BUTANOL PRODUCTION FROM SWITCHGRASS USING NOVEL STRAINS OF *CLOSTRIDIUM BEIJERINCKII*

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BUTANOL PRODUCTION FROM SWITCHGRASS USING NOVEL STRAINS OF *CLOSTRIDIUM BEIJERINCKII*

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Abstract: Sugar substrates from cheap and sustainable feedstock such as lignocellulosic biomass can compete with chemical synthesis-derived butanol. The objective of this study is to evaluate butanol production using novel *Clostridium beijerinckii* strains with improved tolerance to lignocellulosic derived microbial inhibitory compounds (LDMICs) and to evaluate fermentation strategies to enhance butanol production. To achieve this, hydrothermolysis pretreated Alamo switchgrass was hydrolyzed by means of enzyme (Accellerase 1500) to produce enzymatic hydrolysate which contains approximately 65 g/L glucose and 2 g/L xylose. Interestingly, the concentration of furfural and hydroxymethylfurfural (HMF) in the non-detoxified hydrolysate was 60 mg/L and 4 mg/L respectively while the phenolic inhibitory compounds were present within the range of 4 -8 mg/L. Ferulic acid was not detected in the non-detoxified hydrolysate. However, 600 mL of the non-detoxified hydrolysate was detoxified using activated carbon, as a result, 80% of the furan and phenolic compounds were removed from the hydrolysate.

Consequently, ABE fermentation in 150 mL bottles using 50 mL working volume with 6% (v/v) inoculation level was performed anaerobically under N_2 / H_2 (95% / 5%) atmosphere in an anaerobic chamber. The ABE fermentation media used were P2 glucose medium, detoxified switchgrass hydrolysate medium, and non-detoxified switchgrass hydrolysate medium. Furthermore, wild type, AKR and SDR strains of *C. beijerinckii* were used as biocatalysts. The fermentation results show that AKR and SDR strains consumed about 30% more glucose in detoxified hydrolysate medium compared to P2 glucose medium. Consequently, about 20% more glucose was utilized by AKR and SDR strains in the non-detoxified hydrolysate medium with intermittent feeding than without intermittent feeding. Furthermore, about 20 g/L total ABE was produced by the SDR strain in the non-detoxified hydrolysate medium with intermittent feeding while the same strain produced 17 g/L total ABE in the detoxified hydrolysate medium. These results show that using inhibitor tolerant AKR and SDR strains and the intermittent feeding fermentation strategy eliminated the need for detoxification and improved ABE fermentation by about 15% when compared with the results of ABE fermentation in the detoxified switchgrass hydrolysate medium. These results showed there is a high potential of increased butanol yield from non-detoxified hydrolysate, which makes ABE fermentation more feasible.

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CHAPTER I

INTRODUCTION

Energy usage is concomitant to human existence. As a result, the importance of energy cannot be over emphasized. Energy is needed to produce goods, light, cook, and drive vehicles, power industrial engines and to operate domestic appliances. Subsequently, fossil fuel which is currently the cheapest source of energy is non-renewable and can be exhausted (Barreto, 2018; Chen, 2019). Consequently, the increase in world population, economic development and technological advancement has exponentially increased the dependence on energy. As a result, a huge reliability and usage has been placed on fossil fuel and its products, providing almost 80% of global energy demand while renewable energy sources supply about 20% of the world's energy. Hydropower accounts for about 16% of renewable energy sources while renewable biological sources accounts for only 2% (Hussain et al., 2017). Problems related to fossil fuel utilization include high price, environmental factors, nonrenewable nature and unsustainability. These negative factors have justified the reasons to search for an alternative means (Bharathiraja et al., 2017; Liu et al., 2015a; Panwar et al., 2011). Due to this, governments of developed countries, researchers, funding agencies globally are consistently investing funds to find alternatives to fossil fuel derived products and also alleviating their negative effects on the environmental.

According to the U.S. Department of Energy 2016 report, the U.S. energy consumption from coal and petroleum products from 2006 to 2016 decreased by 35 and 7.7%, respectively. However, crude oil production increased by 31% over the same period. It is also important to know

that the United States of America is currently the largest producer of crude oil globally (Barreto, 2018). The different forms of renewable energy include solar, wind, geothermal, hydro thermal and biomass. These forms of energy can provide a long term solution to energy problems but requires intense research efforts.

Brazil and U.S.A. produce bioethanol from sugarcane and corn, respectively which is known first generation bioethanol. As a result, using these food grade feedstocks for bioethanol production gave rise to the food versus fuel tussle. (Jalilnejad and Ghasemzadeh, 2019; Janda and Krištoufek, 2019).

Corn is the major feedstock for bioethanol in the United States, subsequently, about 1.6 billion gallons of bioethanol was produced in 1998, while production increased by about 89% in 2016 to produce 14.5 billion gallons of bioethanol (Robak and Balcerek, 2018) . Furthermore, due to the efforts by the government in terms of subsidy which ranges from 40 to 60 cents per gallon, the ethanol industry has experienced a boom since 2005 with total production per year ranging from less than 265,000 gallons in 1980 to about 11 billion gallons in 2008 (Tyner, 2008). Ethanol in the U.S.A is mostly utilized as a gasoline blend (E10, 10% ethanol and 90% gasoline) due to the fact that utilizing pure ethanol requires combustion engine modification (Phuangwongtrakul et al., 2016).

Despite these great results from these countries on exploring first generation feedstock for ethanol production, this called for an increase in price of corn and sugarcane and a demand to increase production (Hamelinck et al., 2005; Tyner, 2008). Subsequently, due to the hike in corn and sugarcane prices and the food versus fuel debate, the need to explore the second generation lignocellulosic biomass arose. Lignocellulosic biomass consists of cellulose, hemicellulose and

lignin while the cellulose and hemicellulose are linked to a lignin structure through covalent and hydrogen bonds, which is difficult to break (Zabed et al., 2016). The greater challenge with the utilization of lignocellulosic biomass for biofuel production is due to the delignification of the biomass via pretreatment to access the structural carbohydrate and subsequently monomeric sugars (Araújo et al., 2017). Consequently, reduction in overall cost of biofuel production from lignocellulosic biomass is important for its commercialization. Structural carbohydrates such as glucan, xylan, arabinan and mannan are produced from pretreatment of lignocellulosic biomass while enzymatic hydrolysis of pretreated biomass converts the structural carbohydrates to monomeric sugars (Liu et al., 2015a). Lignocellulosic biomass pretreatment methods include hydrothermolysis, alkali pretreatment, acid pretreatment and organosolv pretreatment (Baral and Shah, 2014; Chen et al., 2017; Liu et al., 2015a). Furthermore, enzymatic hydrolysis, which is the breakdown of structural carbohydrates to monomeric sugars precedes pretreatment. The produced hydrolysate after enzymatic hydrolysis consists of primarily glucose which is fermented with clostridia bacteria to acetone, butanol and ethanol (ABE). The delignification of the biomass leads to the release of Lignocellulose Derived Microbial Inhibitory Compounds (LDMICs), which inhibit cell activity during fermentation (Zhang et al., 2014). Due to this, it is important to develop an anaerobic acetogenic microorganism to tolerate LDMICs (Zhang et al., 2014). Several researchers have worked on (ABE) fermentation via biochemical conversion means. Conventional biochemical pathway for ABE production has utilized *clostridia* strains. The drawbacks in utilizing *clostridia* strains for ABE fermentation include cell inhibition by LDMICs, which are generated from the lignin degradation during pretreatment, butanol toxicity, by-products formation and low butanol concentration (Gottumukkala et al., 2017). In the light of this, research interests have been focused on engineering fermentative strains of *Clostridium beijerinckii* to tolerate inhibitors

derived as a result of biomass pretreatment, co utilize pentose and hexose sugar substrates and also to tolerate butanol toxicity (Ezeji et al., 2007a). The overall goal of this project is to examine the characteristics of wild type, and two genetically modified *C. beijerinckii* strains for ABE production from detoxified and non-detoxified switchgrass hydrolysates.

CHAPTER II

LITERATURE REVIEW

2.1 Biofuel definition

Biofuels can be produced from starchy crops like corn and sugar based plants like sugarcane, and from woody and other lignocellulosic biomass. The need for biofuels is becoming increasingly important due to the depletion of fossil fuels reserves. Also, the use of fossil fuels puts the environment at risk. Therefore, utilizing biofuels can reduce greenhouse gas emissions. Biofuels such as ethanol can be blended with gasoline typically at 10 % while butanol can power spark ignition engines without blending with gasoline and does not require engine modification (Phuangwongtrakul et al., 2016). Butanol has an energy density of 29.2 MJ/L while ethanol has an energy value of 21.2 MJ/L (Thakur et al., 2017). Butanol's energy density is about 10 % less than that of gasoline (32.5 MJ/L). Therefore, butanol is a better replacement for gasoline because no engine modification is required (Nanda et al., 2017).

2.2 Feedstocks for biofuel production

2.2.1 First generation biofuels

Starch based crops, sugar bearing plants and oil-bearing seeds are good feedstocks for first generation biofuels such as ethanol, butanol and biodiesel. Corn is the principal feedstock used in the United States for bioethanol production (Manochio et al., 2017). On the contrast, the food

versus fuel tussle has got researchers thinking about commercially viable technologies for biofuel production from a non-edible feedstocks (Araújo et al., 2017; Morone and Pandey, 2014).

Starch is a complex carbohydrate that will be converted into simple sugar through a process known as enzymatic hydrolysis (Felix et al., 2008). Ethanol production from sugarcane is conflicting with edible sugar production from sugarcane. Therefore, a renewable non-edible biofuel feedstock sources remain the alternative.

2.2.2 Second generation biofuels

Lignocellulosic biomass feedstocks are used for the production of second generation biofuels. Feedstocks for second generation biofuels include non-edible plants and agro wastes which are renewable. One way to utilize lignocellulosic biomass to produce biofuel and bio-based products is via the biochemical production pathway. Lignocellulosic biomass is made up of cellulose, hemicellulose and a protective layer called lignin. The cellulose and hemicellulose are rich in C5 and C6 fermentable sugars that can be converted to biofuels. However, some production steps are required to achieve this. Due to the recalcitrance offered by the lignin to access the sugar-rich cellulose and hemicellulose, pretreatment of lignocellulosic biomass is required. Pretreatment breaks down the lignin structure to access cellulose and hemicellulose. Another major drawback of the biochemical production pathway is that during pretreatment, Lignocellulose Derived Microbial Inhibitory Compounds (LDMICs) are released into the hydrolysates, which inhibits microbes' activities during fermentation. According to the publication of the Oklahoma Bioenergy Center in 2016, the predominant lignocellulosic biomass for biofuel production in Oklahoma is switchgrass. Others biomass utilized in the state are redcedar and forage sorghum. Switchgrass, can grow to 12 feet in height and tolerate heat, cold and draught (Eckberg et al., 2015). The grass has both upland and lowland varieties, but much emphasis will be made on the lowland varieties

due to the fact that it has a better yield on Oklahoma soil than the upland variety. The lowland variety was found to yield 2 tons/acres/year more than the upland variety in Chickasha and Haskell, Oklahoma (Plant and soil science department, OSU). The lowland varieties of interests are Alamo and Kanlow switchgrass. Butanol production by *Clostridium acetobutylicum* using Alamo switchgrass was studied by Liu et al. (2015a) and the butanol titer was 11 g/L. Also, Kanlow was studied by other researchers for butanol and ethanol production and was found to yield 8.05 g/L ethanol (Kai and Lars, 2016), 12 g/L butanol (Qureshi et al., 2010) and 22.5 g/L ethanol via simultaneous saccharification and fermentation (SSF) (Pessani et al., 2011).

2.2.3 Synthesis Gas

Second generation biofuel are also made from synthesis gas. Synthesis gas also called syngas is a gaseous mixture produced during the gasification of agricultural and industrial wastes. Syngas can be produced primarily from carbon-rich compounds primarily contains carbon monoxide, hydrogen and carbon dioxide and can include several impurities (Phillips et al., 2017). Syngas fermentation has a competitive edge over saccharification-fermentation route due to the fact that syngas fermentation utilizes the whole part of the biomass, that is cellulose, hemicellulose and lignin for fermentation resulting in higher ethanol yield (Sun et al., 2018). Furthermore, LDMICs that inhibit alcohol production during saccharification-fermentation process are eliminated through gasification to produce syngas (Phillips et al., 2017).

