, 3- Gas administration, 4- Fermentation vessel, 5-Effluent.

Product quantification

The cultures were sampled as indicated. Samples (1 ml) were clarified by centrifugation (14800 × g, 2 min), and frozen for later analyses. The pH and acetate were measured daily. Acetate was quantified by an enzymatic method assay (Roche, Nutley, NJ). Acetate, ethanol, butanol, lactate and formate were quantified by HPLC (Dionex, Sunnyvale, CA). The anion exchange column (Aminex 87H; BioRad) was operated at 50 °C, flow rate 0.4 ml min^{-1}. Eluting compounds were detected by refractive index (Shodex/Showa).

Mathematical Analyses

The experiments were performed in duplicate. The data shown are the means of two replicates unless otherwise indicated. Differences were determined via SAS vs.9.3 with t-test and Tukey's test.

4.4 Results

Preliminary Batch Fermentation

C. thermocellum ATCC 27405 was grown in a sequential culture with *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) in batch cultures containing untreated switchgrass (10% w/w) as substrate in basal media with an initial pH of 6.7. Basal media with Na_2CO_3 (4 mg ml^{-1}) as buffer was called T media, and basal media with phosphate buffer was called TP media. Typical product formation was shown (See numerical values in Appendix Chapter four) for both media type. Acetate and ethanol were the major final products for *C. thermocellum* cultures, but lactate and formate were also produced. *C. saccharoperbutylacetonicum* produced mostly acetate and butyrate in the first days of fermentation. Solvent production was observed after 24 hour of culture, reaching the maximum on day 6 and 7. The sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* grew better in basal media containing carbonate buffer (T media). Even though switchgrass in serum bottles containing phosphate buffer (TP media) was fermentable by both strains, less total product was formed (data not shown, one replication. See Appendix Chapter four).

Survival experiments were carried out to study the ability of *C. saccharoperbutylacetonicum* to survive in the substrate. The viable cell number *C. saccharoperbutylacetonicum* on switchgrass was determined in both pure and sequential cultures (Figure 4.4.1). The viable number in pure cultures decreased from 10^8 cells to 5.5×10^6 cells during the first 24 h, and 99% of the inoculum was dead by day 5. When the switchgrass was sequentially cultured, the initial viable number of *C. saccharoperbutylacetonicum* was maintained for 7 days.

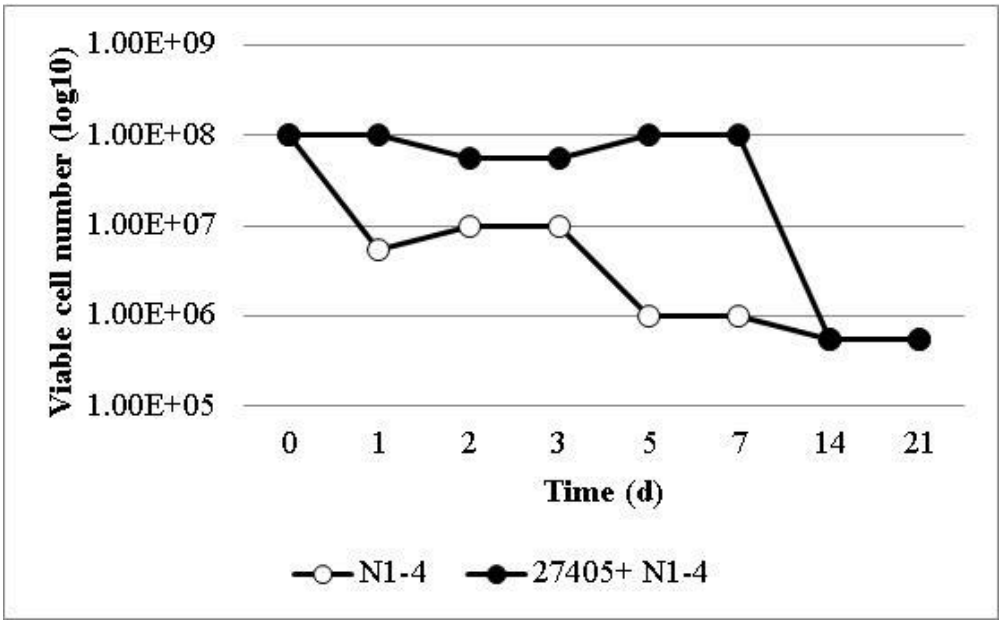


Figure 4.4.1. Viable cell number of *C. saccharoperbutylacetonicum* grown on switchgrass in sequential culture with *C. thermocellum* shown by the solid circles and pure culture of *C. saccharoperbutylacetonicum* shown by the empty circles. See numerical values and standard deviation in Appendix Chapter four.

Continuous Fermentation

To investigate the effect of flow rate on the sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum*, a continuous solid-substrate cultivation system was carried out in basal media with switchgrass as the substrate. Figure 4.4.2 presents temperature and pH during the course of the experiment (see Appendix for daily spreadsheet). To examine the effect of flow rate, a high flow rate (2 ± 0.1 liter per day), medium flow rate (1± 0.1 liter per day) and a low flow rate (0.5±0.1 liter per day) were established. After 96 h of fermentation with *C. saccharoperbutylacetonicum*, there was little metabolism determined by the daily enzymatic acetate assays (data not shown). The temperature was increased to 65 ºC during 24 hours (the flushing was stopped) to activate the cellulosome system and reinoculation with *C. saccharoperbutylacetonicum* was carried out. A comparison of total products obtained during 9 days of fermentation is shown in Figure 4.4.3. There was no statistical difference in the effect of flow rate within the flow range tested.

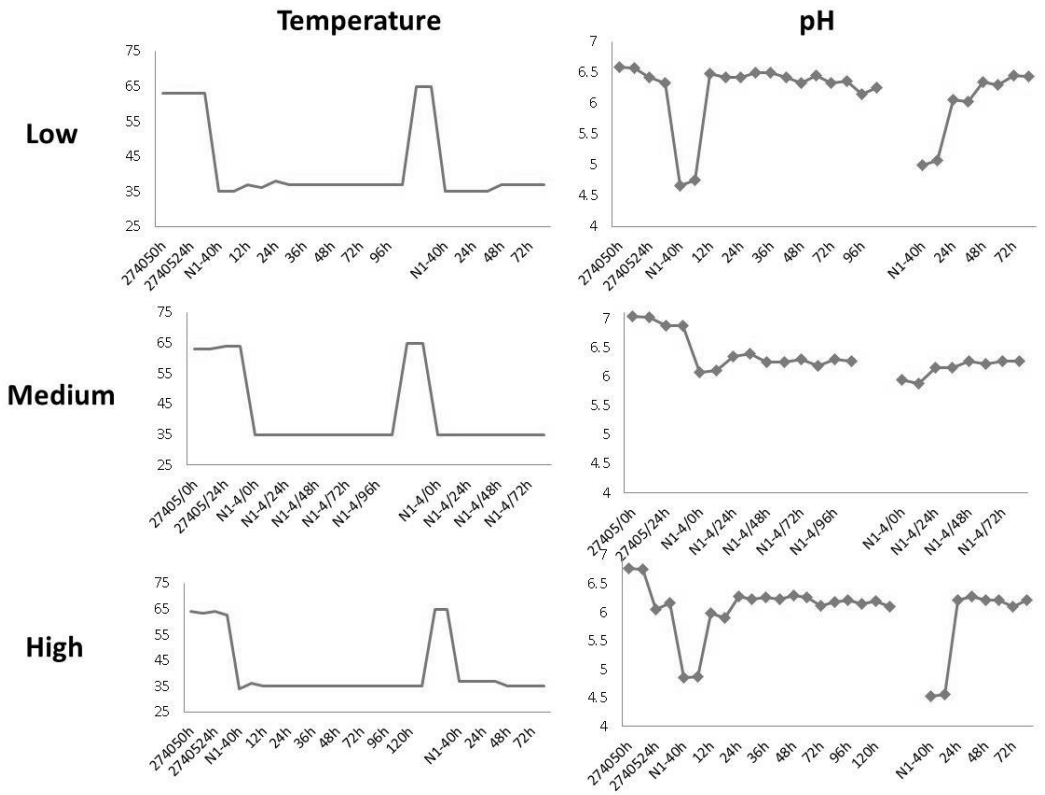


Figure 4.4.2. Controlled parameters during the experiment (temperature and pH at Low, Medium and High flow rates).

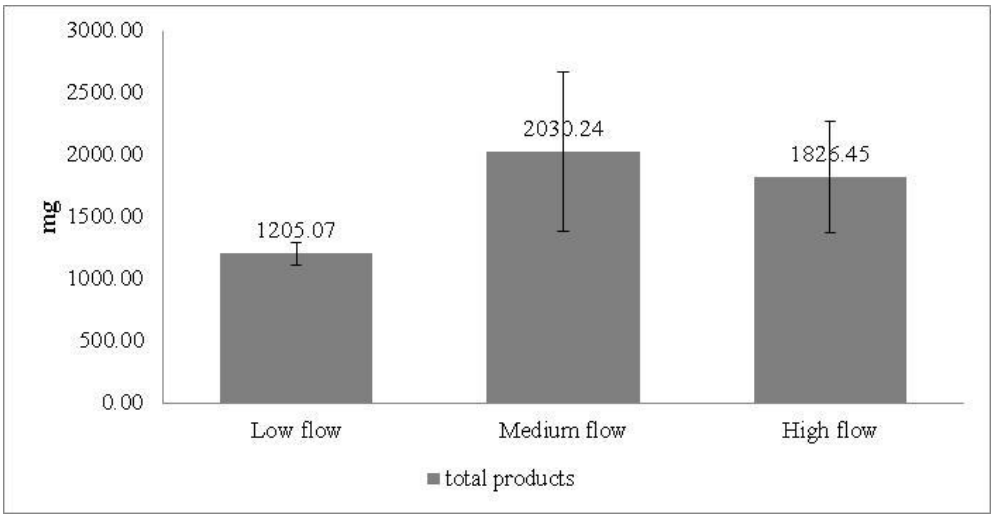


Figure 4.4.3. Total quantity of products collected in a low, medium and high flow rate mode. Switchgrass (25 g, 6.15 % moisture content) was inoculated with a sequential culture of *C.thermocellum* and *C. saccharoperbutylacetonicum* and basal medium was continuously flowed through the system to collect the products.

These data (Figure 4.4.3) indicate that 5.14% of the switchgrass (out of 23.46 g initial substrate) was converted to products on low flow rate system during 9 days of fermentation. Around 8.65 % of conversion was observed at a medium flow rate. Finally, 7.8 % was converted to products, under a high flow rate system. In Figure 4.4.4 is shown the total quantity of acids (acetic, lactic, butyric and formic acid) and solvents (ethanol and butanol) collected under the different flow rates.

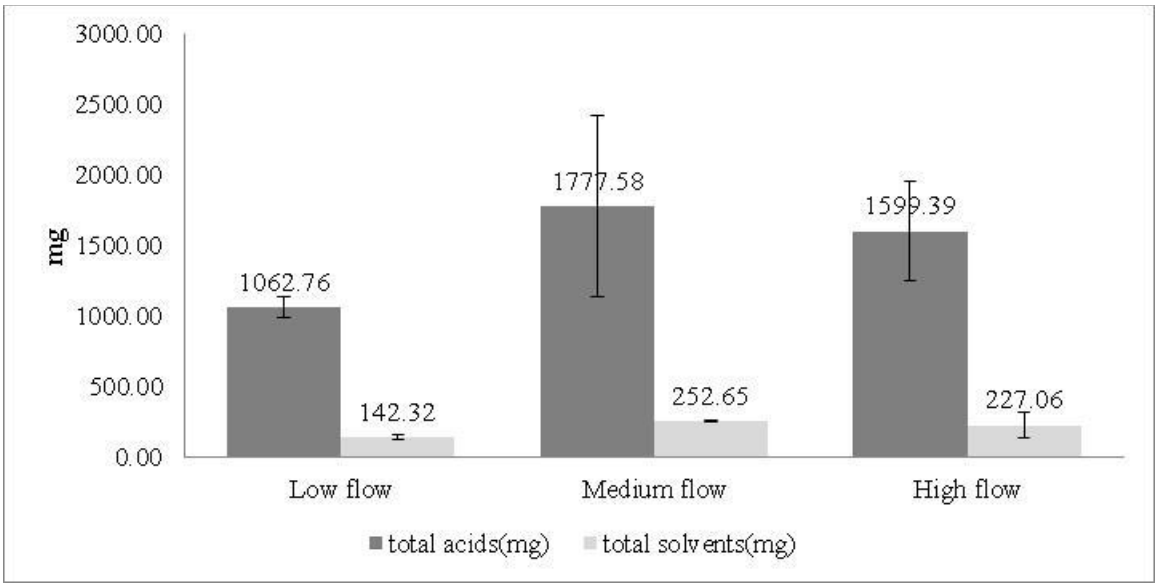


Figure 4.4.4. Total quantity of acids (acetic, lactic, butyric and formic acid) and solvents (ethanol and butanol) collected in a low, medium and high flow rate mode. Switchgrass (25 g, 6.15 % moisture content) was inoculated with a sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* and basal medium was continuously flowed through the system to remove the products.

Acetate and butyrate were the major products (Figure 4.4.5). Ethanol was produced during the initial stage by *C. thermocellum.* On the other hand, butanol production was due to fermentation with *C. saccharoperbutylacetonicum*. Higher lactate and formate yields were observed under medium flow rate. See numerical values and standard error in Appendix Chapter four.

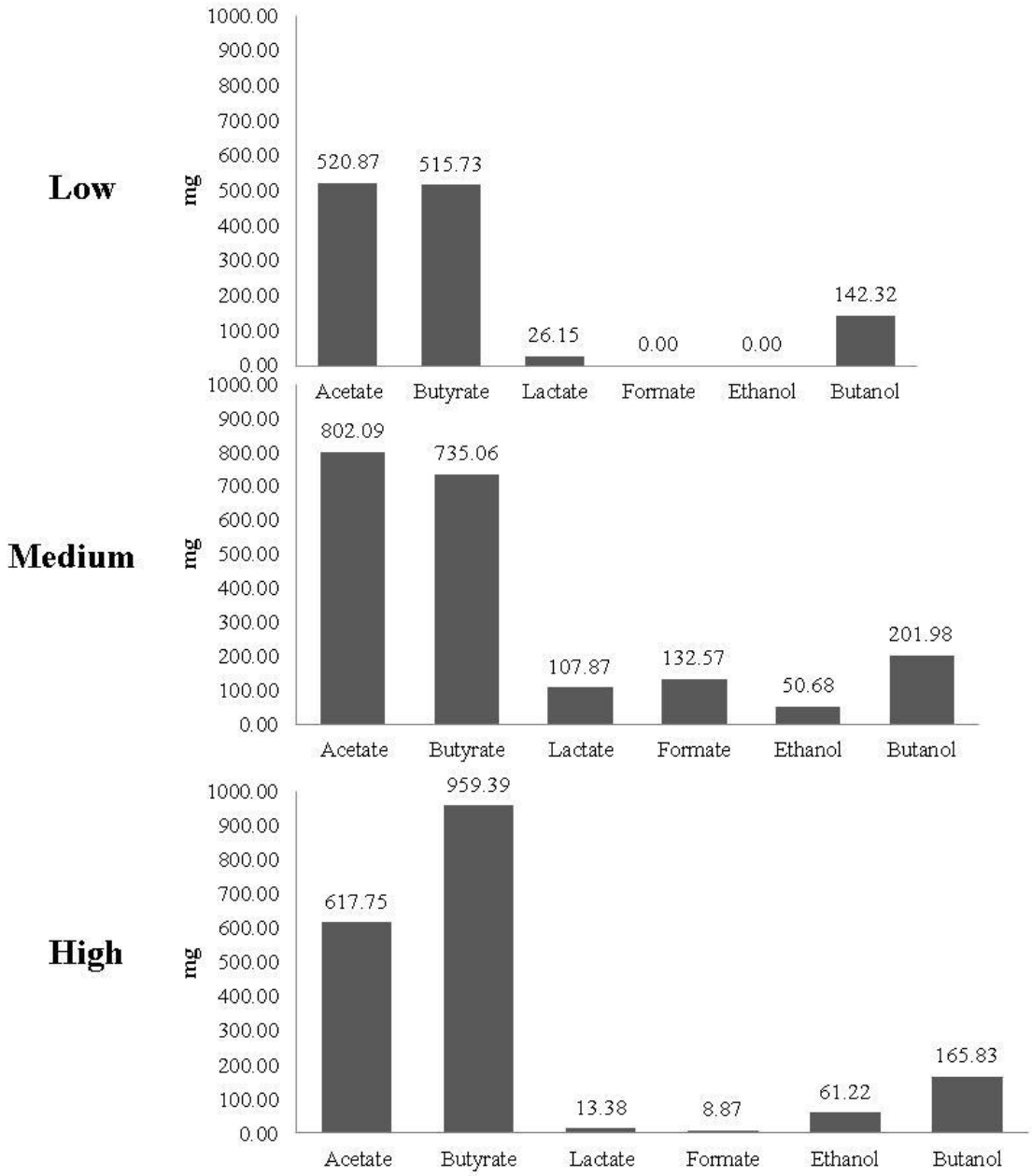


Figure 4.4.5. Product formation by *C. saccharoperbutylacetonicum* at different flow rates: low, medium and high. Switchgrass (25 g, 6.15 % moisture content) was inoculated with a sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* and basal medium was continuously flowed through the system to collect the products. See numerical values and standard error in Appendix Chapter four.

The chemical characterization of switchgrass indicates that the switchgrass used as substrate (Control) had 6.15% moisture content, meaning 23.46 grams out of 25 grams corresponded to dry matter (Table 4.4.1). After fermentation, switchgrass was dried 80 °C until the weight was constant (See weights of switchgrass samples send to Dairy One in Appendix Chapter four). These samples were later analyzed by Forage Testing Laboratory, Dairy One (Table 4.4.1). According to the analysis done on switchgrass after the fermentation at three different flow rates, lignin content remained the same as the control (~11%) (Table 4.4.1). Cellulose content can be determined by subtracting the lignin content from the values of acid detergent fiber (Table 4.4.2). Thus, cellulose content was approximately the same in control switchgrass or switchgrass under different treatments. Moreover, neutral detergent fiber includes hemicellulose, cellulose and lignin; so, hemicellulose was also calculated. Control switchgrass had a 31.5 % of hemicellulose, while hemicellulose content in switchgrass under low, medium and high flow rate was 28.65, 29.6 and 27.6 %, respectively. Non Fiber Carbohydrates decreased by 21.2 % at a low flow rate, 31.5 % at medium flow rate and 20.3 % at a high flow rate. Moreover, crude protein increased by 43.42 % after switchgrass was fermented.

Table 4.4.1. Chemical characterization of switchgrass by Forage Testing Laboratory, Dairy One, INC (Ithaca, New York). NFC: Non Fiber Carbohydrates. ADF: Acid Detergent Fiber. NDF: Neutral Detergent Fiber.

Flow	%moisture	%dry matter	%Crude Protein	%Lignin	% NDF	%ADF	%NFC
High	6.40± 0.3	93.6±0.3	5.7±0.3	12.15±0.5	86.25±0.4	58.65±1.5	8.85±0.1
Medium	5.15± 0.1	94.85±0.1	5.4±0.1	11.10±0.4	87.45±0.2	57.95±0.4	7.60±0.1
Low	6.60± 0.1	93.40±0.1	5.25±0.4	10.75±0.2	85.8±2.4	57.15±1.6	8.75±1.5
Control	6.15±0.2	93.7±0.3	3.8±0	9.45±0.9	87.55±1.9	56.1±1.6	10.6±0.8

Table 4.4.2. Lignocellulose composition of switchgrass (23.425 grams initial) under the different treatments.

Flow rate	%Lignin	%Cellulose	%Hemicellulose
Control	9.45±0.92	46.6	31.5
Low	10.75±0.2	46.4	28.65
Medium	11.1±0.4	46.8	29.6
High	12.15±0.5	46.5	27.6

5. Discussion

Clostridia can ferment a variety of carbon sources, such as glucose, galactose, xylose, mannose, cellobiose and cellodextrins (Gottschalk, 1986). Switchgrass is a lignocellulosic substrate that contains 29-45 % of cellulose, 30-37 % hemicellulose and 12-19% of lignin (Sun and Cheng, 2002). This biomass is a potential carbon source for acids and solvents production by the cellulolytic *C. thermocellum* and the solventogenic *C. saccharoperbutylacetonicum* (Chinn et al., 2006; Al-Shorgani Najeed et al., 2011). Two preliminary experiments were carried out to analyze not only the suitability of switchgrass as substrate but also the sequential culture of these two microorganisms. Batch fermentation having switchgrass as a carbon source was carried out using two different media: T media (carbonate buffer) and TP media (phosphate buffer). The sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* had more productivity on T media than TP media. It was shown in previous experiments (data shown Chapter 2) that *C. saccharoperbutylacetonicum* grew well on TP media having glucose as carbon source but it did not grow as well when switchgrass was the only carbon source. Also, *C. thermocellum* presented difficulties in growing on the phosphate buffer based media as well. It can explain why *C. saccharoperbutylacetonicum* grew better on T media, since *C. thermocellum* did as well. Sequential culture of *C. thermocellum* with the solventogenic bacterium *C. acetobutylicum* has been already studied and the total fermentation products increased about 1.7-2.6 folds (Yu et.al., 1985). Phosphate buffer in the basal media has a higher buffer concentration (13.6 g l^{-1}) compared with carbonate buffer (4 g l^{-1}). Dhamargadda et al. (2010) reported that *C. thermocellum* had metabolic inhibition when osmotic condition was affected by end products. Phosphate buffer concentrations are much higher than carbonate buffer, and this amount of salt (potassium phosphate) may be related with osmotic inhibition. Thus, basal medium with carbonate buffer was tested in the continuous solid-substrate fermentation system. These preliminary experiments showed an increment in the pH (above 7.5); and the deficiency of growth of the microorganisms. The raise in pH could be the combination of the carbonate salt and the carbon dioxide continuously supplied to the system. Consequently, basal medium without buffer was flushed through the system,

and normal behavior of pH values were observed together with metabolic activity of the microorganisms (data not shown).

After the establishment of the basal medium, solid substrate cultivation with continuous removal of products was carried out. Flow rate was tested at three different values (0.5 l d^{-1}, 1.0 l d^{-1} and 2.0 l d^{-1}), but there were fluctuations (± 0.1 l d^{-1}) around these values. Considering total acids, total solvents and total products, the major conclusion of the continuous flow on SSC experiment was that there is no statistical difference in the effect of flow rate within the flow range tested. It is known that the retention time decreases with increasing flow rate, thus at high flow rate the residence time is not enough for the bacteria to convert the substrate and also a wash out in the system is probably to occur (Shuler and Kargi, 2002). At a low flow rate, the conversion was very poor, it can be explained by assuming that the feed media was not reaching the optimum concentrations of nutrients that the bacteria needed to grow or resulted in a high concentration of toxic compounds that may lead to growth inhibition. Solid-substrate cultivation is the growth in conditions with low amounts of water, but it has to be enough to support cell growth. Bacteria and fungi require moisture contents between 30 and 85% (Krishna, 2005). Moisture contents may be responsible of the low conversions rates in all the flow rates evaluated.

The main acid products were acetic acid and butyric acid as it has been described in ABE fermentation (Gottschalk, 1986). Solvent production was poor; butanol is produced in stationary phase since it is non-associated with growth cell (Tashiro et al., 2004). So, the cells may be not growing at optimum rates. It is important to observe that pH values were always in a range between 6 and 7, which suggest that acid accumulation was not enough to have high solvent production. However, the samples were taken every 24 hours; so if there was a change in pH in this time lapse, it has not been recorded.

The chemical characterization of switchgrass indicates that the switchgrass used as substrate (control) had 6.15 % moisture content, meaning 23.46 grams out of 25 grams corresponded to dry matter. The lignocellulose composition (based on dry basis) of the initial switchgrass was 9.45 % lignin, 46.6 % cellulose and 31.5 % hemicellulose (Table 4.4.2). Based on %ADF content, it seems that there was not utilization of cellulose. *C. thermocellum* cultivation (batch mode) during 24 hours may not be enough to produce

enough cellulolytic enzymes. However, hemicellulose content decreased by 9.05, 6.03 and 12.38 % in the low, medium and high flow rate systems, respectively. It is important to mention that the switchgrass was untreated or had not any previous pretreatment. Also, the switchgrass that was sent to Dairy One for Forage analysis was previously dried at 80 ºC (See the values of dry matter of switchgrass in Appendix Chapter four).