2.3 Biofuel production techniques

2.3.1 Syngas fermentation pathway

Syngas fermentation is part of the hybrid gasification-syngas fermentation process to produce alcohols and fatty acids from CO, H_2 and CO_2 (Sun et al., 2018). Biocatalysts such as *Clostridium ljungdahlii, C. carboxidivorans, C. ragsdalei,* and *Alkalibaculum bacchi* convert CO,

H_2 and CO_2 through a the reductive Acetyl-CoA route to alcohols and organic acids (Devarapalli et al., 2016).

Syngas fermentation is still characterized with some limitations such as presence of impurities in the syngas, and high cost of medium (Shen et al., 2018). However, more research has been reported on the development of biorefineries based on the hybrid technology in a recent review paper (Sun et al., 2019).

2.3.2 Biochemical Platform

This route utilizes starchy and lignocellulosic biomass as feedstocks for biofuel production. Biofuels production from lignocellulosic biomass requires pretreatment, enzymatic hydrolysis, fermentation and product recovery. However, it has been reported that lignocellulosic biomass pretreatment costs accounts for about 40% of the total biofuel production cost and it is also the most energy consuming process during biofuel production (Sindhu et al., 2016). Due to this, several pretreatment methods such as acid pretreatment with high pressure steam explosion (Alvira et al., 2010; Hendriks and Zeeman, 2009), ionic pretreatment (Yamada et al., 2017), ammonia fiber explosion (Sun et al., 2016), organosolv pretreatment (Amiri et al., 2014), and hydrothermolysis (Liu et al., 2015a) were developed. Hydrothermolysis was reported to remove 92.2% of the xylan, 16% of the glucan, and 2.4% of the lignin present in the dry biomass (Liu et al., 2015a). The competitive advantage of hydrothermolysis over acid and alkali pretreatment methods is that hydrothermolysis requires no acid or alkali catalyst for biomass pretreatment which in turn reduces pretreatment cost.

After pretreatment, the pretreated biomass is hydrolyzed to release glucose and xylose from cellulose and hemicellulose. The hydrolysate can contain LDMICs depending on the pretreatment

method used, which inhibits ABE fermentation. Enzymatic hydrolysis involves the use of enzymes to convert polysaccharides to monosaccharides or simple sugars. The different enzymes used include Accellerase 1500 (Liu et. al., 2015), Cellic CTec 2 cellulase (Qu et al., 2017), β-glucosidase and xylanase (Zhang and Ezeji, 2014).

LDMICs such as furfural, HMF, vanillic acid, Syringic acid, vanillin, syringaldehyde, p-coumaric acid and ferulic acid are released due to the breakdown of the lignin structure and thus cause cell inhibition during ABE fermentation (Ezeji et al., 2004). Zhang and Ezeji (2014) quantified the LDMICs in *Miscanthus Giganteus* (MG) hydrolysate and discovered that ABE production and growth of *Clostridium beijerinckii* was harshly inhibited by the LDMICs. Similarly, Liu et al. (2015a) found out that LDMICs such as HMF, furfural, cinnamaldehyde, vanillic acid, vanillin, hydroxybenzaldehyde, syringaldehyde, syringic acid, p-coumaric acid, ferulic acid, levulinic acid and formic acid inhibited butanol production in switchgrass hydrolysate and only achieved a butanol titer of 1 g/L. However, adjusting the pH from 5 to 6 in the same hydrolysate increased butanol titer to 4.3 g/L. Furthermore, the same study reported that switchgrass hydrolysate detoxification with activated carbon increased butanol titer to 11 g/L. This shows that the hydrolysate detoxification plays a significant role in increasing butanol production.

Liu et al. (2015a) also found out that addition of $CaCO_3$ and pH adjustment increased butanol titer (5.5 g/L) which conforms to the findings of Zhang and Ezeji (2014) whose finding was to determine the effect of $CaCO_3$ on ABE fermentation in (MG) hydrolysate. It was found that $CaCO_3$ mitigated the effect of LDMICs and facilitated ABE fermentation and the growth of *C. beijerinckii*. However, the cost of $CaCO_3$ increases ABE production cost. This cost can be avoided by developing an economically viable technology for ABE fermentation.

C. beijerinckii and *C. acetobutylicum* were reported to be high butanol producing strains (Qureshi, 2014). Other butanol producing *clostridia* strains include *C. aurantibutyricum, C. cadaveris, C. pasteurianum, C. saccharoperbutylacetonicum, C. saccharobutylicum C. sporogenes,* and *C. tetanomorphum* (Inui et al., 2008; Lin and Blaschek, 1983). The indicator for butanol production for these strains is how tolerant are they to butanol and LDMICs due to the fact that butanol is toxic to the microorganism producing it (Qureshi et al., 2014b). *C. acetobutylicum* was inhibited by 50% at a butanol titer of 7 g/L while 50% cell inhibition was noticed in a butanol-tolerant SA-1 strain at a butanol titer of 15.5 g/L (Lin and Blaschek, 1983). This correlates with the findings of Qureshi (2014) who stated that *C. beijerinckii* P260 cannot produce more than 13-18 g/L butanol. However, to ameliorate the cell inhibition problem, butanol recovery from the fermentation broth was recommended.

2.4 ABE Production Processes

2.4.1 Biomass Milling

Biomass has to be milled once it is harvested. After harvesting, biomass is dried prior to milling. The pre-milling drying step can be sun dried to minimize processing costs (Qu et al., 2017). In order to achieve successful milling, the biomass mechanical properties need to be studied. This is due to the fact that biomass characterization and milling is dependent on its compressive and shear strength (Mayer-Laigle et al., 2018).

2.4.2 Biomass Pretreatment

Lignocellulosic biomass generally consists 40 – 55% cellulose, 24 – 40% hemicellulose and 18 – 25% lignin (Kumar et al., 2009). The physicochemical, structural and compositional properties of biomass make it difficult to access the cellulose and hemicellulose of the biomass for monomeric sugar production which will be fermented for ABE production (Kumar et al., 2009).

The cellulose consists of structural carbohydrate which can be converted to monomeric sugars. This important part of the plant cell wall is held together by β-(1,4) glycosidic bonds (Agbor et al., 2011). The chief goal of pretreatment is to optimally recover the structural carbohydrate prior to enzymatic hydrolysis. The chief bottleneck to this goal is the resistance the lignin offers. Furthermore, LDMICs mainly furfural and phenolic compounds are released during pretreatment. These LDMICs inhibit the activities of microorganisms during ABE fermentation (Bhutto et al., 2017).

Consequently, several strategies have been adapted for optimal lignin removal with minimal cellulose loss and generating LDMICs. Hydrothermolysis is a pretreatment method that was developed about fifty years ago aims at utilizing water at elevated temperature to delignify biomass (Bonn et al., 1983). Different temperature ranges for hydrothermolysis have been previously explored. Hydrothermolysis pretreatment of pure cellulose (Wattman no. 1 filter paper) between 260 and 270ºC resulted in relatively high glucose concentration (about 50%) with water flow rate between 11 – 12 cm^3/min from the storage tank to the reactor vessel while the resident time at this temperature and flow rate was not reported. The experiment was performed in batch mode (Bonn et al., 1983). Elsewhere, the hydrothermolysis of pine wood was found to solubilize about 25% of the lignin at 240ºC in 10 mins (Ståhl et al., 2018). Furthermore, almost all of the pine wood hemicellulose was completely liquefied with an unavoidable 12.5% cellulose loss at 200 ºC (Ståhl et al., 2018). Also, the effects of hydrothermolysis process conditions on ethanol production from switchgrass was determined (Suryawati et al., 2009). Three temperatures of 190, 200 and 210°C and holding times of 10 mins, 15 mins and 20 mins were tested. The results indicated that switchgrass pretreated at 190°C has the highest xylan recovery in the hydrolysate while pretreatment at 210°C with a holding time of 15 mins yielded the highest ethanol

concentration of 16.8 g/L which is 72% of the theoretical yield (Suryawati et al., 2009). In another study, switchgrass was pretreated using hydrothermolysis at 200°C with a holding time of 10 mins resulted in about 55% glucan, 3% xylan, and about 30% lignin (Liu et al., 2015a). Also, the same hydrothermolysis operating conditions yielded about 57.7% glucan (Pessani et al., 2011). Lignin was pretreated to produce bio-oils and phenolic monomers at a temperature range between 200 and 350ºC for one hour. At 300ºC, a yield of 11.8 mg/g phenolic monomers was achieved while a higher yield of 18.8 mg/g was achieved at a temperature of 350ºC (Islam et al., 2018). In addition, another study was carried out to produce monoaromatic phenolic compounds from organosolv lignin which was derived from beech wood through hydrothermolysis. Maximum yield of about 10% of monoaromatics was achieved at 350ºC for 60 min while syringol, guaiacol and syringaldehyde production were optimal at between 270 and 290ºC (Hashmi et al., 2017). Hydrothermolysis is recommended because it is a non-chemical process which is not hazardous to the environment and the operator. In contrast the cost of equipment and electricity is relatively high for hydrothermolysis. Therefore, designing cost effective equipment and is one of the ways of improving this pretreatment process. Dilute acid pretreatment utilizes acid concentration less than 1% to delignify biomass (Amiri and Karimi, 2018). A study shows that different concentrations of sulfuric acid were tested. 0.22, 0.49, and 0.98% w/w at 140, 160, 180 and 200°C to pretreat corn stover were investigated (Lloyd and Wyman, 2005). However, the results indicated that glucose and xylose yields of 56 and 37%, respectively, were achieved (Lloyd and Wyman, 2005). Another study investigated the use of 0.5% sulfuric acid to pretreat switchgrass for 60 mins at 121°C. The results indicated about 47% of the glucan was released (Kshirsagar et al., 2015). Using an increased acid concentration, 5% v/w sulfuric acid was investigated at 121°C for 30, 60, and 90 min (Gonzales et al., 2016). This study aimed at producing sugars from pine tree wood,

empty palm fruit branch, and rice husk for subsequent hydrogen production and fermentation. The results however show that the maximum sugar yield was achieved at 60 min of pretreatment. The researchers found that 46% of glucose was produced from the empty palm fruit bunch and rice husk while 39% glucose was produced from the pine tree wood (Gonzales et al., 2016). Also, pretreatment using dilute NaOH (1%) yielded about 49% glucan for both switchgrass and phragmites (Gao et al., 2014). Organosolv pretreatment utilizes ethanol and dilute acid for biomass pretreatment (Amiri et al., 2014).

2.4.3 Enzymatic Hydrolysis

The solid fraction obtained after pretreatment is rich in cellulose (Amiri and Karimi, 2018). The fractions of the cellulose and hemicellulose contain long chain polymeric sugars like glucan, xylan, arabinan and mannan that needs to be hydrolyzed using enzymes (Liu et al., 2015a). Factors that affect sugar yield during enzymatic hydrolysis are enzyme loading, cellulases activity, temperature and pH (Kumar et al., 2018). The optimal operating time for enzymatic hydrolysis varies. However some studies have shown that 48 h is ideal (Liu et al., 2015a) while other studies performed the operation for 72 h (Gao et al., 2014). After enzymatic hydrolysis, the microbial inhibitors present in the solid fraction after pretreatment are present in the hydrolysate as soluble lignin, otherwise known as soluble lignin content (SLC) (Liu et al., 2015a). The concentration of the SLC, which is a measure of inhibitory compounds in the hydrolysate, can be reduced by detoxifying the hydrolysate (Cho et al., 2009; Ezeji et al., 2007a; Liu et al., 2015a). Different enzymes have been employed for hydrolysis. A study showed that enzymatic hydrolysis performed using Cellic CTec2 enzyme with hydrolysis conditions of 50°C, 150 rpm for 72 h produced 44 and 16 g/L of glucose and xylose respectively from 5 g of oven dried pretreated switchgrass and phragmites (Gao et al., 2014). Furthermore, a different study shows that reducing sugar

concentration up to 94 g/L can be achieved from 120 g/L pretreated rice straw by adding loading the flask with 80 g/L rice straw and 0.05 mol/L acetate buffer (pH 4.8) initially while 20 g/L pretreated rice straw was added intermittently during the enzymatic hydrolysis process. Pretreated solids and enzymes were added at 12 h and 24 h (Li et al., 2018). This enzymatic hydrolysis method is desirable when a high initial concentration of glucose is required for continuous ABE fermentation. Another study utilized Accellerase 1500 to hydrolyze pretreated switchgrass at operating conditions of 50°C at 250 rpm for 48 h producing about 78 g/L of glucose (Liu et al., 2015a). Also, in a different study, pretreated rice straws were subjected to enzymatic hydrolysis using cellulases and β-glucosidase. The pretreated solids were soaked in 50 mM sodium citrate buffer with pH 4.8 prior to enzymatic hydrolysis (Nanda et al., 2014). The hydrolysis which yielded about 40 g of glucose per 100 g of straw was performed at 45 °C and 140 rpm for 72 h (Moradi et al., 2013). Another study showed the utilization of three different enzymes (cellulase, β-glucosidase, and xylanase) at 45°C for 72 h, which converted about 45% glucan and yielded about 60 g/L total sugars (Nanda et al., 2014).