Based on the switchgrass composition on a dry matter basis (46.6% cellulose, 31.5% hemicellulose, and 11% lignin), theoretical yield of monosaccharides, glucose and xylose from cellulose and hemicellulose (assuming just xylose), respectively; and assuming 50% of hydrolysis by the cellulolytic strain is calculated as follows:

$$23.46\text{gswitchgrass} * \frac{0.466\text{ g cellulose}}{\text{gswitchgrass}} * \frac{1.11\text{ g glucose}}{\text{g cellulose}} * .5 = 6.06\text{ g glucose}$$

$$23.46\text{gswitchgrass} * \frac{0.315\text{ g hemicellulose}}{\text{gswitchgrass}} * \frac{1.11\text{ g xylose}}{\text{g hemicellulose}} * .5 = 4.1\text{ g xylose}$$

The approximate stoichiometric equations of an ABE fermentation process from corn starch from a standard strain (Ni et al., 2009):

$$12C_6H_{12}O_6 \rightarrow 6C_4H_{10}O + 4C_3H_6O + 2C_2H_6O + 18H_2 + 28CO_2 + 2H_2O$$

Based on this equation, the theoretical yield of butanol on the basis of the original lignocellulose (based just on cellulose and glucose) would be:

$$6.06\text{g glucose} * \frac{0.2\text{ g butanol}}{\text{g glucose}} = 1.21\frac{\text{g}}{\text{l}}\text{butanol}$$

Lastly, considering the yields obtained from the medium flow rate (high numerical values), 20.89 g (dry matter value considering that 22.03 g of switchgrass were actually given to Dairy One Analysis) were converted to 2.03 g of total products. According to mass balances, 2.56 g corresponds to dry matter disappearance. The mass lost (0.53 g) could be attributed to CO_2 production and cell biomass which can be proved by the increase in protein content of the switchgrass (43.42%). According to survival experiments, the number of cells in the sequential culture remained equal order than the initial inoculum. But, it can be assumed that there was multiplication of cells in the continuous fermentation since it would not be an increase in 43.42% protein with just one

order of magnitude increment in viable cell number. Finally, butanol yield (0.201g) was just 16.6% of the theoretical yield.

The main byproduct of lignocellulose is lignin which can be utilized for chemicals or energy (heat and electricity) production (Yoshida and Matsumura, 2001). Also, the formation of cell biomass with the concomitant increment in nutritional value in the substrate provides the potential as a valuable co-product in feedstock industry. DDGS (Dried Distillers grains with Solubles) is a valuable co-product of the ethanol production process (Sphies et al., 2002). It has a high nutrient feed value for livestock industry. Currently over 80% of DDGS is used to feed ruminants, poultry, and pork. So, high solid substrate cultivation could also provide with valuable coproducts making the whole process more cost effective.

As summary, direct microbial conversion in a consolidated bioprocessing system with continuous removal of products is promising to obtain valuable products from lignocellulose biomass, however, it needs to be optimized in many aspects.

Chapter Five

Future Work

"If the facts don't fit the theory, change the facts" A. Einstein.

If we believed that renewable sources such as cellulosic biomass has the potential not only to supply the energy that we need for replacement of derived petroleum fuel but also to lessening current environmental concerns, then we will have to work hard in changing the facts involved with substrates, low productivity, strict sterility requirements, efficient bioreactors, downstream separation issues, among others.

According to the biomass, one of the great concerns is to have access to the around 80% of sugars enclosed together with lignin, pectin and other compounds in the vegetable macrofibril of switchgrass. So, it may have to be considered in using a different stage of the plant, when the lignin content is lower; even though the high yields of switchgrass will be not reached, having higher product formation could repair this issue. Also, a mixture of lignocellulosic material with other lignocellulosic waste could improve the yields. For instance, *C. thermocellum* uses pectin very efficiently, then combination of food wastes with lignocellulosic biomass can result in a cheap efficient feedstock.

Microorganisms work very efficiently in communities degrading the lignocellulosic material around the world. So, finding the right combination of microorganisms is a challenge that after solved could result in high rate of conversion and different valuable end products.

Fermentation parameters need to be tested to optimize the process. Flow rate was one of them, but starting pH is another parameter to be evaluated. Also, shift conditions in the metabolic pathways can be induced by different compounds such as the addition of butyric acid, or increasing the hydrostatic pressure which it has been already search with *C. thermocellum* (increase in ethanol production after addition of H_2).

Scientists are looking for novel ways to produce fuels using engineered microbes and new feedstocks. There are already cellulosic ethanol pilot plants around the world, so as necessity is the mother of inventions, bringing new technologies will make possible to get cellulosic butanol and ethanol plants providing renewable and cleaner biofuels.

Appendices

Chapter two: **ABE Fermentation in Batch System**

Table related to Figure 2.4.1. Total Product formation during 5 days by *C. beijerinckii* ATCC 51743, *C. saccharoperbutylacetonicum* ATCC 27021 N1-4, *C. acetobutylicum* 824 NC and *C. acetobutylicum* ATCC 824 on RCM and switchgrass.

Strains	Total products on glucose	Total products on switchgrass
C. beijerinckii ATCC 51743	309.55±12.8	93.045±13.43
C. saccharoperbutylacetonicum N1-4	434.7±36.13	102.24±11.33
C. acetobutylicum 824 NC	302.05±20.72	121.51±15.61
C. acetobutylicum ATCC 824	311.5±23.26	113.77±26.89

Table 2.4.1. Product formation on day 0 corresponds to the inoculum of *C. beijerinckii* ATCC 51743, *C. acetobutylicum* ATCC 824, *C. acetobutylicum* 824 NC and *C. saccharoperbutylacetonicum* ATCC 27021 N1-4 on RCM.

Day	glucose	lactate	formate	acetate	butyrate	butanol	acetone
C. beijerinckii ATCC 51743				(mM)			
0	124.5±12.0	0.115±0	-	33±2.8	-	-	-
C. acetobutylicum ATCC 824							
0	123.5±9.1	0.235±0.15	-	34±1.4	-	-	-
C. acetobutylicum 824 NC							
0	119.5±6.3	0.09	-	34±2.8	-	9±0	-
C. saccharoperbutylacetonicum ATCC 27021							
0	120.5±3.5	0.18±0.07	1.1±0	34.5±0.7	-	-	-

Table related to Figure 2.4.3. Cumulative pressure a) psi and b) kPa by *C. beijerinckii* on switchgrass (2 mm, 5 mm and 15 mm).

a) Time(h)	Cumulative pressure (psi)		
	2mm	5mm	15mm
0	-	-	-
5	1.19±0.4	1.17±0.63	0.68±0.49
10.5	1.64±0.2	1.55±0.59	0.74±0.08
15.5	1.8±0.3	1.77±0.78	1.25±0.36
20.5	1.86±0.35	1.83±0.9	1.36±0.51
21.5	1.9±0.38	1.84±0.96	1.363±0.54

b) Time (h)	Cumulative pressure (kPa)		
	2mm	5mm	15mm
0	0	0	0
5	8.2±2.7	6.5±4.3	4.67±3.38
10.5	11.4±1.38	10.69±4.07	5.08±0.55
15.5	12.8±2.07	12±5.38	8.64±2.48
20.5	12.9±2.4	12.2±6.2	9.4±3.5
21.5	13.2±2.6	12.5±6.6	9.4±3.7

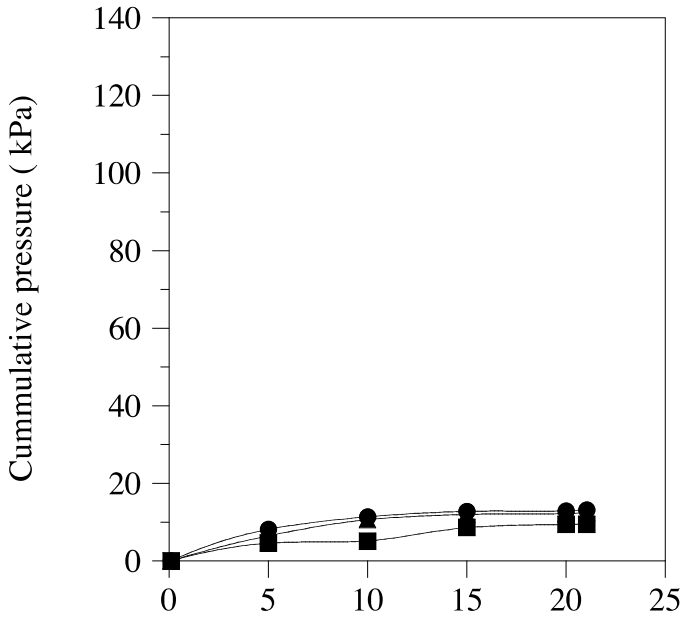


Figure 2.4.3. Gas production by *C. beijerinckii* on switchgrass. 2 mm (black circles), 5 mm (orange triangles) and 15 mm (blue squares) are shown.

Table related to **Figure 2.4.4**. Gas production by sequential culture on switchgrass. *C. beijerinckii* was added after ~ 48 h. a) cumulative pressure in psi and b) in kpa.

a)	Cumulative pressure (psi)		
Time(h)	2mm	5mm	15mm
C. thermocellum			
0.00	-	-	-
10.50	-1.25±1.8	0.21±1.09	0.06±0.87
20.50	0.77±3.15	1.96±2.9	0.72±1.95
30.50	4.77±5.2	5.5±5.2	1.93±3.6
40.50	8.4±6.3	9.02±6.0	2.63±4.7
46.50	10.3±5.9	10.44±6.6	2.55±5.29
C. beijerinckii			
47.00	11.18±5.8	11.75±5.2	3.43±4.3
50.50	12.36±5.9	12.72±4.25	4.46±4.7
60.50	19.26±8.0	17.38±7.7	4.91±4.0
65.50	20.15±7.1	19.2±5.79	5.37±4.75
69.50	20.4±7.0	19.7±5.4	6.01±5.34

b)	Cumulative pressure (kpa)		
Time(h)	2mm	5mm	15mm
C. thermocellum			
0.00	-	-	-
10.50	-8.6±12.4	1.4±7.5	0.4±6.0
20.50	5.3±21.7	13.5±20.4	4.96±13.4
30.50	32.9±35.9	37.9±35.9	13.33±25.1
40.50	58.1±43.6	62.24±41.6	18.2±32.5
46.50	71.1±40.7	72.03±45.8	17.6±36.4
C. beijerinckii			
47.00	77.1±40.1	81.06±35.9	23.7±29.5
50.50	85.22±41.2	87.7±29.3	30.8±32.7
60.50	132.9±55.4	119.9±53.6	33.9±27.9
65.50	138.9±49.1	132.2±39.9	37.0±32.7
69.50	140.7±48.3	135.8±37.6	41.5±36.8

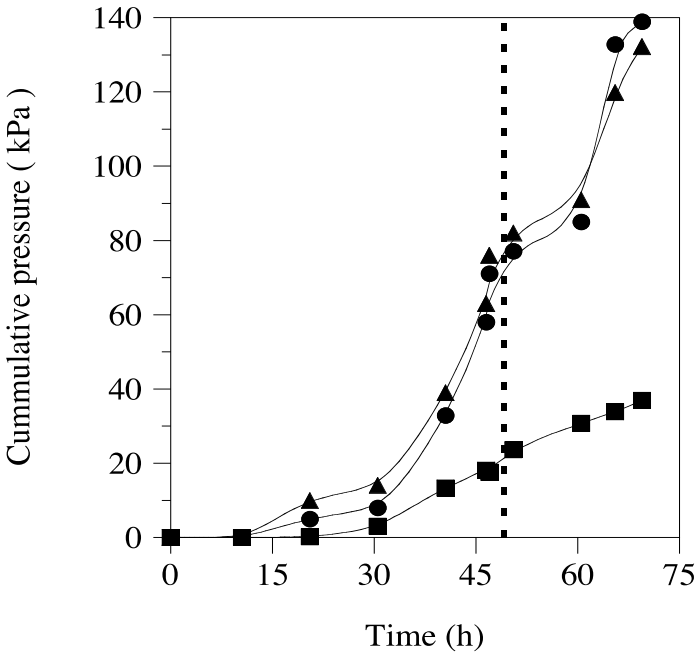


Figure 2.4.4. Gas production by sequential culture on switchgrass. 2 mm (black circles), 5 mm (orange triangles) and 15 mm (blue squares) are shown.

The following formula was used to convert cumulative pressure into moles of gas:

$$n = p\frac{V}{RT}$$

; in which n is moles of gas, p is pressure in kilopascal, V is head space volume (0.2 cm^3), T is temperature in Kelvin and R is the gas constant.

Table related to Figure 3.4.8. Product formation by *C. thermocellum* 27405 on untreated switchgrass.

Day	mM cellobiose	mM glucose	mM xylose	mM lactate	mM formate	mM acetate	mM EtOH
0	-	0.27±0.09	-	0.14±0.01	0.275±0.01	3.1±0.14	5.5±2.1
1	-	-	1.2±0	2.4±0.2	2.2±0	12±1.4	6±4.2
2	-	-	1.25±1.4	2.9±0.2	6.2±0	23±5.6	28±1.4
3	-	-	1.945±1.6	4.65±0.6	6.4±0	24±7.0	29±4.2
4	-	-	2.425±2.6	5.6±1.5	4.35±0.7	26±4.9	33±7.0
5	-	0.34±0	3.3±2.6	5.2±1.5	3.7±0.7	26.5±4.9	34±7.0
6	-	0.65±0	3.7±3.2	6.6±0.8	4.9±0	30.5±7.7	34±7.0
10	-	0.405±0.36	3.85±2.2	6.1±0.9	3.2±0.5	33.5±4.9	36±7.0
12	-	0.455±0.5	3.35±2.8	6.05±1.7	3.8±0.9	33±2.8	34.5±9.1

Table related to Figure 3.4.9. Product formation by *C. thermocellum* 27405 on Fenton-treated switchgrass.

Day	mM cellobiose	mM glucose	mM xylose	mM lactate	mM formate	mM acetate	mM EtOH
0	-	-	0.74±0.2	-	0.76±0.5	5.15±0.5	4.5±0.7
1	0.86±0.03	1.15±0.07	2.5±0.2	-	0.555±0.2	10.5±0.7	4±0
2	0.415±0.04	0.185±0.02	1.3±0	-	1.1±0	15±0	5±0
3	0.52±0.08	0.33±0.02	1.95±0.7	-	0.43±0.03	18±1.4	4±0
4	0.58±0.05	0.42±0.09	1.55±0.07	-	0.455±0.02	19.5±2.1	3.5±0.7
5	0.98±0.02	0.865±0.12	2.4±0.1	-	0.565±0.02	28±1.4	5±0
6	1.1±0	0.93±0.09	2.9±0.2	-	0.67±0.02	31±0	4.5±0.7
10	1.2±0.1	0.96±0.05	3.3±0.2	-	0.96±0.5	34±1.4	5±0
12	0.79±0	0.705±0.1	1.85±0.07	-	0.95±0.07	24±1.4	4±0

Table related to Figure 3.4.7. pH variation along the growth of *C. thermocellum* on untreated and Fenton-treated switchgrass.

	UNTREATED			
DAY	1	1*	AVG	SD
0	6.67	6.66	6.665	0.01
1	6.677	6.65	6.6635	0.02
2	6.5	6.4	6.45	0.07
3	5.99	6.25	6.12	0.18
4	5.9	5.85	5.875	0.04
5	5.9	5.82	5.86	0.06
7	5.64	5.58	5.61	0.04
10	5.57	5.64	5.605	0.05
12	5.38	5.44	5.41	0.04

	TREATED			
DAY	2	2*	AVG	SD
0	6.62	6.65	6.635	0.02
1	6.64	6.65	6.645	0.01
2	6.64	6.55	6.595	0.06
3	6.63	6.62	6.625	0.01
4	6.5	6.47	6.485	0.02
5	6.43	6.54	6.485	0.08
7	6.34	6.26	6.3	0.06
10	6.2	6.2	6.2	0.00
12	6.01	6.06	6.035	0.04

Table related to Figure 3.4.1 . Comparison of gas produced by sequential culture of *C.thermocellum* and *C. beijerinckii* on Fenton-treated and untreated switchgrass.

Untreated	Cumulative Gas pressure (kPa)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0.00	0.00	0.00	0.00	0.00
10h	19.03	19.86	29.37	22.75	5.75
20h	22.55	29.58	39.16	30.43	8.34
30h	33.37	46.13	41.16	40.22	6.43
40h	42.68	55.50	64.19	54.12	10.82
46h	49.92	54.47	77.77	60.72	14.94
50h	47.16	54.40	77.77	59.78	16.00
60h	75.77	86.80	78.05	80.21	5.82
65h	75.22	87.56	78.53	80.44	6.39
70h	74.74	86.80	79.01	80.19	6.12

Fenton-Treated	Cumulative Gas pressure (kPa)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0	0	0	0	0
10h	18.55	22.89	25.86	22.43	3.68
20h	19.79	24.06	24.34	22.73	2.55
30h	19.51	26.34	0.48	15.44	13.40
40h	18.82	26.41	30.82	25.35	6.07
46h	20.55	29.10	44.88	31.51	12.35
50h	18.82	29.85	45.64	31.44	13.48
60h	112.66	60.67	55.16	76.16	31.73
65h	115.42	108.59	103.42	109.14	6.02
70h	115.63	108.66	103.42	109.24	6.12

Table related to Figure 3.4.1 in psi . Comparison of gas produced by sequential culture of *C.thermocellum* and *C. beijerinckii* on Fenton-treated and untreated switchgrass. *Inoculation with *C.beijerinckii*.

Untreated	Ccumulative Gas pressure (psi)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0.00	0.00	0.00	0	0
10h	2.76	2.88	4.26	3.30	0.8335466
20h	3.27	4.29	5.68	4.41	1.2097245
30h	4.84	6.69	5.97	5.83	0.9325413
40h	6.19	8.05	9.31	7.85	1.5695859
46h*	7.24	7.90	11.28	8.81	2.1672409
50h	6.84	7.89	11.28	8.67	2.3204956
60h	10.99	12.59	11.32	11.63	0.8447682
65h	10.91	12.70	11.39	11.67	0.9265168
70h	10.84	12.59	11.46	11.63	0.8872993
Fenton-Treated	Cumulative Gas pressure (psi)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0.00	0.00	0.00	0.00	0
10h	2.69	3.32	3.75	3.25	0.5331354
20h	2.87	3.49	3.53	3.30	0.370045
30h	2.83	3.82	0.07	2.24	1.9433734
40h	2.73	3.83	4.47	3.68	0.8800758
46h*	2.98	4.22	6.51	4.57	1.7908378
50h	2.73	4.33	6.62	4.56	1.9551726
60h	16.34	8.80	8.00	11.05	4.6015794
65h	16.74	15.75	15.00	15.83	0.8727543
70h	16.77	15.76	15.00	15.84	0.8879377

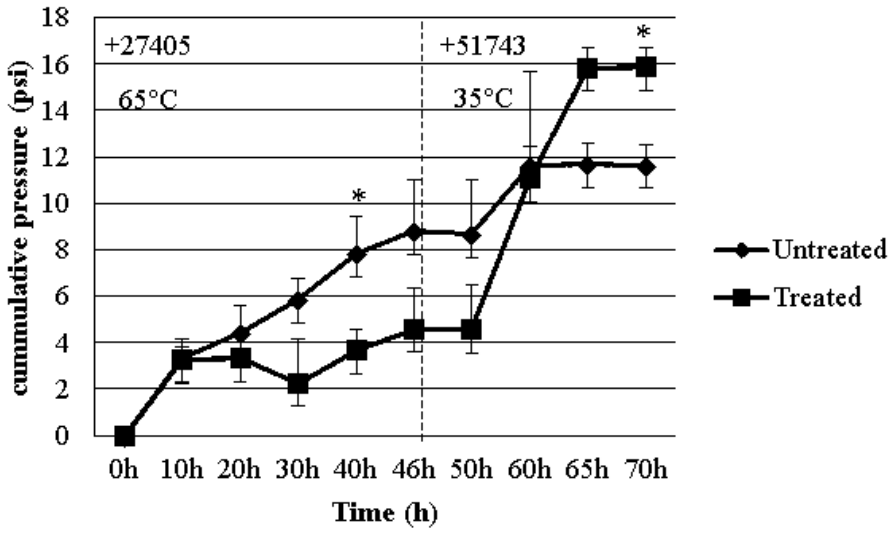


Figure 3.4.1. Gas production by the sequential culture of *C. thermocellum* followed by *C. beijerinckii* on untreated and Fenton-treated switchgrass. The dashed line indicates the inoculation with the mesophile and the associated switch in temperatures. * indicates significance difference. Error bars indicate ± one standard deviation. After 70 hours, the cumulative pressure levels out.

Table related to Figure 3.4.2. Comparison of gas produced by *C. beijerinckii* on Fenton-treated and untreated switchgrass. A)kPa. B)psi

A)Untreated	Cumulative Gas pressure (kPa)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0.00	0.00	0.00	0.00	0.00
5h	15.58	9.03	-17.31	2.44	17.41
10h	17.10	9.51	-17.37	3.08	18.12
15h	17.58	11.31	-16.06	4.27	17.89
20h	19.03	9.03	-15.79	4.09	17.93
24h	18.06	8.76	-14.82	4.00	16.95
Fenton-Treated	Cumulative Gas pressure (kPa)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0	0	0	0	0
5h	-0.48	11.79	4.41	5.24	6.18
10h	0.76	13.58	14.75	9.70	7.76
15h	8.00	13.79	24.34	15.38	8.28
20h	27.30	14.62	25.86	22.59	6.94
24h	31.10	13.79	25.86	23.58	8.87

B)Untreated	Cumulative Gas pressure (psi)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0	0	0	0	0
5h	2.26	1.31	-2.51	0.35	2.5248036
10h	2.48	1.38	-2.52	0.45	2.6274195
15h	2.55	1.64	-2.33	0.62	2.5949759
20h	2.76	1.31	-2.29	0.59	2.6001603
24h	2.62	1.27	-2.15	0.58	2.4587192
Fenton-Treated	Cumulative Gas pressure (psi)				
Time	**1**	**2**	**3**	**AVG**	**SD**
0h	0	0	0	0	0
5h	-0.07	1.71	0.64	0.76	0.8960469
10h	0.11	1.97	2.14	1.41	1.1261587
15h	1.16	2	3.53	2.23	1.2016239
20h	3.96	2.12	3.75	3.28	1.0071908
24h	4.51	2	3.75	3.42	1.2871286

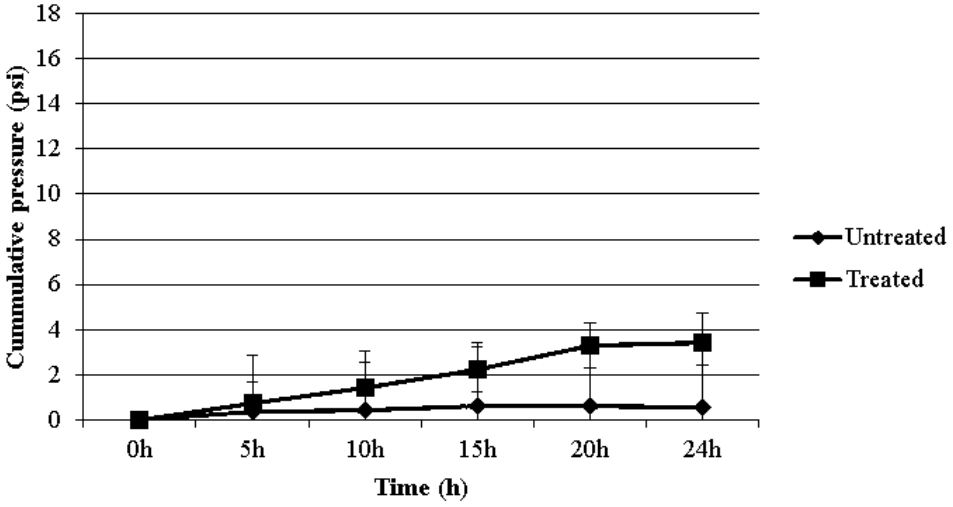


Figure 3.4.2. Gas production by *C. beijerinckii* on untreated and Fenton-treated switchgrass. There was no significance difference between the values at any time. Error bars indicate ± one standard deviation. After 24 hours, the cumulative pressure levels out.

Table related to Figure 3.4.3. Comparison of gas produced by *C. beijerinckii* on Fenton-treated (light grey squares) and untreated switchgrass (grey rhomboid).

Time(h)	untreated	Fenton-treated
0	-	-
5	-0.94±1.6	-0.07±1.4
15	19.4±16.9	44.65504±42.4
20	19.72±16.8	77.63±18.2
25	19.47±16.2	77.73±18.3

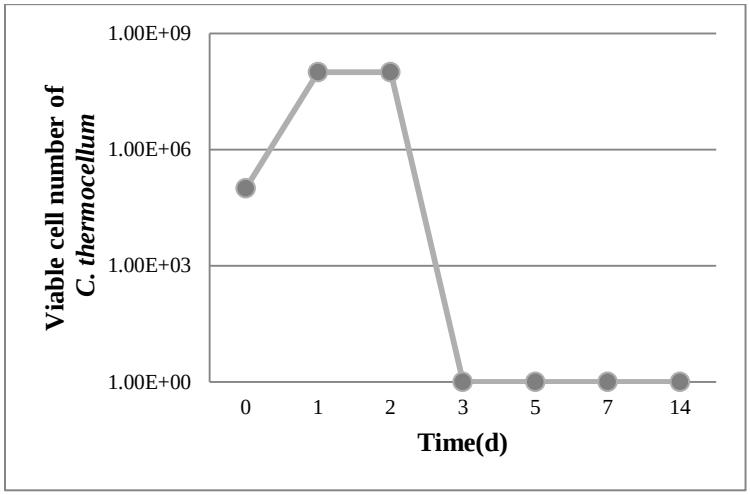


Figure 3.4.3. *C. thermocellum* viable cell number on untreated switchgrass determined using serial dilution technique.