2.4.4 Acetone, Butanol, and Ethanol (ABE) Fermentation with mono-culture techniques

Acetone, butanol and ethanol (ABE) fermentation is the center of the butanol production value chain. The sugars namely glucose and xylose which were produced after pretreatment and enzymatic hydrolysis lignocellulosic biomass are fermented by anaerobes to produce solvents and fatty acids. ABE fermentation can either be a batch process (Ezeji et al., 2007a; Gao et al., 2014; Lin and Blaschek, 1983; Liu et al., 2015a) or continuous fermentation (Ezeji et al., 2005; Qureshi et al., 2014a; Survase et al., 2011). During batch ABE fermentation, the substrate and limiting nutrients are added at 0 h and products are not recovered during the fermentation. Operating parameters such as pH are not controlled. On the other hand, during the continuous process, the

limiting nutrient and substrate are fed continuously into the bioreactor. The dilution rate is adjusted to prevent cell and product washout. Also, products can be recovered during fermentation and operating parameters such as pH can be instantaneously controlled (Lee et al., 1999). Solventogenic *clostridia* species has gained much research attention due to its ability to produce solvents like butanol and other industrially important chemicals (Ezeji et al., 2007b).

Different clostridia species has been studied. *C. beijerinckii* BA101 was used for butanol production from glucose produced from agricultural residues as substrate (Ezeji et al., 2007a; Ezeji et al., 2005). Also, *C. saccharobutylicum* DMZ 13864 was used for butanol production from alkali pretreated switchgrass (Gao et al., 2014). Furthermore, an investigation also researched the butanol production from extruded corn broth using *C. acetobutylicum* (Lin and Blaschek, 1983; Moradi et al., 2013; Park et al., 1989). *C. acetobutylicum* ATCC 824 was also used for butanol production from switchgrass (Liu et al., 2015a). Also, in another study, 12 *clostridia* strains were screened to select the ideal strain for butanol production from lignocellulosic hydrolysate. The strains were *C. acetobutylicum* DSM 1731, 1732, 1733, 1738, 4685, 6228 and ATCC 824, *C. beijerinckii* DSM 1739 and 6422, *C. saccharobutylicum* DSM 13864, *C. saccharoperbutylacetonicum* DSM 14923 and 2125 (Magalhães et al., 2018). However, *C. saccharobutylicum* DSM 13864 was the best strain. This strain consumed about 95% of the sugars in the sugarcane straw hydrolysate while producing 10.33 g/L total ABE (Magalhães et al., 2018).

Butanol concentration and yield from solventogenic clostridia species differ based on operating parameters and medium formulation. It is important to note that, the prime limiting factor to butanol yield is the presence of microbial inhibitory compounds (Ezeji et al., 2007a). A study investigated ABE fermentation using alkali pretreated non-detoxified switchgrass and phragmites hydrolysate with pure glucose medium as control while *C. saccharobutylicum* was used as a

biocatalyst. The results showed a total ABE of about 23 g/L from the pure glucose medium (acetone 9.6 g/L, ethanol 1.3 g/L and butanol 12.2 g/L). The non-detoxified hydrolysate from switchgrass shows a similar result to the control with about 22 g/L total ABE (acetone 9.1 g/L, ethanol 0.6 g/L, butanol 13 g/L) while phragmites hydrolysates yielded 19.8 g/L total ABE (Gao et al., 2014). In another study, hydrolysates were produced from wood pulp and detoxified by overliming, activated carbon adsorption, resin adsorption and evaporation (Lu et al., 2013). They showed a total ABE of 11.4 g/L was produced with the wood pulp hydrolysate detoxified with resin adsorption and evaporation (Lu et al., 2013). Furthermore, another study investigated and screened 12 *clostridia* strains using glucose from sugarcane straw hydrolysate (Magalhães et al., 2018). The hydrolysate was not detoxified, supplemented with minerals and vitamins and was also buffered with MES. The butanol titer can be improved with the use of detoxification techniques because the hydrolysate contains 0.6 g/L, 0.5 g/L, and 500 mg/L of HMF, furfural and phenolic compounds, respectively (Magalhães et al., 2018).

Studies have shown that supplementing hydrolysate with some minerals and elements improved ABE titer. The supplementation of non-detoxified hydrolysate with $CaCO_3$ improved cell growth, glucose utilization and ABE titer (Han et al., 2013; Liu et al., 2015a). Also, adjusting the pH of non-detoxified hydrolysate to 6 and supplementing it with 4 g/L $CaCO_3$ increased butanol titer from 1 to 6 g/L (Liu et al., 2015a).

Another study examined the use of cornstarch as substrate to produce butanol in a 3 L bioreactor with a dilution rate of 0.02 h^{-1} using *C. beijerinckii* BA 101. ABE titer was compared at 19 and 37°C. ABE titers were 6 and 7.2 g/L at 19 and 37°C, respectively, after 260 h (Ezeji et al., 2005). Also, continuous ABE fermentation of concentrated cassava bagasse hydrolysate was reported using immobilized *C. acetobutylicum* in a fibrous bed reactor. Continuous gas stripping

was used for product recovery (Lu et al., 2012). They shows that the concentrated cassava bagasse hydrolysate contains 584.4 g/L glucose while a total ABE of 108.5 g/L was produced (acetone 27 g/L, butanol 76.4 g/L and ethanol 5.1 g/L) with no acid accumulation (Lu et al., 2012). Another study investigated the use of spent liquor from spruce chips for batch and continuous ABE fermentation using *C. acetobutylicum* DSM 792 (Survase et al., 2011) . During the batch process, 8.8 g/L total ABE was achieved from 35 g/L of glucose. Furthermore, the effect of dilution rates (0.21, 0.36, 0.64 and 1.07 h^{-1}) on solvent production during the continuous process in column reactors was studied. The results showed that dilution rate of 0.21 h^{-1} was the most effective with total ABE production of 12 g/L (Survase et al., 2011). Higher butanol or ABE titers can be achieved under continuous conditions if the solvents are recovered from the reactor as they are produced to prevent cell inhibition (Lin and Blaschek, 1983).

2.4.5 Acetone, Butanol and Ethanol Fermentation with two-stage and co-culture techniques

Optimizing ABE production from lignocellulosic hydrolysate is important to increase titer and yield (Ezeji et al., 2007a). Different strategies have been employed to optimize carbon conversion efficiency for optimum ABE yield. Furthermore, another study investigated the co-culture of *C. beijerinckii* and *C. tyrobutyricum* as a means of enhancing butanol production, yield and volumetric productivity in a continuous fermentation system with 0.144 h^{-1} dilution rate (Li et al., 2013). Results of this study shows butanol production of 6.66 g/L using cassava starch as a substrate with an ABE yield of 0.36 g/g (Li et al., 2013). This promising technique however can be improved by varying the inoculum ratio in the co-culture. Elsewhere, a study investigated ABE fermentation with *C. acetobutylicum* and *S. cerevisiae* co-culture system (Luo et al., 2015). The aim of the co-culture was to enhance glucose consumption by *S. cerevisiae* which has better

glucose utilization ability than that of *C. acetobutylicum*. The experiment was conducted in a fed-batch 7L reactor which yielded about 15 g/L butanol (Luo et al., 2015).

2.5 Factors affecting ABE fermentation

2.5.1 pH and temperature effect on ABE fermentation

The pH at which fermentation occurs determines cell viability, organic acid and solvent production. The pH of the fermentation medium should be buffered to about 6 to avoid excessive accumulation of acid which could lead to acid crash. Furthermore, this factor also influences the microbial pathway shift from acetogenesis to solventogenesis. In a recent study, the effect of initial pH 5.0 to 7.0 on butanol production was studied (Al-Shorgani et al., 2018). It was observed that a pH range of 6.0 and 6.2 favored cell growth and butanol production. Butanol concentration with an initial fermentation pH of 6.2 was at least 15% better than the other above listed initial pH values (Al-Shorgani et al., 2018). Furthermore, for the fermentation of pure glucose, it was found that solventogenesis was switched at a pH of 4.8 from an initial pH of 6 (Jiang et al., 2014). For optimal butanol production, the initial pH of the fermentation broth should be close to neutral between 6.0 and 6.8 while the accumulation of organic acids during the exponential cell growth phase will lower the pH between 4.8 and 5.0 to switch to solventogenesis (Jiang et al., 2014). Furthermore, a study to determine the optimal pH for butanol production showed that a pH of 5.0 favored butanol production (Saini et al., 2016). As a consequence of the above discussion, for optimal butanol production, an initial pH of between 6.0 and 6.2 should be adopted while the accumulation of organic acids will lower the pH to 4.8 – 5.0 for a switch to solventogenesis. To further support the selected pH range for butanol production, another study revealed that organic acid assimilation and accumulation will drop the pH within the range of 4.5 – 5.0. Therefore, at this pH range, butanol is being produced (Lee et al., 2008). It is also important to note that reaching

this pH without accumulating enough organic acid will lead to low ABE production (Lee et al., 2008).

Temperature is an important factor to consider during fermentation. Different microbes adapt to different temperature environment which ultimately affect their growth and productivity. *Clostridia* species survive at a mesophilic temperature range between 30 to 38ºC and are consequently regarded as anaerobic mesophiles (Lee et al., 2008). A previous study shows that butanol fermentation at 30ºC produced about 13.5 g/L butanol using an initial pH of 6.0 (Al-Shorgani et al., 2018).

2.5.2 Effects of inhibitors on ABE fermentation

Lignocellulosic biomass pretreatment generates microbial inhibitory compounds, which make it difficult for microbes to thrive during ABE fermentation. The inhibitory compounds are generated from lignin breakdown. Classes of microbial inhibitory compounds are furan and phenolic compounds, acids, and aldehyde (Ezeji et al., 2007a; Liu et al., 2015a; Pienkos and Zhang, 2009). These microbial inhibitory compounds have distinct inhibition mechanism. Weak acids such as formic acid, acetic acid and levulinic acid which are formed by the degradation of furfural and hydroxymethylfurfural (HMF) are mainly responsible for acid crash and result in little amount of ABE production (Qi et al., 2017). Formic acid concentration of 0.5 g/L in a 50 g/L sugar medium resulted in 0.75 g/L total ABE (Qi et al., 2017). Subsequently, 11.4 g/L total ABE was produced when the medium was supplemented with 4 g/L $CaCO_3$. This result shows about 93% increase in ABE production while highlighting the inhibition effects of formic acid (Qi et al., 2017). Also, microbial inhibitors such as furan and phenolic compounds and weak acids are referred to as process inhibitors while inhibition due to solvents such as butanol is referred to as product inhibitors (Baral and Shah, 2014). Furfural and HMF are formed degradation of sugars (pentose

and hexose) (Baral and Shah, 2014). Other studies showed that there was no cell mass of about 2.7 g/L was achieved in a medium containing 2 g/L of furfural and 2 g/L of HMF (Ezeji et al., 2007a). In contrast, cell inhibition occurred in medium with about 1.0 g/L of ferulic acid and 1.0 g/L of p-coumaric acid. Ferulic acid and p-coumaric acid were found to be the most toxic compounds and resulted in acid crash (Ezeji et al., 2007a). Liu et al. (2015a) investigated butanol production using *C. acetobutylicum* from non-detoxified switchgrass hydrolysate using which has microbial inhibitors such as HMF, furfural, coumaric acid, syringic acid, vanillin, vanillic acid, and cinnamaldehyde. Furfural and HMF concentrations were about 4.5 g/L and 0.3 g/L respectively while phenolic inhibitor concentration ranged from 0.02 to 0.04 g/L. ABE fermentation in this non-detoxified switchgrass hydrolysate showed a low butanol titer of less than 1 g/L due to acid crash with undissociated acid concentration greater than 60 mM (Liu et al., 2015a).