Table related with Figure 3.4.3. Viable cell number of *C.thermocellum* on untreated switchgrass (duplicates).

Day	**Viable cell number**	**SD**
0	1.00E+05	0
1	1.00E+08	0
2	1.00E+08	0
3	1.00E+00	0
5	1.00E+00	0
7	1.00E+00	0
14	1.00E+00	0

Table related to Figure 3.4.5. Viable cell number of sequential culture on untreated (a) and treated (b) switchgrass.

a)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**	**21**
1	1000	1000	1000000	1000000	10000	10000	10	10	0	0
2	1000	1000	100	1000000	1000	100	100	10	0	0
3	1000	1000	100	100	1000	100	100	10	0	0
Avg	**1.00E+03**	**1.00E+03**	**3.33E+05**	**6.67E+05**	**4.00E+03**	**3.40E+03**	**7.00E+01**	**1.00E+01**	**0**	**0**

b)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**	**21**
1	1.00E+07	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+07	1.00E+07	1.00E+06
2	1.00E+06	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+07	1.00E+07	1.00E+06
3	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+07	1.00E+07	1.00E+07	1.00E+06
Avg	**7.00E+06**	**7.00E+07**	**1.00E+07**	**1.00E+07**	**1.00E+07**	**7.00E+06**	**4.00E+06**	**1.00E+07**	**1.00E+07**	**1.00E+06**

Table related with Figure 3.4.6. Viable cell number of pure culture on untreated (a) and treated (b) switchgrass.

a)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**
1	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+06	1.00E+06	1.00E+06
2	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+06	1.00E+06	1.00E+06	1.00E+06
3	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+06	1.00E+06	1.00E+07
Avg	**7.00E+07**	**1.00E+07**	**1.00E+07**	**1.00E+07**	**7.00E+06**	**1.00E+06**	**1.00E+06**	**1.00E+06**	**4.00E+06**

b)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**
1	1.00E+08	1.00E+08	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+07
2	1.00E+08	1.00E+08	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+07
3	1.00E+07	1.00E+08	1.00E+09	1.00E+08	1.00E+07	1.00E+07	1.00E+07	1.00E+06	1.00E+06
Avg	**7.00E+07**	**1.00E+08**	**4.00E+08**	**4.00E+07**	**1.00E+07**	**1.00E+07**	**1.00E+07**	**7.00E+06**	**7.00E+06**

Table related to Figure 3.4.8 and 3.4.9. Product formation by *C. thermocellum* ATCC 27405 on a) untreated switchgrass and b)Fenton-treated switchgrass during 12 d at 65 ºC.

a)

Day	glucose	xylose	lactate	formate	acetate	EtOH
			(mM)			
0	0.27±0.09	-	0.14±0.01	0.28±0.01	3.1±0.14	5.5±2.12
1	-	1.2±0	2.4±0.28	2.2±0	12±1.4	6±4.2
2	-	1.25±1.48	2.9±0.28	6.2±0	23±5.6	28±1.41
3	-	1.95±1.63	4.65±0.63	6.4±0	24±7.0	29±4.2
4	-	2.43±2.6	5.6±1.55	4.35±0.7	26±4.9	33±7.0
5	0.34±0	3.3±2.6	5.2±1.55	3.7±0.7	26.5±4.9	34±7.0
6	0.65±0	3.7±3.2	6.6±0.84	4.9±0	30.5±7.7	34±7.0
10	0.41±0.36	3.85±2.19	6.1±0.98	3.2±0.56	33.5±4.9	36±7.0
12	0.46±0.5	3.35±2.89	6.05±1.76	3.8±0.98	33±2.8	34.5±9.2

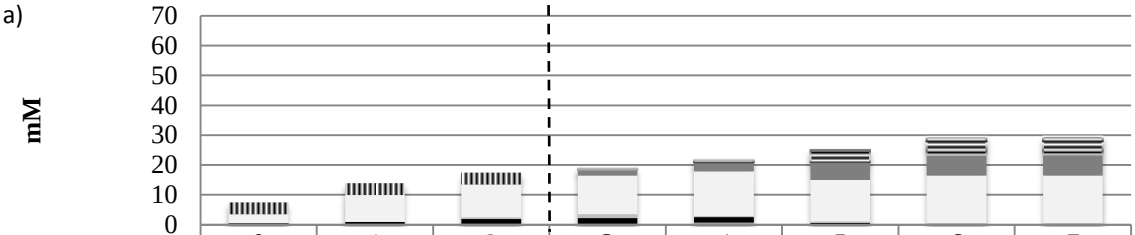
b)

Day	cellobiose	glucose	xylose	lactate	formate	acetate	EtOH
				(mM)			
0	-	-	0.74±0.18	-	0.76±0.48	5.15±0.49	4.5±0.7
1	0.86±0.03	1.15±0.07	2.5±0.28		0.56±0.21	10.5±0.7	4±0
2	0.42±0.04	0.19±0.02	1.3±0	-	1.1±0	15±0	5±0
3	0.52±0.08	0.33±0.03	1.95±0.77	-	0.43±0.02	18±1.41	4±0
4	0.58±0.05	0.42±0.09	1.55±0.07	-	0.46±0.02	19.5±2.11	3.5±0.7
5	0.98±0.02	0.87±0.12	2.4±0.14	-	0.57±0.02	28±1.44	5±0
6	1.1±0	0.93±0.09	2.9±0.28	-	0.67±0	31±0	4.5±0.7
10	1.2±0.14	0.96±0.05	3.3±0.28	-	0.96±0.48	34±1.41	5±0
12	0.79±	0.71±0.11	1.85±0.07	-	0.95±0.07	24±1.41	4±0

Chapter four: **ABE Fermentation in a Solid Substrate Cultivation with Continuous Product Removal.**

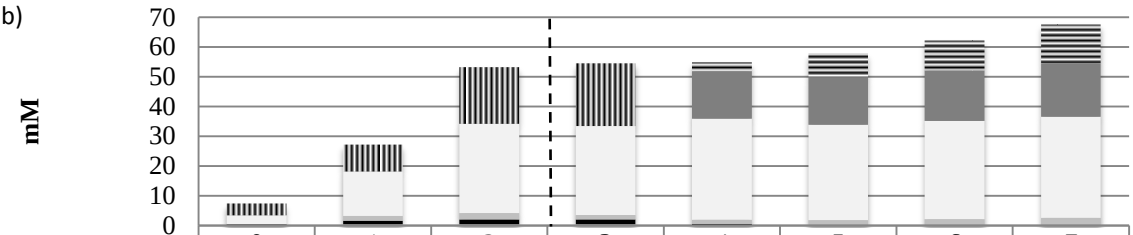
Preliminary Batch fermentation

a)



	0	1	2	3	4	5	6	7
mM butanol	0	0	0	0.56	0.8	4	6	6
mM butyrate	0	0	0	1.8	3	6	7	7
mM EtOH	4	4	4	0	0	0	0	0
mM acetate	3	9	11	13	15	14	16	16
mM formate	0	0	0.52	1.2	0.33	0.42	0.47	0.52
mM lactate	0.48	0.6	1.7	2	1.9	0.6	0	0
mM xylose	0	0.31	0.23	0.25	0.66	0	0	0

b)



	0	1	2	3	4	5	6	7
mM butanol	0	0	0	0	3	8	10	13
mM butyrate	0	0	0	0	16	16	17	18
mM EtOH	4	9	19	21	0	0	0	0
mM acetate	3	15	30	30	34	32	33	34
mM formate	0	1.6	2.2	1.5	1.4	1.8	2.1	2.5
mM lactate	0.41	1.2	1.6	1.6	0.44	0	0	0
mM xylose	0	0.37	0.39	0.35	0	0	0	0

Figure 1. Product formation of sequential culture of *C. thermocellum* (10 %w/w) followed by *C. saccharoperbutylacetonicum* (10 %w/w) in TP media (a) and in T media (b)both with switchgrass (10 %w/w) as substrate. Dashed line indicates inoculation of *C. saccharoperbutylacetonicum* and temperature decrease to 35 °C.

Table related to Figure 4.4.1. Viable cell number of *C. saccharoperbutylacetonicum* grown on switchgrass in sequential culture with *C. thermocellum* and pure culture of *C. saccharoperbutylacetonicum* with the corresponding standard deviation.

Day	Pure Avg	SD	Seq Avg	SD
0	1.00E+08	0.00E+00	1.00E+08	0.00E+00
1	5.50E+06	6.36E+06	1.00E+08	0.00E+00
2	1.00E+07	0.00E+00	5.50E+07	6.36E+07
3	1.00E+07	0.00E+00	5.50E+07	6.36E+07
5	1.00E+06	0.00E+00	1.00E+08	0.00E+00
7	1.00E+06	0.00E+00	1.00E+08	0.00E+00
14	5.50E+05	6.36E+05	5.50E+05	6.36E+05
21	5.50E+05	6.36E+05	5.50E+05	6.36E+05

Table 1. Preliminary experiment evaluating toxicity of *C. thermocellum's* inoculum grown either filter paper or switchgrass on *C. saccharoperbutylacetonicum* at different concentrations. Optical density and pH were evaluated (duplicates).

OD		27405 grown on FILTER			27405 grown on SWITCH		
Time	Control	10%	25%	50%	10%	25%	50%
0h	0.65±0.03	0.44±0.02	0.47±0.2	0.89±0.1	0.58±0.09	0.53±0	0.84±0
4h	0.75±0.3	0.72±0.04	0.65±0.19	1.29±0.1	0.82±0.4	0.72±0.01	0.97±0
7h	2.65±0.06	3.15±0.02	2.44±1.0	3.81±0.02	2.13±0.3	2.92±0.01	3.49±0.02
12.5h	3.14±0.09	3.27±0.2	2.51±0.5	2.27±0.1	1.64±0.6	2.17±0	2.86±0.02
24h	2.81±0.14	2.85±0.04	2.31±0.6	1.78±0.17	2.22±0.15	2.94±0.01	2.19±0.01
pH							
0h	5.81±0.03	6.02±0.01	6.29±0.12	6.42±0.14	5.69±0.18	5.73±0.07	5.72±0.02
4h	5.8±0	5.97±0.02	6.37±0.18	6.41±0.14	5.7±0.11	5.68±0.11	5.7±0
7h	6.79±0.09	6.91±0.04	7.48±0.45	7.16±0.01	6.9±0.01	6.70±0	6.74±0.08
12.5h	5.1±0	5.13±0.01	6±0.9	5.91±0.1	5.31±0.29	5.1±0	5.15±0.07
24h	5.11±0.01	5.16±0.01	5.48±0.22	5.94±0.04	5.35±0.35	5.08±0.02	5.15±0.07

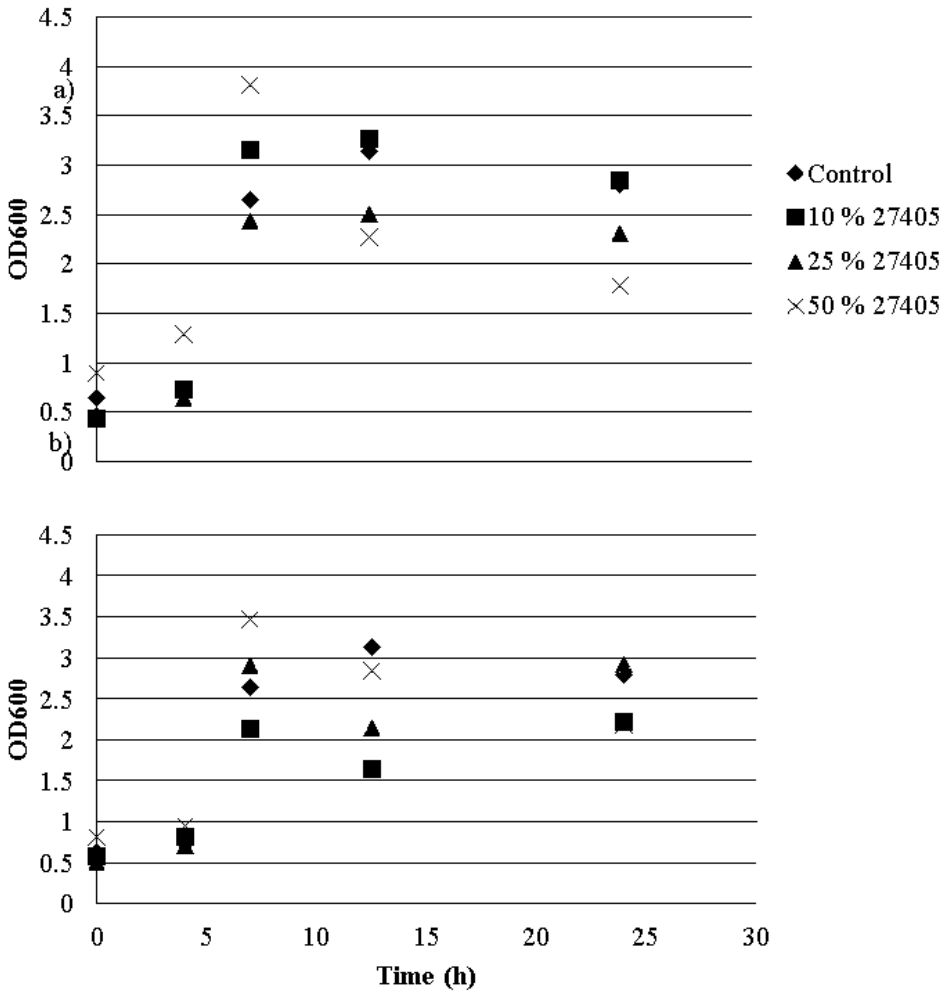


Figure 2. Preliminary experiment evaluating toxicity of *C. thermocellum's* inoculum grown either filter paper (a) or switchgrass (b) on *C. saccharoperbutylacetonicum* at different concentrations.