However, supplementing the fermentation medium with 4 g/L $CaCO_3$, which acted as a buffer for the fermentation medium, increased the butanol titer to about 6 g/L (Liu et al., 2015a). The detoxification of the hydrolysate with activated carbon removed all the inhibitors except cinnamaldehyde. ABE fermentation in the detoxified switchgrass hydrolysate resulted in 11 g/L butanol and 17 g/L total ABE (Liu et al., 2015a). Also, Liu et al. (2015b) examined butanol production from redcedar hydrolysate using *C. acetobutylicum* and *C. beijerinckii*. Unlike the switchgrass hydrolysate study, non-detoxified redcedar hydrolysate inhibitor concentrations were in mg/L range. HMF and furfural concentrations in the non-detoxified redcedar hydrolysate were about 70 and 25 mg/L, respectively, while phenolic compounds concentration ranges from 0.3 to 202 mg/L. Both strains produced about 1 g/L butanol in the non-detoxified hydrolysate while detoxifying the hydrolysate increased butanol titer from 1 to 13 g/L (Liu et al., 2015a)

CHAPTER III

RESEARCH OBJECTIVES

The goal of this research is to explore the use of switchgrass for butanol production. Acetone, butanol and ethanol (ABE) will be produced from Alamo switchgrass using engineered *Clostridium beijerinckii* strains, notably aldo/keto reductase (AKR) strain, short chain dehydrogenase reductase (SDR) strain and the wild type strain (Okonkwo et al., 2019). The AKR and SDR strains were genetically modified by the overexpression of some hydrogenases to tolerate LDMICs notably furfural up to 5 g/L during ABE fermentation (Okonkwo et al., 2019).

The main research objective is to evaluate butanol production using novel stains of *C. beijerinckii* (Wild type, AKR and SDR strains) with improved tolerance to lignocellulose derived microbial inhibitory compounds LDMICs. Also, fermentation strategies to enhance butanol production will be evaluated. This is achieved by multiple tasks that include pretreatment of biomass, hydrolysis of pretreated biomass, detoxification of hydrolysate and ABE fermentation.

CHAPTER IV

MATERIALS AND METHODS

ABE production from Alamo switchgrass requires several unit operations like biomass collection and storage, biomass milling, pretreatment, enzymatic hydrolysis, hydrolysate detoxification and ABE fermentation as shown in Figure 4.1

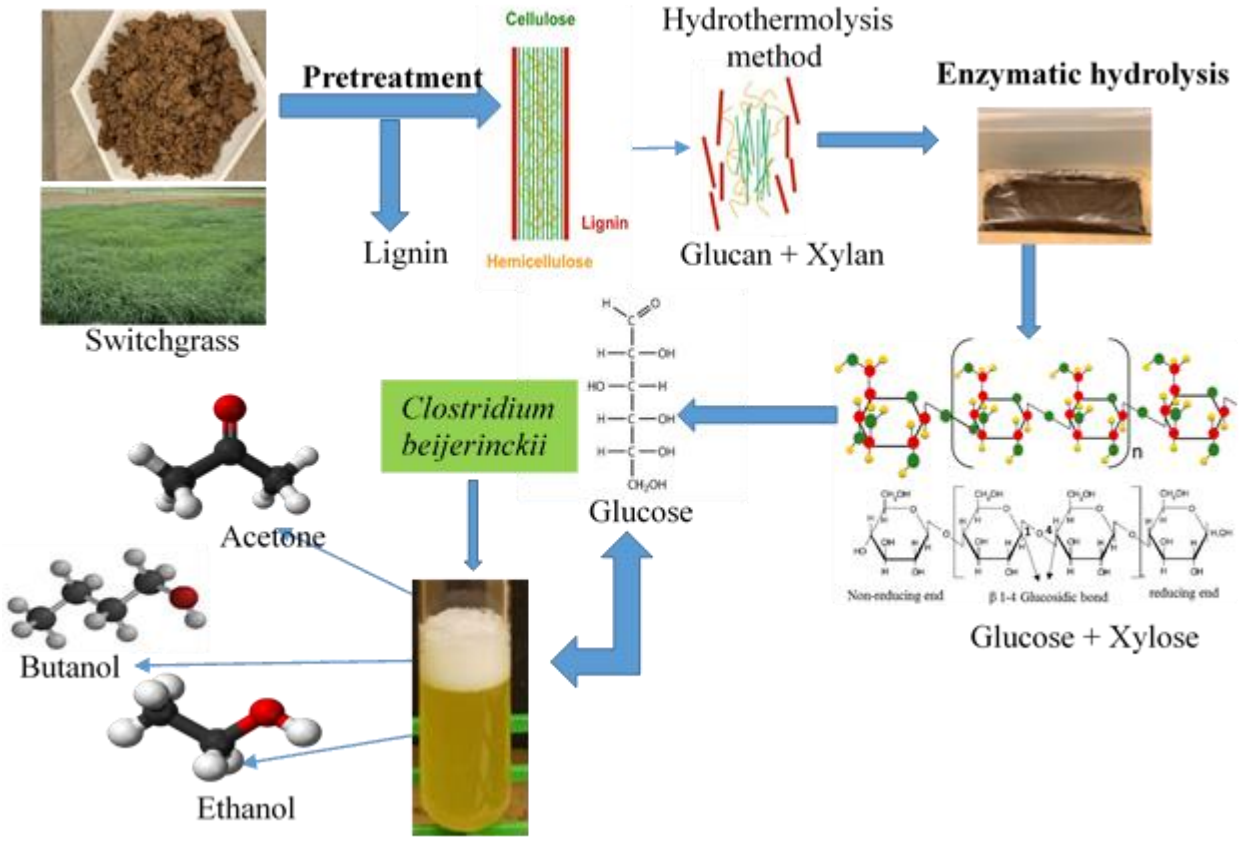


Figure 4.1: Typical butanol production process from switchgrass

4.1 Biomass Collection, Storage and Particle Size Determination

Alamo switchgrass was stored in 7 boxes which weighed about 1 kg prior to milling. To determine the particle size distribution of the biomass prior to milling, 100 g of biomass sample was placed in the sieve collection with five different mesh sizes. The vibrating sieves separated the particles according to their sizes. The mass of retained biomass on each of the sieves was measured.

4.2 Biomass Milling

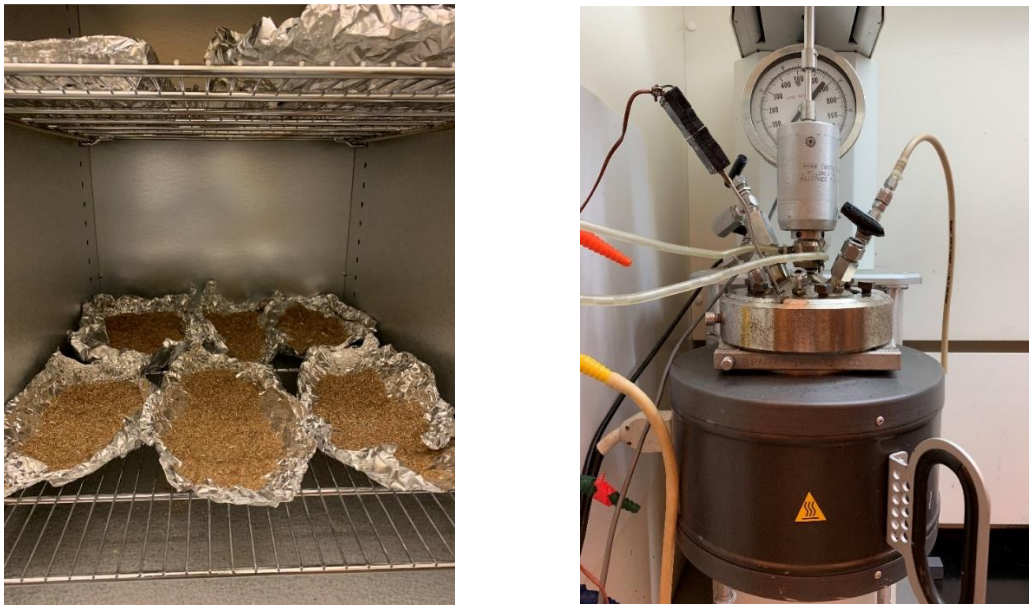
The biomass was milled using a hammer mill in a milling company at Ponca City, Oklahoma. Particle size distribution was determined after milling as described above.

4.3 Hydrothermolysis Pretreatment

For the pretreatment, 65.53 g of biomass was kept in the oven at 105ºC in aluminum pans as shown in Figure 4.2. The weight of the biomass was taken before and after drying to calculate the moisture content.

65.53 g of dried biomass with 10 % (w/w) loading and 534.47 g of DI water were transferred into the reactor. The Parr reactor was tightly secured with wrenches and placed on the heating station under the fume hood. Furthermore, a heating jacket was firmly secured at the base of the Parr reactor. A thermocouple was placed in its position in the Parr reactor as shown in Figure 4.2. The agitation speed was set at 500 rpm. Temperature and pressure readings were recorded every 2 min until 45ºC. It took about 8 min to attain 45ºC. Furthermore, the temperature setting was adjusted to 200ºC and held at this temperature for 10 min.

Afterwards, the reactor was transferred immediately into an ice bath. The temperature of the reactor dropped to below 60ºC with continuous stirring, after which the pressure was released in the fume hood. The contents of the reactor were filtered using vacuum filtration while the retained solids were weighed. The pre-hydrolysate was stored at 4ºC for further analysis. Consequently, the pretreated biomass was washed with 500 g of DI water and mixed using the magnetic stirrer. Then it was filtered using vacuum filtration. The washing steps were performed four times while the wash water samples were taken at all washing for pH analysis. Samples of pretreated solids before and after washing were kept in the oven to determine the percentage of dry solids. The washed pretreated solids were kept in a well labelled zip lock bag and kept in the cold room for further analysis. A typical pretreatment process is shown in Figure 4.3.



(a) (b)

Figure 4.2: (a) Biomass drying in the oven at 105°C and **(b)** 1 L parr reactor used for switchgrass pretreatment.

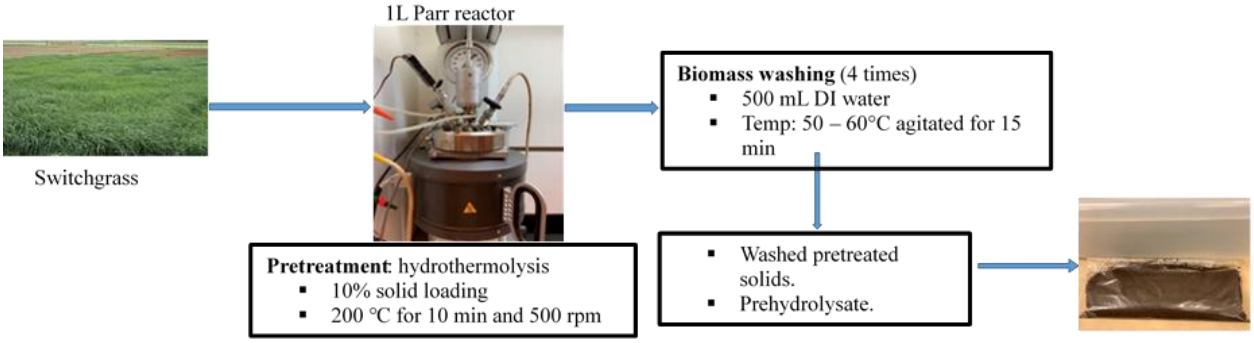


Figure 4.3: Typical switchgrass hydrothermolysis pretreatment process.

4.4 Compositional Analysis

Compositional analysis was performed to determine the amount of structural carbohydrate present in the sample using a method that has been previously reported (Sluiter et al., 2008). To achieve this, the following items are required. 30°C water bath, pressure tubes with stirrers and Teflon caps, vacuum oven, Sugar Recovery Standards (SRS) and 72% sulfuric acid. Prior to the experiment, 5 g of the pretreated switchgrass was kept in the vacuum oven and allowed to dry for about 24 h. 0.3 g of the dried sample was measured in the pressure tubes; each sample was prepared in duplicate. In addition, about 0.9 g of the dried sample was transferred to an aluminum pan of known weight to measure the moisture content.

3 mL of 72% sulfuric acid was added to the pressure tubes with pretreated switchgrass samples. The acid and sample were evenly mixed using the stirrer stick to ensure that all the biomass has been immersed in the acid. After achieving this, the pressure tubes were incubated in the 30°C water bath for 1 h while the content was stirrer at 10 mins interval. After 1 h incubation period, 84 ml of DI water was added to each tube while ensuring that the stirrer stick was well rinsed not to lose any solids. Consequently, 10 ml of SRS was also transferred into two pressure tubes while adding 348 µL of 72% sulfuric acid. All tubes were fitted with the Teflon caps with O ring while the tubes were tightly sealed not to lose any vapor prior to autoclaving. All tubes were autoclaved using cycle 4 for 60 min at 121°C. Then, the tubes were left in the fume hood to cool down to room temperature for 30 min.

The content of the tubes was vacuum filtered to separate the solids from the aliquot. The solids were collected in crucibles of known weight and dried in the 105ºC oven for at least 4 h. Samples of the aliquot was taken for absorbance measurement at 205 nm to estimate the Acid Soluble Lignin (ASL) while another sample was taken for pH adjustment between 5 and 6 using

pure $CaCO_3$ prior to analysis using HPLC. The samples were analyzed on the HPLC using Aminex HPX-87P column utilizing DI water as mobile phase flowing at 0.6 ml/min at 80ºC.

4.5 Enzyme Activity

Cellulases activity determination is an essential procedure because it determines the quantity of the enzyme needed for enzymatic hydrolysis. The reagents used during enzyme activity measurement are 10 mg/ml glucose, 0.05M citrate buffer solution (pH 4.8), dinitrosalicylic (DNS) reagent, and Whatmann No 1 filter paper. The Whatmann No 1 filter paper was cut into 50 mg size strips rolled and then placed into 8 long test tubes which were labeled from T_1A, T_1B, T_2A, T_2B, T_3A, T_3B to T_4A, T_4B. 1 ml 0.05M citrate buffer (pH 4.8) was placed in each of the test tubes to soak the filter papers.

In addition, enzyme dilutions from Accellerase 1500 were prepared in four separate tubes. These enzyme dilutions were prepared from an enzyme stock which was diluted 20 times with 0.05M citrate buffer. A 0.5 ml of each diluted enzyme was added to the 8 test tubes while 0.5 mL was also added to the enzyme control tubes (four tubes: EC 1, EC 2, EC 3, EC 4). Each replicate had its own control tube. Furthermore, a reagent blank (1.5 ml citrate buffer), and substrate control tubes (filter paper + 1.5 ml citrate buffer) were also prepared. Then, two sets of glucose dilution were also prepared. The first set was the glucose dilution in four separate tubes with 1 ml of 10 mg/ml glucose concentration in each tube and 0.5, 1, 2 and 4 mL of 0.05M citrate buffer were added to the glucose dilution tubes, respectively. Furthermore, the second set was the glucose standard tubes (four tubes) that were prepared by adding 0.5 mL of each of the glucose dilution to 1 mL 0.05M citrate buffer in each of the glucose standard tubes.

All 18 tubes, blanks, controls, glucose standard tubes and enzymes assay tubes were incubated at 50ºC for 60 min. The reaction was stopped by adding 3 mL of DNS reagent in each tube. The tubes were then placed in a boiling water bath for 5 min. After 5 min, all the tubes were transferred to a cold ice bath for at least 30 min until all the pulps settled as per NREL protocol for cellulases activity determination. The absorbance of the samples is measured at 540 nm against the reagent blank.

4.6 Enzymatic Hydrolysis

Enzymatic hydrolysis protocol previously described by Liu et al. (2015a) was employed. The following materials were used, a 250 mL flasks (seven), a shaker, Accellerase 1500, acetate buffer (pH 5.5) and DI water. 47.27 g of pretreated switchgrass on wet basis was measured and transferred into the Erlenmeyer flasks while 40.38 mL of DI water was also added. The pretreated biomass and water were autoclaved using cycle 4 at 121°C for 1 h. After autoclave, 7352 µL of enzyme with 35.15 FPU/mL cellulase activity and 5 mL of 1M acetate buffer were added into the flask while the lost water during autoclave was replenished using autoclaved DI water. The total working volume in the flask was 100 g.

The flasks were labelled Flasks 1 through 7. After autoclave, sterilized DI water was added in the biosafety cabinet to compensate for the lost water. Enzyme and acetate buffer were added in the biosafety cabinet. Consequently, the flask was transferred to the incubating shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA). The shaking conditions were 250 rpm at 50°C. Samples were taken every 6 h until 54 h. After a sample was withdrawn from each flask, all samples were centrifuged at 13,000 rpm for 10 min and filtered with a 0.2 µm nylon filter. The pH of the sample was also measured. The samples were analyzed by Agilent 1100 series HPLC (Agilent technologies, Santa Clara, CA USA) using Aminex HPX-87P column utilizing DI water

as mobile phase flowing at 0.6 ml/min at 80ºC. This column was used to measure glucose, cellobiose, xylose, galactose arabinose and mannose. The Aminex HPX-87H column was used with diluted sulfuric acid as mobile phase flowing at 0.6 ml/min at 60ºC to measure cellobiose, glucose, xylose, HMF and furfural. The concentrations of glucose and xylose in the hydrolysate were about 65 and 2 g/L, respectively. After enzymatic hydrolysis, the hydrolysate was immediately centrifuged in the Beckman Coulter centrifuge (Avanti J-E, Beckman Coulter, Inc., Brea, CA, USA) four times at 20,000 rpm for 15 min using rotor 20 to ensure that all the solids were removed from the hydrolysate.

4.7 Detoxification of Enzymatic Hydrolysate

Some of the produced hydrolysates was detoxified using (10% w/v) Calgon rod shaped activated carbon (AP4-60, Calgon Carbon Corporation, Pittsburgh, PA, USA). The activated carbon was measured on the weighing scale and boiled in hot water for 3 h. After boiling, the activated carbon was dried overnight. Subsequently, the activated carbon was measured and added to an Erlenmeyer flask along with the hydrolysate. This mixture was then transferred to the shaker at 250 rpm and 30°C for 1 h. Then, the mixture was centrifuged 4 times at 20,000 rpm for 15 min to remove the fine activated carbon particles from the detoxified hydrolysate. After centrifugation, the detoxified hydrolysate was stored at -20°C prior to fermentation. Detoxification steps are shown in Figure 4.4.

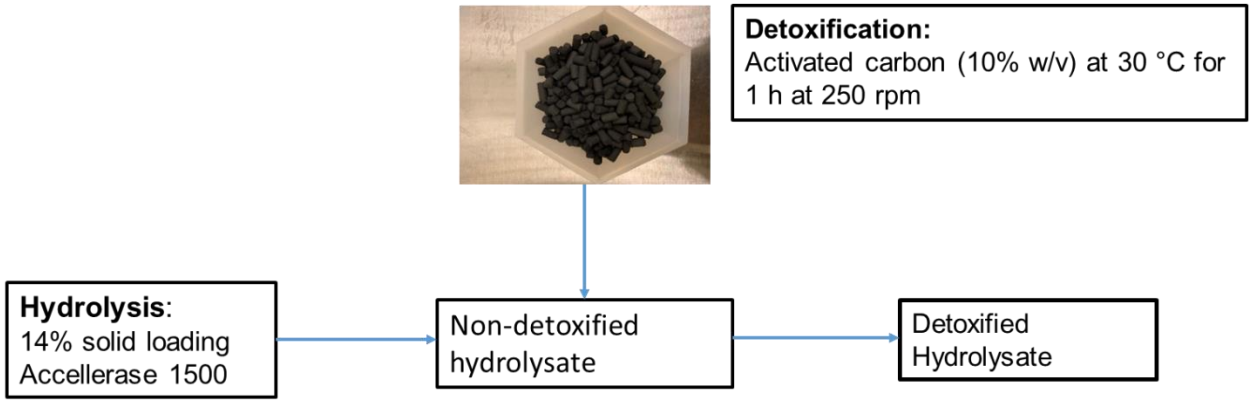


Figure 4.4: Hydrolysate detoxification steps.

4.8 ABE Fermentation

There are two important work areas that are utilized during ABE fermentation. They are the biosafety cabinet and the anaerobic chamber. The biosafety cabinet is required for sterile transfer of the spores of the strain used from the storage tubes to the centrifuge tubes. The centrifuge tubes and pipette tips must be autoclaved prior to this operation. The anaerobic chamber is required for ABE fermentation experiments. Pipette tips, centrifuge tubes, tryptone-glucose-yeast extract (TGY) medium, and glucose (P2) medium were autoclaved using cycle 2 at 121℃ for 20 min before use in the anaerobic chamber. The stock solutions (P2 buffer stock solution, vitamins stock solution, and minerals stock solutions) were added filter sterilized in medium in the anaerobic chamber.

4.8.1 Bacteria and Inoculum Preparation

For inocula preparation, 200 µL spores (wild type, AKR and SDR) were transferred using a pipette from the original stock spores to 2 mL centrifuge tubes in the biosafety cabinet. The transfer was done in duplicates. Furthermore, the transferred spores were heat shocked in the 2 mL centrifuge tubes at 75°C for 5 min for the wild type and 3 min for AKR and SDR strains. The modified strains were heat shocked for 3 min due to their relatively more heat sensitivity compared to the wild type. After heat shock of spores, the centrifuge tubes containing spores were transferred into an ice bath for 2 min.

The heat shocked spores were transferred using the pipette into 40 mL test tubes containing 10 mL TGY medium prepared in duplicate in the anaerobic chamber. Growth was observed at an inoculum age between 24 - 26 h (OD 0.9-1.1 at 600 nm) for the AKR strain while growth was observed at an inoculum age between 12 – 14 h for the SDR and wild type strains. Inoculum was transferred from the test tube with the highest OD to fermentation bottles. For the second passage

of the subculture, 4 mL of the AKR strain, 3.4 mL of the wild type and 3 mL of the SDR strain into 30 mL TGY medium in the anaerobic chamber and was prepared in duplicate. The inoculation level for the second passage was 10% (v/v) as previously reported (Liu et al., 2015a). It took 3-5 h for the cells to reach an OD of 0.9-1.1 in the second passage. The test tube with the best growth was used for fermentation.

4.8.2 Fermentation Medium.

Liquid media and stock solutions were prepared prior to ABE fermentation experiments. Glucose P2 medium, TGY medium, P2 buffer, vitamins and mineral stock solutions were all prepared according to compositions shown in Table 4.1. The composition of P2 glucose medium and detoxified hydrolysate medium used in ABE fermentations with the three strains is shown in Table 4.2. The composition of the non-detoxified hydrolysate medium used in intermittent feeding ABE fermentation is shown in Table 4.3.

Table 4.1: Composition of glucose (P2) medium, TGY medium and stock solutions for inocula and ABE fermentation using *Clostridium beijerinckii*.

Medium Component	Formula	Amount g/L
Glucose P2 medium		
Glucose	$C_6H_{12}O_6$	63.83
Yeast Extract	-	1
TGY medium		
Tryptone	-	30
Glucose	$C_6H_{12}O_6$	20
Yeast Extract	-	10
L-Cysteine	$C_3H_7NO_2S$	1
P2 buffer stock solution		
Potassium phosphate monobasic	KH_2PO_4	50
Potassium phosphate dibasic	K_2HPO_4	50
Ammonium acetate	$NH_4CH_3CO_2$	220
Vitamins		
p-(4)-Aminobenzoic acid	$C_7H_7NO_2$	0.1
Thiamine	$C_{12}H_{17}N_4OS^+$	0.1
Biotin	$C_{10}H_{16}N_2O_3S$	0.01
Minerals stock solution		
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	20
Manganese sulfate heptahydrate	$MnSO_4 \cdot 7H_2O$	1
Ferrous sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	1
Sodium chloride	NaCl	1

Table 4.2: The composition of P2 glucose medium, AKR detoxified, SDR detoxified and wild type detoxified hydrolysate media with P2 buffer.