Continuous Fermentation

Daily Data Sheet

Table 1. Low Flow Rate.

Daily	Time	ml collected	T ºC	pH
3	274050h	200	63	6.59
4	274050h	200	63	6.58
13	2740524h	200	63	6.42
14	2740524h	200	63	6.33
23	N1-40h	100	35	4.67
24	N1-40h	100	35	4.75
33	12h	270	37	6.49
34	12h	270	36	6.42
43	24h	250	38	6.42
44	24h	250	37	6.5
53	36h	300	37	6.5
54	36h	310	37	6.43
63	48h	250	37	6.33
64	48h	250	37	6.45
73	72h	580	37	6.33
74	72h	610	37	6.36
83	96h	600	37	6.15
84	96h	610	37	6.26
93	N1-40h	100	35	5
94	N1-40h	100	35	5.07
103	24h	600	35	6.06
104	24h	600	35	6.03
113	48h	610	37	6.34
114	48h	610	37	6.3
123	72h	620	37	6.45
124	72h	620	37	6.44

Table 2. Medium Flow Rate.

Sample	Time	ml collected	T ºC	pH
10	27405/0h	200	63	7.03
20	27405/0h	200	63	7.02
11	27405/24h	200	64	6.88
21	27405/24h	200	64	6.88
12	N1-4/0h	100	35	6.08
22	N1-4/0h	100	35	6.1
13	N1-4/24h	1150	35	6.35
23	N1-4/24h	1150	35	6.39
14	N1-4/48h	1000	35	6.25
24	N1-4/48h	1000	35	6.25
15	N1-4/72h	1000	35	6.29
25	N1-4/72h	1000	35	6.19
16	N1-4/96h	1000	35	6.3
26	N1-4/96h	1000	35	6.27
17	N1-4/0h	100	35	5.95
27	N1-4/0h	100	35	5.88
18	N1-4/24h	1100	35	6.15
28	N1-4/24h	1100	35	6.15
19	N1-4/48h	1000	35	6.26
29	N1-4/48h	1000	35	6.21
110	N1-4/72h	1000	35	6.26
210	N1-4/72h	1000	35	6.26

Table 3. High Flow Rate.

Sample	Time	ml collected	T ºC	pH
1	274050h	200	64	6.77
2	274050h	200	63.5	6.76
11	2740524h	200	64	6.05
12	2740524h	200	62.5	6.17
21	N1-40h	100	34	4.86
22	N1-40h	100	36	4.87
31	12h	1170	35	5.98
32	12h	1160	35	5.91
41	24h	850	35	6.28
42	24h	840	35	6.23
51	36h	1100	35	6.26
52	36h	1100	35	6.23
61	48h	810	35	6.29
62	48h	810	35	6.26
71	72h	1960	35	6.11
72	72h	1920	35	6.18
81	96h	1950	35	6.21
82	96h	1900	35	6.15
111	N1-40h	100	37	4.53
112	N1-40h	100	37	4.57
121	24h	1920	37	6.22
122	24h	1850	37	6.28
131	48h	2100	35	6.22
132	48h	2000	35	6.21
141	72h	2200	35	6.1
142	72h	2000	35	6.22

Table 4. Low, Medium and High Flow Rate data with the corresponding standard error.

Flow Rate	Acetate mg	Butyrate mg	Lactate mg	Formate mg	Ethanol mg	Butanol mg	Total acids	Total solvents	Total products
Low	520.87±45.55	515.73±31.27	26.15±3.27	0.00	0.00	142.32±15.51	1062.76±73.55	142.32±15.81	1205.07±89.36
Medium	802.08±354.5	735.05±256.44	107.87±33.44	132.57±2.6	50.677±32.6	201.98±26.73	1777.58±646.88	252.65±5.85	2030.23±641.04
High	617.75±206.5	959.39±137.3	13.38±15.5	8.87±7.75	61.2±24.74	165.83±70.99	1599.39± 351.50	227.06±95.73	1826.45±447.23

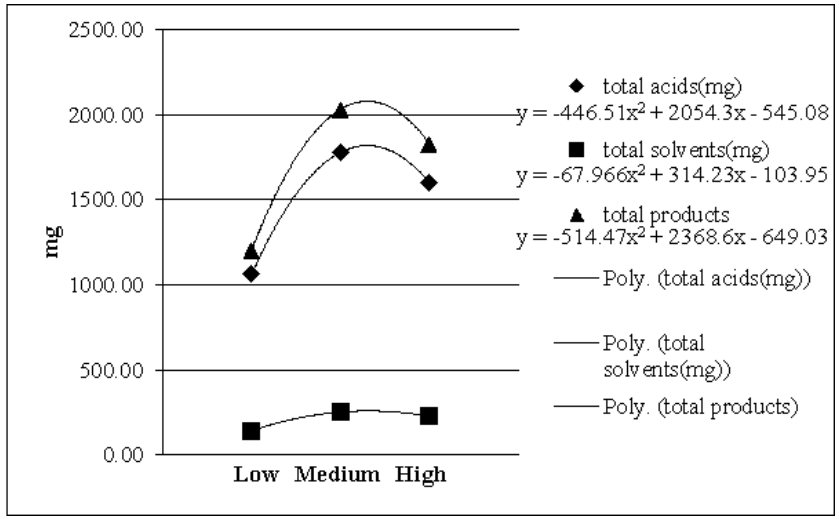


Figure 3. Distribution of total products, total acids and total solvent at different flow rates (low, medium, high) during 9 days of fermentation of switchgrass in a continuous mode. It has to be point out that there was no significance difference between treatments.

Calculations

Moisture content
Switchgrass (25 g, 6.15% moisture content based on Dairy One Analysis) dried in the oven after fermentation.
Considering that we started with 23.46 g of dry matter:

Flow rate	Dry matter	Water loss	%moisture
Control	23.46		6.15
Low	22.26±0.7	1.21	5.16
Medium	22.03±0.41	1.43	6.09
High	21.47±0.36	1.99	8.48

Moisture content calculations:

$$\%= \frac{m_w}{m_w+m_{dm}}$$

Low:

$$5.16\%=100* \frac{1.21}{1.21+22.26}$$

Medium

$$6.09\%=100* \frac{1.43}{1.43+22.03}$$

High

$$8.48\%=100* \frac{1.99}{1.99+21.47}$$

If the moisture contents are added, the following values of moisture are found (but not sure if it is correct to do it since environmental humidity could affect the water content between the time that the samples were analyzed):

Flow rate	Dry matter	Water loss	%moisture corrected
Control	23.46		6.15
Low	22.255±0.7	1.21+1.46	10.71
Medium	22.03±0.41	1.43+1.135	10.42
High	21.465±0.36	1.99+1.373	14.63

Corrected dry matter (based on the weights obtained after drying at 80 ºC):

Flow rate	%dry Matter (Dairy One analysis)	Corrected Dry matter (g)	DMD (g)	% (DMD)
Control	93.7	23.46		
Low	93.40±0.14	20.79	2.67	11.38
Medium	94.85±0.07	20.9	2.56	10.91
High	93.60±0.28	20.10	3.36	14.32

References

- Afschar A., Biebl S., Schaller K. and Schügerl K. 1985. Production of acetone and butanol by *Clostridium acetobutylicum* in continuous culture with cell recycle Appl Microbiol Biotechnol 22(6):394-398.
- Alizadeh H., Teymouri F., Gilbert T. and Dale B. 2005. Pretreatment of switchgrass by ammonia fiber explosion (AFEX). Appl Biochem Biotechnol 124:1133-1141.
- Al-Shorgani Najeed K.N., Kalil Mohd S. and Yusoff Wan Mohtar Wan. 2011. The effect of different carbon sources on butanol production using *Clostridium saccharoperbutylacetonicum* N1-4. Biotechnol 10:280-285.
- Al-Shorgani N. K., Kalil M.S. and Yusoff W.M. 2012. Biobutanol production from rice bran and de-oiled rice bran by *Clostridium saccharoperbutylacetonicum* N1-4. Bioprocess Biosyst Eng 35(5):817-826.
- Anastassiadis S. and Morgunov I.G. 2007. Gluconic acid production. Recent Pat Biotechnol 1(2):167-180.
- Andersen V.F., Anderson J.E., Wallington T.J., Mueller S.A. and Nielsen O. J. 2010. Distillation curves for alcohol–gasoline blends. Energ Fuel 24:2683–2691.
- Arantes V., Jellison J. and Goodell B. 2012. Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl Microbiol Biotechnol 94:323-338.
- Bahl H. and Gottshalk G. 1988. Microbial production of butanol/acetone. Biotechnology. H.J. Rehm and J.Reed (Ed).1-30.
- Bahl H., Andersch W., Braun K. and Gottschalk G. 1982. Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. Appl Microbiol Biotechnol 14:17-20.
- Banerjee G., Car S., Liu T., Williams D., López M., Walton J. and Hodge D. 2011. Scale-up and Integration of Alkaline Hydrogen Peroxide Pretreatment, Enzymatic Hydrolysis, and Ethanolic Fermentation. Biotechnol Bioeng 109(4):922-931.
- Bayer E.A., Kenig R. and Lamed R. 1983. Adherence of *Clostridium thermocellum* to cellulose. J Bacteriol 156:818-27.
- Bayer E.A., Belaich J.P., Shoham Y. and Lamed R. 2004. The cellulosomes: multienzymatic machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol 58:521-554.
- Bayer E. A., Morag E. and Lamed R. 1994. The cellulosome: a treasure-trove for biotechnology. Trends Biotechnol 12:378-386.

- Bentivenga G., Bonini C., D'Auria M. and Bona A.D. 2003. Degradation of steam-exploded lignin from beech by using Fenton's reagent. Biomass Bioenerg 24:233-238.
- Bhardwaj R. and Mohanty A.K. 2007. Advances in the properties of polylactides based materials: A review. J Biobased Mat Bioen 1(2):191-209.
- Briens C., Piskorz J. and Berutti F. 2008. Biomass valorization for fuel and chemicals production-A review. Internat J of Chem React Eng (6).
- Brown R.C. 2003. Biorenewable Resources: Engineering New Products from Agriculture. Blackwell Publishing. Ames, IA. 1st ed..109-134.
- Cato E.P., George W.L. and Finegold S.M. 1986. Genus Clostridium. In Bergey's Manual of Systematic Bacteriology. (2):1141±1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Chang V.S., Holtzapple M.T. 2000. Fundamental factors affecting biomass enzymatic reactivity. Appl Biochem Biotechnol 84(6):5-37.
- Chang V.S., Kaar W.E., Burr B. and Holtzapple M.T. 2001. Simultaneous saccharification and fermentation of lime-treated biomass. Appl Biochem Biotechnol 23:1327-1333
- Chen C.K. and Blaschek H.P. 1999. Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. Appl Microbiol Biotechnol 52:170–173.
- Chevanan N., Womac A.R., Bitra V.S.P., Igathinathane C., Yang Y.T., Miu P. I. and Sokhansanj S. 2010. Bulk density and compaction behavior of knife mill chopped switchgrass, wheat straw, and corn stover. Bioresource Technol 101(1): 207-214.
- Chinn M.S.,Nokes S.E. and Strobel H.J. 2006. Screening of thermophilic anaerobic bacteria for solid substrate cultivation on lignocellulosic substrates. Biotechnol Prog 22(1):53-59
- Chinn M.S., Nokes S.E. and Strobel H.J. 2007. Influence of process conditions on end product formation from *Clostridium thermocellum* 27405 in solid substrate cultivation on paper pulp sludge. Bioresource Technol 98(11):2184-2193.
- Collins M.D., Lawson P.A., Willems A., Cordoba J.J., Fernandez-Garayzabal J., Garcia P., Cai J., Hippe H. and Farrow J.A.E. 1994. The phylogeny of the genus *Clostridium:* Proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 44:812-826.
- Currie M.A., Adams J.J., Ali S., Smith S.P. and Jia Z.C. 2010. Purification and crystallization of a multimodular heterotrimeric complex containing both type I and type II cohesin-dockerin interactions from the cellulosome of *Clostridium thermocellum*. Acta Crystallograph Sect F Struct. Biol Cryst Commun 66: 327-329.