Components	**P2 glucose medium**	**AKR detox[a]**	**SDR detox[a]**	**WT detox[a]**
Glucose, g/L	60	58	58	58
Yeast Extract, g/L	1.0	1.0	1.0	1.0
Stocks	mL/50mL			
(P2) medium[b]	45.5	-	-	-
Non-detoxified hydrolysate[c]	-	44.5	44.5	44.5
P2 buffer	0.5	0.5	0.5	0.5
Vitamin	0.5	0.5	0.5	0.5
Mineral	0.5	0.5	0.5	0.5
50 g/L Yeast Extract	0.0	1.0	1.0	1.0
Inoculum[d]	3	3	3	3

[a]Soluble lignin content in the detoxified hydrolysate was about 0.85 g/L
Contains 60 g/L glucose and 1 g/L yeast extract.
Hydrolysate diluted with sterilized DI water to obtain a final glucose concentration in the hydrolysate with all added stock solutions to 60 g/L
[d]Values based on 6% inoculation rate

Table 4.3: The composition of AKR non-detoxified, SDR non-detoxified and wild type non-detoxified hydrolysate media with P2 buffer.

Components	**AKR Non-detox[a]**	**SDR Non-detox[a]**	**WT Non-detox[a]**
Glucose, g/L	65	65	65
Yeast Extract., g/L	1.0	1.0	1.0
Stocks	mL/50mL		
Non-detoxified hydrolysate[c]	44.5	44.5	44.5
P2 buffer	0.5	0.5	0.5
Vitamin	0.5	0.5	0.5
Mineral	0.5	0.5	0.5
50 g/L Yeast Extract	1.0	1.0	1.0
Inoculum[d]	3	3	3

[a]Soluble lignin content in the non-detoxified hydrolysate was about 1.05 g/L
[b]Contains 60 g/L glucose and 1 g/L yeast extract.
Hydrolysate diluted with sterilized DI water to obtain a final glucose concentration in the hydrolysate with all added stock solutions to 60 g/L
[d]Values based on 6% inoculation rate

4.8.3 ABE Fermentation

The anaerobic chamber is an oxygen free environment where anaerobes can be cultured, grown and used for fermentation. The chamber was kept anaerobic by vacuuming and introducing 95% Nitrogen and 5% hydrogen gas mix. An oxygen and hydrogen measuring meter was placed in the anaerobic chamber to measure the amount of oxygen present (ppm) and hydrogen present (%) in the anaerobic chamber. Furthermore, 20 g of calcium chloride was placed in the chamber as a desiccant for moisture absorption while 20 g of sodium bicarbonate was also placed to absorb odor.

For the ABE fermentation experiments, 150-mL bottles were used for ABE fermentations with a working volume of 50 mL. Table 4.2 shows the composition of the fermentation medium containing P2 glucose medium and the detoxified hydrolysate with P2 buffer. The control was glucose P2 medium consisting 63.83 g/L glucose and 1 g/L yeast extract while the experimental study is non detoxified hydrolysate contained 60 g/L glucose. The P2 glucose medium was prepared by adding 63.83 g/L glucose and 1 g/L yeast extract into 1 L DI water. The P2 glucose medium was autoclaved using cycle 2 at 121ºC for 20 min. After autoclave, the sterilized medium was cooled to 40ºC and allowed to reach anoxic conditions in the anaerobic chamber. Furthermore, 50 g/L yeast extract stock solution was also prepared, which was added to the detoxified switchgrass hydrolysate. The 50 g/L yeast extract was autoclaved under the same conditions as glucose P2 medium.

Subsequently, mineral, vitamin and P2 buffer stock solution was added to the P2 glucose medium and the detoxified hydrolysate in the biosafety cabinet as described in Table 4.2. The pH of the P2 glucose medium after the addition of the stock solutions was 6.50 while the pH of the detoxified hydrolysate after the addition of the stock solution was 5.15. However, the pH of the

detoxified hydrolysate medium was adjusted to 6.55 using 1.3 mL 8N NH_4OH in about 450 mL hydrolysate. After pH adjustment, the P2 glucose and the detoxified hydrolysate media were transferred into anaerobic chamber prior to inoculation. ABE fermentations in P2 glucose and detoxified hydrolysate media were prepared in triplicates for each strain. Before inoculation, 47 mL of P2 glucose and the detoxified hydrolysate media was transferred into eighteen 150 mL bottles using 50 mL pipette, three bottles for each strain. Afterwards, each three sets of bottles were inoculated with 3 mL of the wild type, AKR and SDR stains of *C. beijerinckii*. Samples were taken from each bottle at various time intervals to analyze for pH, cell mass, sugars, fatty acids and solvents concentrations.

Non-detoxified hydrolysate medium was used in ABE fermentations with and without intermittent feeding. After the addition of the stock solutions, P2 buffer and the yeast extract medium, the pH of the non-detoxified hydrolysate medium was 5.21. The pH of the non-detoxified hydrolysate medium was adjusted to 6.50 using 1.3 mL 8N NH_4OH in 450 mL hydrolysate. Afterwards, the non-detoxified hydrolysate medium was transferred into the anaerobic chamber. For the non-intermittent feeding fermentation, 47 mL of the non-detoxified hydrolysate medium was transferred into nine 150-mL bottles. Each set of three bottles were inoculated with 3 mL of the wild type, AKR and SDR stains of *C. beijerinckii*. Samples was taken from each bottle at 0 h to analyze for pH, cell mass, sugars, fatty acids and solvents concentrations. However, for the intermittent feeding fermentation, 30% of the non-detoxified hydrolysate was added at 0 h and the remaining 70% of the non-detoxified hydrolysate was added after 6 h. A 14.1 mL of the non-detoxified hydrolysate was transferred into nine 150 mL bottles. Subsequently, each set of three bottles were inoculated with 3 mL of the wild type, AKR and SDR stains *C. beijerinckii*. Samples was taken from each bottle at 0 h to analyze for pH, cell mass, sugars, fatty acids and solvents

concentrations. After 6 h of fermentation, samples were taken from each bottles of the treatment to monitor fermentation progress and then 32.9 mL of the non-detoxified hydrolysate was added to each fermentation bottle. Samples were also taken after the addition of the 70% non-detoxified hydrolysate and other time intervals to measure pH and cell mass, sugars, fatty acids and solvents concentrations.

4.9 Product Analysis

Glucose, xylose, arabinose, cellobiose, and galactose were quantified using, the Agilent 1100 series High Performance Liquid Chromatography (HPLC) equipped with an AMINEX HPX-87P column (Biorad, Sunnyvale, CA, USA) at 85ºC and 60 bars with DI water running as effluent at 0.6 mL/min. Also, to quantify furfural, HMF, acetic acid and ethanol Aminex HPX – 87H column was used at 60ºC and 60 bars with 0.001 M sulfuric acid running as the mobile phase at 0.6 mL/min. Furthermore, acetone, butanol, ethanol, acetic acid and butyric acid were measured using Agilent Gas Chromatography (GC) 6890 (Agilent Technologies, Wilmington, DE, USA) equipped with thermal conductivity detector and Supelco PLOT 1010 column. Samples were acidified with 0.1M HCl prior to GC analysis. Also, LDMICs were analyzed at Ohio State University on the HPLC using Waters XBridge® C18 3.5 µm x 4.6 mm x 150 mm column. Each sample was analyzed in duplicate to determine the inhibitor concentration.

To determine the cell mass, the liquid samples for each treatment was withdrawn and diluted accordingly. The cell mass was measured at 600 nm using UV-1800 spectrophotometer (Shimadzu, Houston, TX, USA). Samples with the absorbance above 0.5 diluted to be within the linear range.

4.10 Statistical Analysis

The statistical analysis was performed using Tukey's honest significant difference (HSD) multiple comparison of means at 95% confidence level using SAS JMP Pro 14 analytical software. This was to determine the pairwise statistical differences of cell mass, solvents, organic acids and inhibitors between various media and the three different strains used.

CHAPTER V

RESULTS AND DISSCUSSION

5.1 Biomass Pretreatment and Compositional Analysis

Six switchgrass sub-batches (batches 20 to 25) were pretreated using the pretreatment method described in chapter IV. Sub-batches 20-22 and 23-25 were combined and named here as Batch 1 and Batch 2, respectively, that were used for enzymatic hydrolysis. Sample calculations for estimation of percentage switchgrass solids recovered and amount of pretreated solids obtained after pretreatment for the sub-batches are shown in appendix A2. The results showed that the glucan contents of the pretreated switchgrass solids were 54.6% (Batch 1) and 55.4% (Batch 2) as shown in Table 5.1. These values are similar to what was previously reported by other researchers (Liu et al., 2015a; Pessani et al., 2011). In the present study, the xylan content in Batches 1 and 2 were 2.7% and 1.6%, respectively. Previous studies with switchgrass showed a higher xylan content of 5% (Pessani et al., 2011).

Table 5.1: Composition of switchgrass before and after pretreatment on dry basis

Compound	Raw ASG[a]	Pretreated Batch 1	Pretreated Batch 2	Pretreated Pessani (2011)	Pretreated Liu (2015)
Glucan (%)	35.46 ± 0.63	54.62 ± 0.01	55.40 ± 0.09	57.70 ± 0.50	55.68 ± 0.59
Xylan (%)	23.48 ± 0.16	2.68 ± 0.04	1.56 ± 0.07	5.00 ± 0.50	3.14 ± 0.09
Arabinan (%)	2.41 ± 0.03	0.44 ± 0.05	0.38 ± 0.11	-	0.30 ± 0.01
Lignin (%)	19.77 ± 0.24	35.38 ± 0.52	34.37 ± 0.42	35.10 ± 0.70	36.05 ± 0.27

ASG[a]: Raw Alamo switchgrass compositional analysis before pretreatment (Liu et al., 2015a).

5.2 Enzymatic Hydrolysis

The glucose profile for the enzymatic hydrolysis experiment is shown in Figure 5.1. Enzymatic hydrolysis resulted in glucose concentrations of 65.64 g/L and 67.22 g/L in non-detoxified hydrolysates from Batches 1 and 2, respectively, with a glucan to glucose conversion efficiency of about 75%. The xylose concentration in both batches was about 2 g/L. Also, Batch 1 hydrolysate was detoxified using activated carbon (10% w/v) before fermentation and used in the experiment that compared ABE in this hydrolysate and P2 glucose medium. Hydrolysate from Batch 2 was not detoxified and was used in the ABE fermentations with and without intermittent feeding. The detoxification step for Batch 1 hydrolysate resulted in about 10% and 15% reduction in glucose and xylose concentrations, respectively. Subsequently, the resultant glucose and xylose concentrations after detoxification were about 59 g/L and 1.7 g/L, respectively.

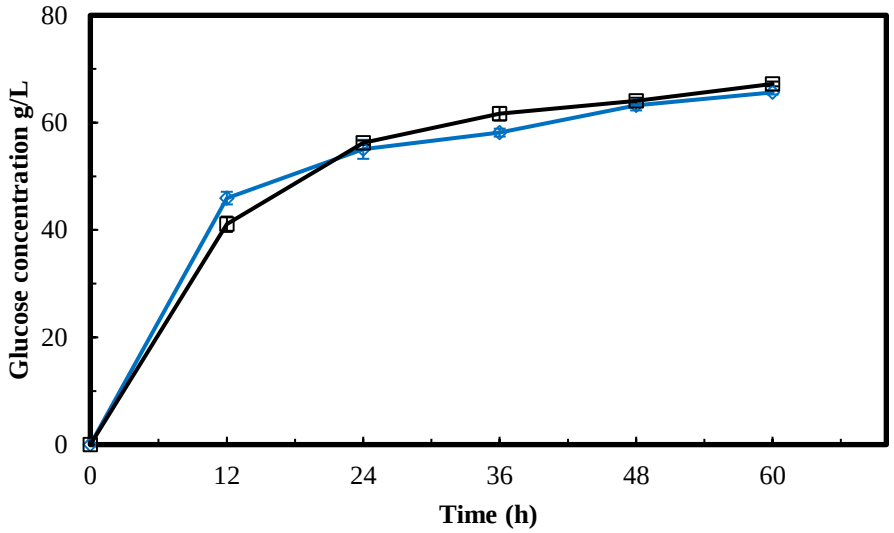


Figure 5.1: Glucose profiles for enzymatic hydrolysis of switchgrass (◇) Batch 1 (□) Batch 2. Error bar represents values from 7 replications for each batch (n=7).