- Daniel G. 2003. Microview of wood under degradation by bacteria and fungi. Wood Deterioration and Preservation: Advances in Our Changing World. B. Goodell, D. D. Nicholas and T. P. Schultz. 845:34-72.
- Davin L.B. and Lewis N.G. 1992. Phenylpropanoid metabolism: Biosynthesis of monolignols, lignans and neolignans, lignins and suberins. Rec Adv Phytochem 26-325-375.
- Demain A., Newcomb M. and Wu J.H.D. 2005. Cellulase, Clostridia, and Ethanol. Microbiol Mol Biol Rev 69:124–54.
- Demain A. and Solomon N.A. 1981. Industrial Microbiology. Sci Am 245(3)8:67-75.
- Demirbas A. 2012. Fuels for petroleum, coal and biomass. Energy Educ Sci and Tech 29(1):701-705.
- Demirbas T. and Demirbas A.H. 2010. Bioenergy, Green Energy. Biomass and Biofuels. Energy Sources Part a-Recovery Utilization and Environmental Effects 32(12):1067-1075.
- Demirbas A. 2002. Biodiesel from vegetable oils via transesterification in supercritical methanol. Energ Convers Manage 43(17):2349–2356.
- Dharmagadda V.S., Nokes S.E., Strobel H.J. and Flythe M.D. 2010. Investigation of the metabolic inhibition observed in solid substrate cultivation of *Clostridium thermocellum* on cellulose. Bioresource Technol 101(15):6039-6044.
- Dickinson J.R. 1999. Carbon metabolism. In J. R. Dickinson and M. Schweizer. The metabolism and molecular physiology of *Saccharomyces cerevisiae*. Philadelphia, PA: Taylor & Francis. ISBN 978-0748407316.
- Durre P. 2008. Fermentative butanol production: Bulk chemical and biofuel. Incredible Anaerobes: From Physiology to Genomics to Fuels. J. Wiegel, R. J. Maier and M. W. W. Adams. Oxford, Blackwell Publishing. 1125: 353-362.
- Evans G. 2008. International Biofuels Strategy Project. Liquid Transport Biofuels Technology Status Report. NNFCC 08-017.
- Ezeji T.C., Qureschi N. and Blaschek H.P. 2004. Butanol fermentation research: upstream and downstream manipulations. Chem Rec 4:305-314.
- Felix C.R. and Ljungdahl L.G. 1993. The cellulosome: the exocellular organelle of Clostridium. Annu Rev Microbiol 47:791-819.
- Fenton H.J.H. 1894. Oxidation of tartaric acid in the presence of iron. J Chem Soc 65:899-910.
- Flythe M.D. and Russell J.B. 2006. Fermentation acids inhibit amino acid deamination by *Clostridium sporogenes* MD1 via a mechanism involving a decline in intracellular glutamate rather than protonmotive force. Microbiol-Sgm 152:2619-2624.
- Forsberg C.W. and Cheng K.J. 1990. Integration of rumen microorganisms and their hydrolytic enzymes during the digestion of lignocellulosic materials.

Microbial and Plant opportunities to improve lignocellulose utilization by ruminants. Elsevier Science Publishing Co., Inc., New York.

- Gaughran E.R.L. 1947. The thermophilic microorganisms. Microbiol Mol Biol Rev 11(3):189-225.
- George H.A., Johnson J.L., Moore W.E.C., Holdeman L.V. and Chen J.S. 1983. Acetone, Isopropanol,and Butanol Production by *Clostridium beijerinckii*(syn *Clostridium butylicum)* and *Clostridium aurantibutyricum*. Appl Environ Microbiol 45:1160-1163.
- Gevo Inc Web site :http://www.gevo.com/.
- Godfray C.J., Beddington J.R. and Crute I.R. 2010. Food Security: The Challenge of feeding 9 billion People. Science 327(5967): 812-818.
- Gottschalk G. 1986. Bacterial metabolism. Second Edition, Springer-Verlag, New York.
- Halliwell G., Philips T.M. and Halliwell N. 1995. Microcrystalline forms of cellulose as substrates for strains of *C.thermocellum* and cellulose formation. Proc Biochem 30:243-250.
- Hammel K.E., Kapich A.N., Jensen K.A. and Ryan Z.C. 2002. Review: Reactive oxygen species as agents of wood decay by fungi. Enzyme Microb Technol 30:445-453.
- Herrero A.A., Gomez R.F. and Roberts M.F. 1985. 3lP NMR studies of *Clostridium thermocellum.* Mechanism of end product inhibition by ethanol. J Biol Chem 260:7442–7451.
- HFCs, the Montreal Protocol and the UNFCCC. 2012. Eliminating 1 of the 6 Kyoto gases *Bangkok climate change conference, August-September.*
- Himmel M., Tucker M., Baker J., Rivard C., Oh K. and Grohmann K. 1985. Comminution of biomass: hammer and knife mills. Biotechnol Bioeng 15: 39-58.
- Himmel M.E., Ding S., Johnson D.K., Adney W.S., Nimlos M.R., Brady J.W.and Foust T.D. 2007. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science 315:804-807.
- Holdeman L.V., Cato E.P. and Moore W.E.C. 1977. Anaerobe Laboratory Manual, 4th ed. Blacksburg, VA: Virginia Polytechnic Institute and State University.
- Hongo M. 1960. Process for producing butanol by fermentation.US Patent 2945786.
- Hutkins R.W. and Kashket E.R. 1986. Phosphotransferase activity in *Clostridium acetobutylicum* from acedogenic and solvetogenic phases of growth. Appl Environ Microbiol 51:1121–1123.
- Jacobsen S. and Fritz H.G. 1996. Filling of poly(lactic acid) with native starch. Polym Eng Sci 36(22):2799-2804.

- Jang Y.S., Malaviya A., Cho C., Lee J. and Lee S. Y. 2012. Butanol production from renewable biomass by clostridia. Bioresource Technol 123(0):653-663.
- Jeffries T.W. 1994. Biodegradation of lignin and hemicelluloses. In: Ratledge C (ed). Biochemistry of microbial degradation. Kluwer, Dordrecht. 233-277.
- Jones D.T. and Keis S. 1995. Origins and relationships of industrial solvent-producing clostridial strains. FEMS Microbiol Rev 17(3):223-232.
- Kamm B. and Kamm M. 2004. Principles of Biorefineries. Appl Microbiol Biotechnol 64:137-145.
- Keis S., Bennett C.F., Ward V.K. and Jones D.T. 1995. Taxonomy and phylogeny of industrial solvent-producing clostridia. Int J Syst Bacteriol 45:693:705.
- Kim S. and Dale B. 2004. Global potential bioethanol production from wasted crops and crop resideus. Biomass Bioeng 26:361-375.
- Kirk T.K., Connors W.J. and Zeikus J.G. 1977. Advances in understanding the microbial degradation of lignin in: Recent Advances in Phytochemistry. Plenum Press, New York. 11:369-394.
- Kirk T.K., Schulz E., Connors W.J., Lorenz L.F. and Zeikus J.G. 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium.* Arch Microbiol 117:277-285.
- Kirk T.K., Higuchi and Chang H.M. 1980. Lignin Biodegradation: Microbiology, Chemistry and Potential Applications. CRC Press, Boca Raton, Fl. 1:236.
- Klein D.W., Lansing M., Harley J. 2004. Microbiology (6^{th} ed.). New York: McGraw-Hill. ISBN 978-0072556780.
- Klyosov A.A. 1983. Bioconversion of Plant Raw Material by Microorganisms. Prhinen T. and Hatakka A. Eds. Helsinki.152-178.
- Klyosov A.A. 1986. Role of the activity and adsorption of cellulases in the efficiency of the enzymatic hydrolysis of amorphous and crystalline cellulose. Biochem 25:540-542.
- Krishna C. 2005. Solid-state fermentation systems: an overview. Crit Rev Biotechnol 25:1-30.
- Lam P.S., Sokhansanj S., Bi X., Mani S., Womac A.R., Hoque M., Peng J., JayaShankar T., Naimi L.J., Narayan S. 2007. Physical Characterization of Wet and Dry Wheat Straw and Switchgrass-Bulk and specific Density, Paper 076058. ASABE. St. Joseph, Michigan.
- Larsson P.T., Wickholm K. and Iversen T. 1997. A CP/MAS C-13 NMR investigation of molecular ordering in celluloses. Carbohydr Res 302:19-25.
- Lee S.Y., Park J.H., Jang S.H., Nielsen L.K., Kim J. and Jung K.S. 2008. Fermentative butanol production by *Clostridia*. Biotechnol Bioeng 101 (2):209-228.

- Lekanda J.S. and Perez-Correa J.R. 2004. Energy and water balances using kinetic modeling on a pilot-scale SSF bioreactor. Process Biochem 39(11):1793-1802.
- Lenz T.G. and Moreira A.R. 1980. Economic-evaluation of the acetone-butanol fermentation. Abstracts of Papers of the American Chemical Society 179(MAR): 33-INDE.
- Lin Y.L. and Blaschek H.P. 1983. Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl Environ Microbiol 45:966–973.
- Liu S. Q. and Qureshi N. 2009. How microbes tolerate ethanol and butanol. New Biotechnol 26(3-4):117-121.
- Ljungdahl L.G. and Eriksson K.E. 1985. Ecology of microbial cellulose degradation. Adv Microb Ecol 8:237-99.
- Lu Y.P., Zhang Y.H.P. and Lynd L.R. 2006. Enzyme-microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*. Proc Natl Acad Sci USA 103:16165–16169.
- Luterbacher J.S., Froling M., Vogel F., Marechal F., Tester J.W. 2009. Hydrothermal gasification of waste biomass: Process design and life cycle assessment. Environ Sci Technol 43:1578-1583.
- Lynd L., Zyl W., Mc Bride J., Laser M. 2005. Consolidated bioprocessing of cellulosic biomass: An update. Curr Opin Biotechnol 16:577-583.
- Lynd L.R, Weimer PJ., van Zyl W.H. and Pretorius I.S. 2002. Microbial cellulose utilization fundamentals and biotechnology. Microbiol Mol Biol Rev 66(3):506-577.
- Lynd L.R., Grethlein H.G. and Wolkin R.H. 1989. Fermentation of cellulose substrates in batch and continuous culture by *Clostridium thermocellum.* Appl Environ Microbiol 55:3131-3139.
- Lynd L.R. 1996. Overview and evaluation of fuel ethanol production from cellulosic biomass: technology, economics, the environment, and policy. Annu Rev Energ Environ 21:403–465.
- Madigan M.T. and Martinko J.M. 2006. Brock: Biology of Microorganisms. 11th edition .Pearson Education, Inc. 11th edition. Upper Saddle River, NJ.
- McBee R.H. 1954.The characteristics of *Clostridium thermocellum*. J Bacteriol 67(4):505-506.
- McCutchan W.N. and Hickey R.J. 1954. Industrial Fermentations. Chem publishing Co., New York. 1:347-388.
- McLaughlin S.B. and Kszos L.A. 2005. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. Biomass Bioenergy 28:515-535.