5.3 Inhibitor Analysis

Lignocellulose derived microbial inhibitory compounds (LDMICs) measured in non-detoxified and detoxified hydrolysates are shown in Table 5.2. Furfural and HMF concentrations in the non-detoxified switchgrass hydrolysate were about 62.9 and 4.4 mg/L respectively, while the concentrations of furfural and HMF in the detoxification were reduced by about 90% and 50%, respectively. Furthermore, the concentrations of phenolic compounds in the non-detoxified hydrolysate namely 4-hydroxybenzaldehyde, vanillic acid and syringic acid were found to be 4.7, 2.4 and 5.7 mg/L, respectively, while their concentrations were reduced by detoxification by about 63, 27 and 76%, respectively. Also, vanillin and p-coumaric acid concentration in the non-detoxified hydrolysate were about 7.8 and 4 mg/L, respectively, while they were completely removed by detoxification. Also, syringaldehyde concentration was reduced by 86% from an initial concentration in the non-detoxified hydrolysate of 6.72 mg/L after detoxification.

Consequently, it has been previously shown by Liu et al. (2015a) and Liu et al. (2015b) that detoxification of switchgrass and redcedar hydrolysate was required to increase butanol titer. The reason for the detoxification is the presence of phenolic compounds, though in small concentrations within the range of 0.03 to 0.3 g/L, inhibited cell growth and caused sudden fermentation shutdown (Yao et al., 2017). Another study reported that ferulic acid, 4-hydroxybenzaldehyde and p-coumaric acid has the ability to exert inhibitory effects during fermentation at low concentrations of 0.8, 0.5 and 0.3 g/L, respectively (Yao et al., 2017; Zhang and Ezeji, 2014a). Unlike phenolic compounds, furan compounds such as furfural and HMF are less toxic to cells even at concentrations as high as 3 g/L because they are converted to their respective alcohols during fermentation (Yao et al., 2017; Zhang and Ezeji, 2014a).

Table 5.2. LDMICs concentrations in non-detoxified and detoxified switchgrass hydrolysate samples. Standard deviation represents two replicates (n=2).

LDMIC	Non-detoxified Hydrolysate (mg/L)	Detoxified hydrolysate (mg/L)
Furfural	62.86 ± 3.37	6.50 ± 0.05
HMF	4.35 ± 0.18	2.19 ± 0.11
4-Hydroxybenzaldehyde	4.68 ± 0.26	1.73 ± 0.04
Vanillic acid	2.41 ± 0.12	1.77 ± 0.05
Syringic acid	5.66 ± 0.33	1.38 ± 0.01
Vanillin	7.84 ± 0.30	0.00 ± 0.00
Syringaldehyde	6.48 ± 0.35	0.87 ± 0.06
p-Coumaric acid	4.34 ± 0.18	0.00 ± 0.00
Ferulic acid	0.00 ± 0.00	0.00 ± 0.00

5.4 ABE Fermentation with P2 glucose medium and detoxified hydrolysate

Fermentations were performed in P2 glucose medium and detoxified switchgrass hydrolysate. P2 glucose medium otherwise known as P2 medium was the control with an initial glucose concentration of 60 g/L, while the initial glucose in the hydrolysate after detoxification was about 58 g/L.

Figure 5.2 (A) shows the optical density (OD) at 600nm changes for *C. beijerinckii* wild type, AKR and SDR in P2 medium and detoxified hydrolysate. The growth profile shows that all the three stains grew in both media while the maximum OD achieved by all three strains was measured at 24 h. There were no statistically significant differences in growth profiles among the three strains irrespective of the medium as shown in Table 5.3 ($p > 0.05$). However, the OD values for all three strains in the detoxified hydrolysate were slightly higher than in P2 medium.

The pH profiles for the three strains in both media decreased after inoculation because of acid production in the first 12 h of fermentation (Figure 5.2B). However, a switch to solventogenesis was triggered in the detoxified switchgrass hydrolysate medium with the three strains, which is evident by the pH increase to about 6 after 24 h due to the conversion of the produced acids to their corresponding alcohols. This is similar to the findings by Liu et al. (2015a) who observed an increase in solvent production when pH increased to 6. On the contrary, further acid assimilation was evident in the AKR and SDR strain until 36 h as shown by a further pH drop to about 5.1.

Glucose consumption was achieved by three strains in both media as shown in Figure 5.2C. However, almost all of the glucose in the detoxified hydrolysate medium was consumed by the genetically modified AKR and SDR strains after 72 h of fermentation. The three strains consumed

about 70% of glucose in P2 medium. Also, more glucose consumption was observed in the detoxified hydrolysate due to the extra nutrients present in the hydrolysate. However, statistical analysis showed no significant differences in glucose consumed among the three *C. beijerinckii* strains in P2 medium ($p > 0.05$).

Butanol profile showed an increase in butanol production until 60 h for all strains in both media (Figure 5.2E). However, there was a slight increase in butanol production in all strains until 72 h. Slight insignificant differences in butanol production were observed with the three strains at 24 h of fermentation in both media ($p > 0.05$). However, a significant increase ($p > 0.05$) in butanol production by the AKR and SDR strains was observed in the detoxified hydrolysate medium compared the P2 medium from 36 to 72 h of fermentation . Furthermore, wild type, AKR and SDR strains produced about 1, 12 and 28% more butanol, respectively, in the detoxified switchgrass hydrolysate compared to P2 medium. About 3, 10 and 26% more ABE was produced at 72 h by the wild type, AKR and SDR strains, respectively in the detoxified hydrolysate compared to the P2 medium (Figure 5.2I). However, the SDR strain performed better than the wild type and AKR strain in the detoxified hydrolysate in terms of total ABE production by at least 13%.

Figure 5.2 (G) shows the acetic acid profile. The results showed that about 51% more acetic acid was assimilated in the detoxified hydrolysate medium than in P2 medium. This implies that more acetic acid was available to be converted into ethanol. Similarly, Figure 5.2 (H) shows the butyric acid profiles. After 72 h, AKR and SDR strains in the detoxified hydrolysate medium assimilated 59% more butyric acid than the three strains in P2 medium.

Furthermore, the acid crash threshold concentration was not reached during fermentation in both media as shown in Figure 5.3. The concentrations of undissociated acids were about 10 and 25 mM in P2 medium and detoxified medium, respectively. However, the undissociated acid concentration of 60 mM was previously reported to cause acid crash (Maddox et al., 2000).

Table 5.3: Fermentation parameters using *C. beijerinckii* wild type (WT), AKR and SDR strains in P2 glucose medium (P2) containing 59.04 g/L glucose and in detoxified hydrolysate (DETOX) containing 57.02 g/L glucose and 1.70 g/L xylose.

Treatment	P2- WT	P2-AKR	P2-SDR	DETOX-WT	DETOX-AKR	DETOX-SDR
Time (h)	72	72	72	72	72	72
Maximum OD[a] (24h)	5.71 ± 0.51^{A}	5.60 ± 0.36^{A}	5.94 ± 0.99^{A}	6.30 ± 0.44^{A}	6.12 ± 0.65^{A}	6.13 ± 0.10^{A}
Glucose used, g/L	40.90 ± 0.61^{A}	42.96 ± 0.57^{A}	39.23 ± 1.15^{A}	45.90 ± 1.70^{B}	56.32 ± 0.36^{C}	57.86 ± 0.12^{C}
Glucose conversion, %	68.62 ± 1.06	70.86 ±0.98	68.94 ± 2.34	80.79 ± 3.21	100.00 ± 0.00	99.88 ± 0.20
Xylose used, g/L	-	-	-	1.63 ± 0.21^{A}	1.67 ± 0.06^{A}	1.63 ± 0.25^{A}
Xylose conversion, %	-	-	-	92.22 ± 4.10	100.00 ± 0.00	97.78 ± 3.85
Total sugars used, g/L	40.90 ± 0.61	42.96 ± 0.57	39.23 ± 1.15	47.53 ± 2.53	57.99 ± 0.21	59.50 ± 0.70
Final acetone, g/L	2.12 ± 0.17^{B}	2.67 ± 0.47^{AB}	2.28 ± 0.58^{B}	2.21 ± 0.18^{B}	3.57 ± 0.20^{A}	3.51 ± 0.58^{A}
Final butanol, g/L	10.96 ± 1.13^{AB}	10.07 ± 0.58^{AB}	8.70 ± 0.86^{B}	11.05 ± 1.23^{AB}	11.46 ± 0.20^{A}	11.89 ± 1.14^{A}
Final ethanol, g/L	1.55 ± 0.19^{A}	1.97 ± 0.31^{A}	1.99 ± 0.62^{A}	1.80 ± 0.30^{A}	1.41 ± 0.47^{A}	1.93 ± 0.16^{A}
Final total ABE, g/L	14.63 ± 1.37	14.71 ± 1.05	12.97 ± 1.66	15.06 ± 1.43	16.44 ± 0.27	17.33 ± 1.88
ABE yield, g/g	0.36 ± 0.03	0.34 ± 0.02	0.33 ± 0.03	0.32 ± 0.03	0.28 ± 0.01	0.29 ± 0.04
Final acetic acid, g/L	0.75 ± 0.18^{B}	0.97 ± 0.11^{B}	1.29 ± 0.22^{B}	2.86 ± 0.37^{A}	2.52 ± 0.17^{A}	2.68 ± 0.34^{A}
Final butyric acid, g/L	0.43 ± 0.11^{B}	0.45 ± 0.30^{B}	1.34 ± 0.31^{A}	1.90 ± 0.50^{A}	1.56 ± 0.17^{A}	1.57 ± 0.25^{A}
Final total acids, g/L	1.17 ± 0.29	1.43 ± 0.41	2.63 ± 0.53	4.76 ± 0.87	4.08 ± 0.23	4.25 ± 0.59

Same capital letter in each row represents no significant differences between treatments ($p > 0.05$)
[a]Statistical differences for optical density was performed at 24 h when OD was maximum