- Mehdi Dashtban, Heidi Schraft, Tarannum A. Syed, Wensheng Qin. 2010. Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 1(1):36-50.
- Minister Marcin Korolec, President of COP19/CMP9, Conference of Paris and climate change conference, November 2013.
- Mohagheghi A., Dowe N., Schell D., Chou YC., Eddy C. and Zhang M. 2004. Performance of a newly developed integrant of *Zymomonas mobilis* for ethanol production on corn stover hydrolysate. Biotechnol Lett 26:231-235.
- Monique H., Faaij A., van den Broek R., Berndes G., Gielen D., Turkenburg W. 2003. Exploration of the ranges of the global potential of biomass for energy. Biomass Bioenergy. 25:119–133.
- Moser L.E. and Vogel K.P. 1995. Switchgrass, big bluestem, and indiangrass. In R.F Barnes(ed.) Forages: An introduction to grassland agriculture. Iowa State Univ. Press, Ames, IA.409-420.
- Mosier N., Wyman C., Dale B., Elander R. , Lee Y.Y., Holtzapple M., Ladisch M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technol 96:673-686.
- Ni Y. and Sun Z. H. 2009. Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl Microbiol Biotechnol 83(3):415-423.
- Nigam P. and Singh D. 1994. Solid-state (substrate) fermentation systems and their applications in biotechnology. J Basic Microbiol 34(6):405-423.
- Nisizawa K. 1973. Mode of action of cellulases. J Ferment Technol. 51:267-304.
- Olsson L., Jörgensen H., Krogh K., and Roca C. 2005. Bioethanol production from lignocellulosic material. In Polysaccharides: Structural Diversity and Functional Versatility, Dumitriu S. 2nd (ed.). 957:993.
- Paredes C.J., Alsaker K.V. and Papoutsakis E.T. 2005. A comparative genomic view of clostridial sporulation and physiology. Nature Rev Microbiol 3:969-978.
- Parekh M., Formanek J. and Blaschek H.P. 1999. Pilot-scale production of butanol by *Clostridium beijerinckii* BA101 using a low-cost corn steep water based fermentation medium. Appl Microbiol Biotechnol 51:152-157.
- Parrish D.J. and Fike J.H. 2005. The biology and agronomy of switchgrass for biofuels. Crit Rev Plant Sci. 24:423-459.
- Perlack R.D., Lynn L., Wright A.F. and Turhollow R.L. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion ton annual supply. USDOE.USDA.
- Qureshi N. and Blaschek H.P. 2000. Economics of butanol fermentation using hyperbutanol producing *Clostridium beijerinckii* BA101. Food Bioprod Process 78:139–144.

- Qureshi N. and Blaschek H.P. 2001. Recent advances in ABE fermentation: hyper-butanol producing *Clostridium beijerinckii* BA101. J Ind Microbiol Biotechnol 27:287–291.
- Qureshi N. and Ezeji T.C. 2008. Butanol, 'a superior biofuel' production from agricultural residues (renewable biomass): recent progress in technology. Biofuel Bioprod Bior 2(4):319-330.
- Rosenberg S.L. 1980. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enzyme Microb Tech 2:185-193.
- Rydzak T., Levin D.B., Cicek N. and Sparling R. 2009. Growth phase-dependant enzyme profile of pyruvate catabolism and end-product formation in *Clostridium thermocellum* ATCC 27405. J Biotechnol 140(3-4):169-175.
- Samson R., Lem C.H., Bailey Stamler and Dooper J. 2008. Developing energy crops for thermal applications. Optimizing fuel quality, energy security and GHG mitigation, in Biofuels, Solar and Wind as Renewable Energy Systems.Pimentel D. Springer, Dordrecht, The Netherlans. 395-423.
- SAS.SAS User's Guide: Statistics vs 9.3. SAS Institute Inc., Cary, NC.
- Schmer M.R., Vogel K.P., Mitchell R.B. and Perrin R.K. 2008. Net energy of cellulosic ethanol from switchgrass. Proc Natl Acad Sci USA. 105:464-469.
- Schmer M.R.,Vogel K.P., Mitchell R.B, Moser L.E., Eskridge K.M. and Perrin R.K. 2006. Establishment Stand Thresholds for Switchgrass Grown as a Bioenergy Crop. J Agron Crop Sci. 46:157-161.
- Shuler M. and Kargi F. 2002. Bioprocess engineering. Part3. Chapter 9: Operating Considerations for Bioreactors for suspension and immobilized cultures. Second Edition. 248-263.
- Sills D.L. and Gossett J.M. 2011. Assessment of commercial hemicellulases for saccharification of alkaline pretreated perennial biomass. Bioresource Technol 102:1389-1398.
- Silzer T. 2000. *Panicum virgatum L*. Switchgrass, prairie switchgrass, tall panic grass. *Rangeland Ecosystems & Plants Fact Sheets*. University of Saskatchewan Department of Plant Sciences. Retrieved 2007-12-08.
- Sjöström E. 1993. Wood chemistry: fundamentals and applications, 2nd ed. Academic, San Diego.
- Smith L.D.S. and Hobbs G. 1974. Genus III. *Clostridium prazmowski* 1880. In Bergey's Manual of Determinative Bacteriology, 8th ed. 551-572. Edited by R. E. Buchanan and N. E. Gibbons. Baltimore: Williams & Wilkins.
- Spiehs M.J., Whitney M.H., Shurson G.C. 2002. Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota. J Anim Sci 80(10):2639-2645.
- Spivey M.J. 1978. Acetone-butanol-ethanol fermentation. Process Biochem 13(11):2-5.

- Sun Z. and Liu W. 2012. Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by *Clostridium acetobutylicum* ATCC 824. Biomass Bioenergy 39:39-47.
- Sun Y. and Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technol 83:1-11.
- Swana J., Yang Y., Behnam M. and Thompson R. 2011. An analysis of net energy production and feedstock availability for biobutanol and bioethanol. Bioresource Technol 102:2112-2117.
- Tashiro Y., Takeda K., Kobayashi G., Sonomoto K., Ishizaki A. and Yoshino S. 2004. High butanol production by *C. sacchraperbutylacetonicum* N1-4 in a fed-batch culture with pH-sta continuous butyric acid and glucose feeding method. J Biosci Bioeng 98:263-268.
- Thang, V. H., Kanda, K. and Kobayanshi, G. 2010.Production of Acetone-Butanol-Ethanol (ABE) in Direct Fermentation of Cassava by *Clostridium saccharoperbutylacetonicum* N1-4. Appl Biochem Biotechnol 161(1-8): 157-170.
- van den Brink J., de Vries R.P. 2011. Fungal enzymes sets for plant polysaccharide degradation. Appl Microbiol Biotechnol 91:1477-1492.
- van Soest P.J. and Wine R.H. 1967. Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. J Assoc Off Anal Chem 50:50-55.
- Vidal B.C., Dien B.S., Ting K.C. and Singh V. 2011. Influence of Feedstock particle size on Lignocellulose Conversion: A review. Appl Biochem Biotechnol 164(8):1405-1421.
- Viljoen J.A., Fred E.B. and Peterson W.H. 1926. The fermentation of cellulose by thermophilic bacteria. J Agr Sci 16:1-17.
- Waeonukul R., Kyu K.L., Sakka K., Ratanakhanokchai K. 2009. Isolation and characterization of a multienzyme complex(cellulosome) of the *Paenibacillus curdlanolyticus* B-6 grown in Avicel under aerobic conditions. J Biosci Bioeng 107:610-614.
- Wallner T., Miers S.A. and McConnell S. 2009. A Comparison of Ethanol and Butanol as Oxygenates Using a Direct-Injection, Spark-Ignition Engine. Journal of Engineering for Gas Turbines and Power-Transactions of the Asme 131(3).
- Wang L. and Chen H.Z. 2011. Increased fermentability of enzymatically hydrolyzed steam- exploded corn stover for butanol production by removal of fermentation inhibitors. Process Biochem 46(2):604-607.
- Weimer P.J. and Zeikus J.G. 1977. Fermentation of cellulose and cellobiose by C*lostridium thermocellum* in the absence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol 33(2):289-97.
- Weizmann C. 1919. Production of acetone and alcohol by bacteriological processes. US Patent 1315585.

- Weizmann C. and Rosenfeld B. 1937. The activation of the butanol-acetone fermentation of carbohydrates by *Clostridium acetobutylicum*. Biochem J 31:619-639.
- Wu M., Wang M., Liu J.and Huo J. 2008. Assessment of potential life-cycle energy and greenhouse gas emission effects from using corn-based butanol as a transportation fuel. Biotechnol Progr 24 (6):1204–12.
- Wyman C.E. 1999. Biomass ethanol: Technical progress, opportunities, and commercial challenges. Annu Rev Energy Environ 24:189-226.
- Yang Y., Sharma-Shivappa R.R., Burns J.C. and Cheng J. 2009. Energy Fuels. 23:5626-5635.
- Yin H., Haselbeck R., Niu W., Pujol-Baxley C., Burgard A., Boldt J., Khandurina J., Trawick J.D., Osterhout R.E., Stephen R., Estadilla J., Teisan S. Schreyer H.B., Andrae S,m Yang TH., Lee SY., Burk MJ, Van Dien S. 2011. Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. Nat Chem Biol 7(7):445-452.
- Yoshida T. and Matsumura Y. 2001. Gasification of cellulose, xylan, and lignin mixtures in supercritical water. Ind Eng Chem Res 40:5469-5474.
- Zhang M., Eddy C., Deanda K., Finkelstein M., Picataggio S. 1995. Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic *Zymomonas mobilis.* Science 267:240-243.
- Zhang Y.H.P. and Lynd L.R. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol Bioeng 88(7):797-824.
- Zhou S., Iverson A.G. and Grayburn WS. 2008. Engineering a native homoethanol pathway in *Escherichia coli* B for ethanol production. Biotechnol Lett 30:335-342.

Vita

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Education

2009- Degree of Microbiologist. National University of Río Cuarto, Río Cuarto, Córdoba. Argentina. GPA: 9.14 Out of 10.
2004- Bachelor of Natural Sciences, Degree: Health and Environment. Secondary Institute General Levalle, Gral. Levalle, Córdoba. Argentina. GPA: 9.61 Out of 10.

Related Experience

Attendance at Conferences and Scientific Meetings

12/2007 Participed in IX Argentine Congress of Microbiology. Asociación Argentina de Microbiología. Cordoba, Argentina
09/2008 Participed in Conference Transmitted Disease Epidemiology for Food. National University of Río Cuarto. Rio Cuarto, Cordoba. Argentina
10/2008 Participed in XIII Argentine Microbiology Jorney. Asociación Argentina de Microbiología Filial Rosario. Rosario, Santa Fe Argentina
2004-2009 Assistant in Biosafety Symposium organized by Safety Area and Environment of Technical Secretariat. National University of Rio Cuarto, Río Cuarto, Córdoba.
05/2009 Assistant in III National Meeting of Biology Students, III JoNEBI. National University of Rio Cuarto, Río Cuarto, Córdoba.
10/2009 Participant in VI Argentine Congress of General Microbiology. SAMIGE. Villa Carlos Paz, Cordoba, Argentina.
05/2013 American Society for Microbiology General Meeting. (ASM 2013).Denver, Colorado, USA. Poster presentation: Switchgrass (*Panicum virgatum*) fermentation by sequential culture of *Clostridium thermocellum* and *Clostridium beijerinckii*: effect of particle size on gas production.

Independent Activities

1998-2003 Participation in Argentine Biology Olympiad.
2001: National Instance Argentine Biology Olympiad.
2003: National Instance Argentine Biology Olympiad.
2000-2001 Competitor in Zonal Instance Computer Olympiad.Laboulaye, Córdoba, Argentina.
2000-2001 Competitor in Provincial Instance Computer Olympiad, Laboulaye, Córdoba, Argentina.
2003 Mention as owner Category Student Computer Argentine Olympiad utilities.
10/2005 Helper in the Committee of Support for the XIV Argentine Biology Olympiad. Río Cuarto, Córdoba, Argentina
11/2006 Helper of the Facilitation Committee of the XV Argentine Biology Olympiad. Río Cuarto, Córdoba, Argentina.
7/2006 Participed as a guide in the 17th International Biology Olympiad, National University of Río Cuarto. Río Cuarto, Córdoba, Argentina
10/2007 Helper in the Facilitation Committee at the XVI Argentine Biology Olympiad. Río Cuarto, Córdoba, Argentina.
10-11/2008 Participed in Extracurricular Course: "First steps in university Teaching "organized by School of Natural, Physical-Chemical, and Exact Sciences. National University of Río Cuarto.
09/2009 Participant the First Student Convention 2009 Río Cuarto Fitness. Board of Physical Education, Sport and Recreation Welfare Department National University of Rio Cuarto, Río Cuarto, Córdoba.
05-06/2010 Online Biofuels course organized by G-Tek. Buenos Aires.

Honors

Bearer (the honor of being on the payroll flag) of the Secondary Institute General Levalle in 2004.General Levalle, Córdoba, Argentina.

Bearer (the honor of being on the payroll flag) of the School of Natural, Physical-Chemical, and Exact Sciences. National University of Río Cuarto, Río Cuarto, Córdoba. Argentina.