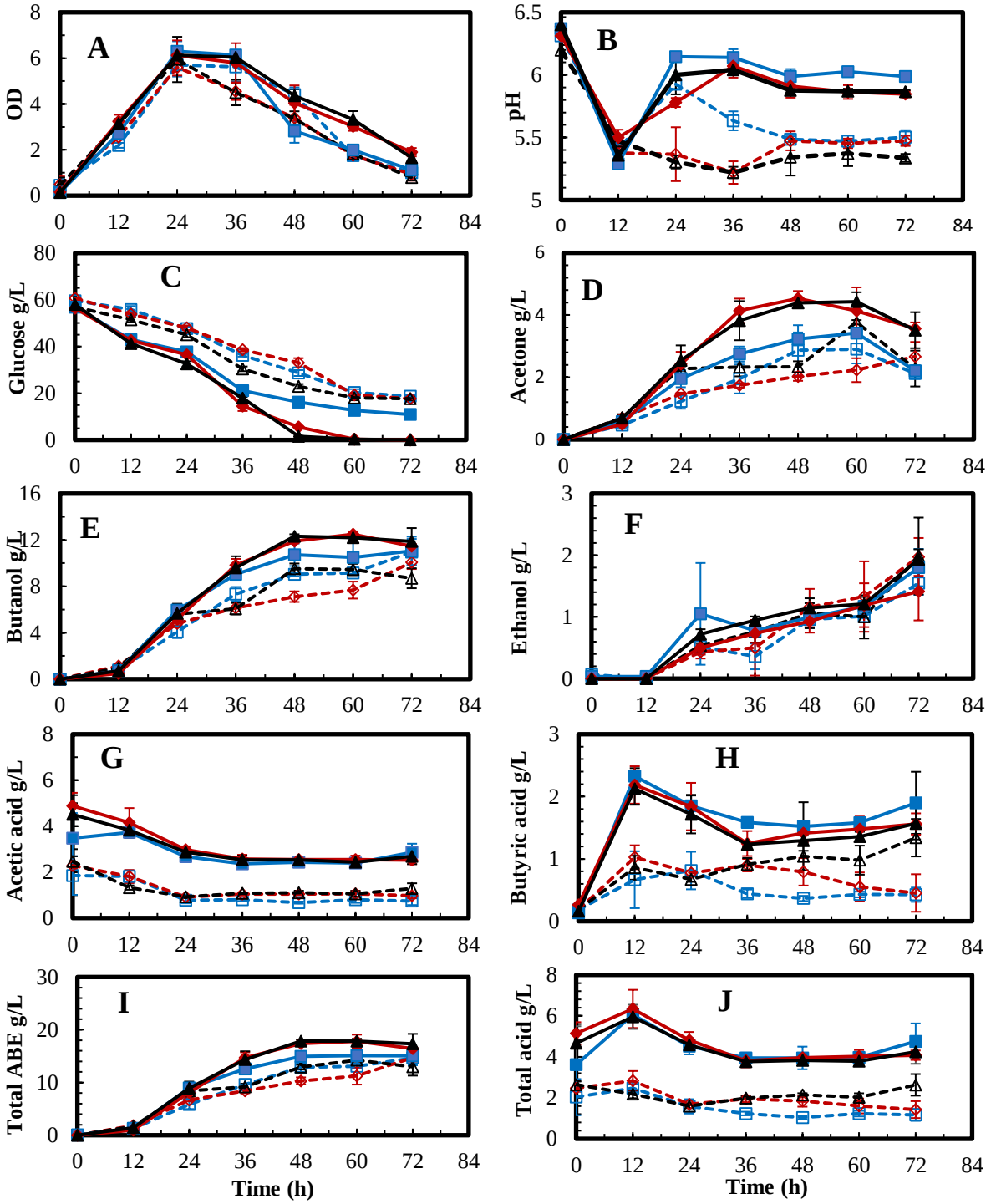


Figure 5.2: Profiles of OD, pH, glucose consumption, productions of acetone, butanol, ethanol, acetic acid and butyric acid for treatments wild type (■), AKR (◆) and SDR (▲) strains of *Clostridium beijerinckii* in P2 glucose and detoxified hydrolysate media during 72 h of fermentation. Solid symbols and lines represent detoxified hydrolysate medium while open symbols and dashed lines represents P2 glucose medium. Error bar represents standard deviation of three replicates (n=3).

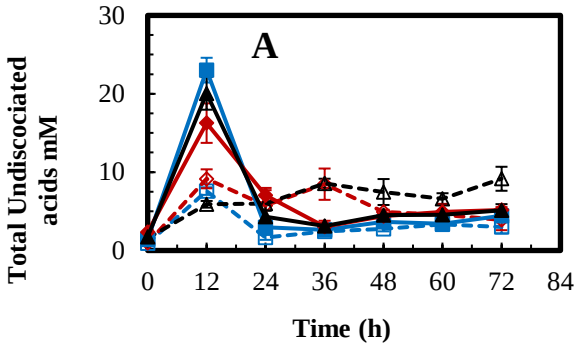


Figure 5.3: Total undissociated acids profile using wild type ( ), AKR ( ) and SDR ( ) strains of *Clostridium beijerinckii* in P2 glucose and detoxified hydrolysate media during 72 h of fermentation. Solid symbols and lines represent detoxified hydrolysate medium while open symbols and dashed lines represents P2 glucose medium. Error bar represents standard deviation of three replicates (n=3).

5.5 ABE fermentation with non-detoxified switchgrass hydrolysate with and without intermittent feeding

ABE fermentation in the non-detoxified hydrolysate with the wild type, AKR and SDR strains of *C. beijerinckii* can determine inhibitor tolerance ability of these stains. In addition, the intermittent feeding technique can enable the strains to gradually adapt to the toxic inhibitory compounds in the fermentation medium.

Results of ABE fermentations with and without intermittent feeding in non-detoxified switchgrass hydrolysate medium are shown in Figures 5.4 and 5.5. In fermentation with intermittent feeding, 30% of the non-detoxified hydrolysate was added at 0 h while the remaining 70% was added at 6 h of fermentation. A preliminary investigation to determine optimal intermittent feeding times of 6 h and 10 h is shown in appendix B. The 6 h intermittent feeding resulted in better ABE yield from non-detoxified hydrolysate.

Growth of the three strains was measured during fermentation with and without intermittent feeding. Figure 5.4 (A) shows the optical density profiles. The maximum OD was achieved at 24 h in the medium with intermittent feeding. On the other hand, an extended lag phase (24 h) occurred with all three strains without intermittent feeding. The extended lag phase was due to the additional time required for the three strains to adapt and tolerate the presence of inhibitory compounds in the non-detoxified hydrolysate. No lag phase was observed in the intermittent feeding treatment due to the higher ratio of inoculum to medium and less concentration of inhibitory compounds with only addition of 30% non-detoxified hydrolysate. The maximum OD values for all the three strains in the non-detoxified hydrolysate medium without intermittent feeding were achieved at 48 h. Furthermore, there were no statistical significance differences in the maximum OD between all strains with intermittent feeding ($p > 0.05$) as shown in Table 5.4.

However, the AKR strain was significantly different in maximum OD compared to the wild type and SDR strain without intermittent feeding ($p > 0.05$). However, at least 14% greater growth was achieved with the SDR strain with intermittent feeding when compared with all three strains without intermittent feeding. The pH profiles showed that the all three stains in the medium with intermittent feeding were in acetogenesis stage from 0 h to 6 h (Figure 5.4B). Supplementing the remaining 70% non-detoxified hydrolysate at 6 h increased the pH to about 6 which favored a switch to solventogenesis stage after 12 h. The pH for the AKR and SDR strains in the medium without intermittent feeding was stable around 6 for over 24 h of fermentation before a switch to acetogenesis occurred. A pH drop was evident after 24 h while solvents production increased the pH after 36 h in the medium without intermittent feeding. Also, Figure 5.4 (C) shows the glucose consumption profiles. The three strains in the medium with intermittent feeding consumed about 20% more glucose than in the medium without intermittent feeding. This shows that the intermittent feeding fermentation strategy enabled the strains to adapt and ferment more sugars from the non-detoxified hydrolysate.

Figure 5.4 (E) shows the butanol production profiles in the non-detoxified hydrolysate with and without intermittent feeding. With intermittent feeding, butanol production gradually increased until 48 h and butanol concentrations slightly changed until 72 h. It was observed that butanol production did not start in the medium without intermittent feeding until after 24 h due to an extended lag phase. Intermittent feeding favored butanol production with all three strains. Butanol production in the medium without intermittent feeding was slow until 36 h while it increased gradually afterwards until 72 h. Table 5.4 showed that about 25 and 16% more butanol were produced with the AKR strain without intermittent feeding compared with the wild type and SDR strains without intermittent feeding, respectively ($p > 0.05$). Similarly, about 4 and 15%

more butanol were produced by the AKR strain compared to the wild type and SDR strains, respectively, with intermittent feeding($p > 0.05$). Ethanol production profiles showed that ethanol production range was between 5 – 7 g/L with the three stains with intermittent feeding (Figure 5.4F). However, another alcohol presumably isopropanol was produced, which was quantified with ethanol because the peak showed up as an overlapping peak with ethanol. In broader terms, Table 5.4 showed that, about 28, 14, and 20% more total ABE was produced using intermittent feeding fermentation strategy compared to no intermittent feeding with the wild type, AKR and SDR strains. Furthermore, comparing total ABE produced in the detoxified hydrolysate and the non-detoxified hydrolysate with intermittent feeding, it was shown that about 20 and 15% more ABE were produced with intermittent feeding strategy with non-detoxified hydrolysate compared to detoxified hydrolysate using AKR and SDR strains, respectively. These results showed that the detoxification step during ABE fermentation can be eliminated when intermittent feeding fermentation technique is implemented with the genetically modified strains. Furthermore, Figure 5.4J shows that about 19, 4 and 22% more total acids were accumulated in the treatments without intermittent feeding with the wild type, AKR and SDR strains, respectively, compared with the treatments with intermittent feeding. This also signifies that more acids were converted to their respective alcohols in treatments with intermittent feeding compared to the no intermittent feeding. Also, the total undissociated acids concentrations in both treatments with and without intermittent feeding were below the acid crash threshold of 60 mM previously reported (Maddox et al., 2000)

Table 5.4: Fermentation parameters using wild type (WT), AKR and SDR *C.beijerinckii* strains in non-detoxified hydrolysate medium containing 65.28 g/L glucose and 1.89 g/L xylose without intermittent (No-INT) and with intermittent (INT) feeding strategies.

Treatment	WT – NO INT	AKR – NO INT	SDR – NO INT	WT -INT	AKR - INT	SDR - INT
Time, h	84	84	84	84	84	84
Maximum OD	6.30 ± 0.89^{A}*	4.77 ± 0.42^{B}*	6.23 ± 0.21^{A} *	6.73 ± 0.15^{A}**	7.37 ± 0.40^{A}**	7.30 ± 0.52^{A}**
Glucose used, g/L	42.03 ± 1.95^{C}	47.90 ± 2.25^{B}	42.60 ± 0.92^{BC}	55.30 ± 0.63^{A}	59.91 ± 2.32^{A}	59.48 ± 2.65^{A}
Glucose conversion, %	64.67 ± 3.00	73.69 ± 3.47	65.54 ± 1.41	85.07 ± 0.97	92.16 ± 3.57	91.51 ± 4.07
Xylose used, g/L	1.69 ± 0.18^{A}	1.67 ± 0.10^{A}	1.49 ± 0.14^{AB}	1.14 ± 0.18^{BC}	0.91 ± 0.06^{C}	1.07 ± 0.07^{C}
Xylose conversion, %	87.11 ± 5.17	86.79 ± 6.67	85.86 ± 5.64	86.04 ± 3.91	84.60 ± 1.12	86.83 ± 4.16
Total sugars used, g/L	43.72 ± 2.12	49.57 ± 2.15	44.09 ± 1.03	56.44 ± 0.81	60.81 ± 2.28	60.55 ± 2.71
Final acetone, g/L	1.79 ± 0.30^{B}	2.56 ± 0.36^{AB}	2.39 ± 0.67^{AB}	2.63 ± 0.45^{AB}	3.15 ± 0.18^{A}	2.61 ± 0.36^{AB}
Final butanol, g/L	7.79 ± 0.59^{C}	10.32 ± 1.40^{ABC}	8.65 ± 1.81^{BC}	11.54 ± 0.86^{AB}	11.97 ± 0.46^{A}	10.12 ± 0.58^{ABC}
Final ethanol, g/L	4.34 ± 0.79^{B}	4.89 ± 0.56^{B}	5.15 ± 0.32^{B}	5.07 ± 0.52^{B}	5.57 ± 1.02^{AB}	7.57 ± 1.25^{A}
Final total ABE, g/L	13.91 ± 1.55	17.77 ± 1.97	16.17 ± 2.19	19.23 ± 1.33	20.69 ± 1.34	20.30 ± 2.16
ABE yield, g/g	0.32 ± 0.02	0.36 ± 0.03	0.37 ± 0.04	0.34 ± 0.02	0.34 ± 0.03	0.34 ± 0.05
Final acetic acid, g/L	3.00 ± 0.22^{AB}	3.09 ± 0.16^{AB}	3.37 ± 0.01^{A}	2.65 ± 0.27^{B}	2.99 ± 0.06^{AB}	2.98 ± 0.02^{AB}
Final butyric acid, g/L	3.41 ± 0.13^{AB}	3.48 ± 0.35^{AB}	4.05 ± 0.76^{A}	2.57 ± 0.36^{B}	3.32 ± 0.37^{AB}	2.78 ± 0.26^{B}
Final total acids, g/L	6.45 ± 0.14	6.57 ± 0.43	7.42 ± 0.77	5.22 ± 0.60	6.31 ± 0.31	5.76 ± 0.24

Same capital letter in each row represents no significant differences between treatments($p > 0.05$).

**Statistical differences for optical density was performed at 24 h for treatment with intermittent feeding

*Statistical differences for optical density was performed at 48 h for treatment without intermittent feeding

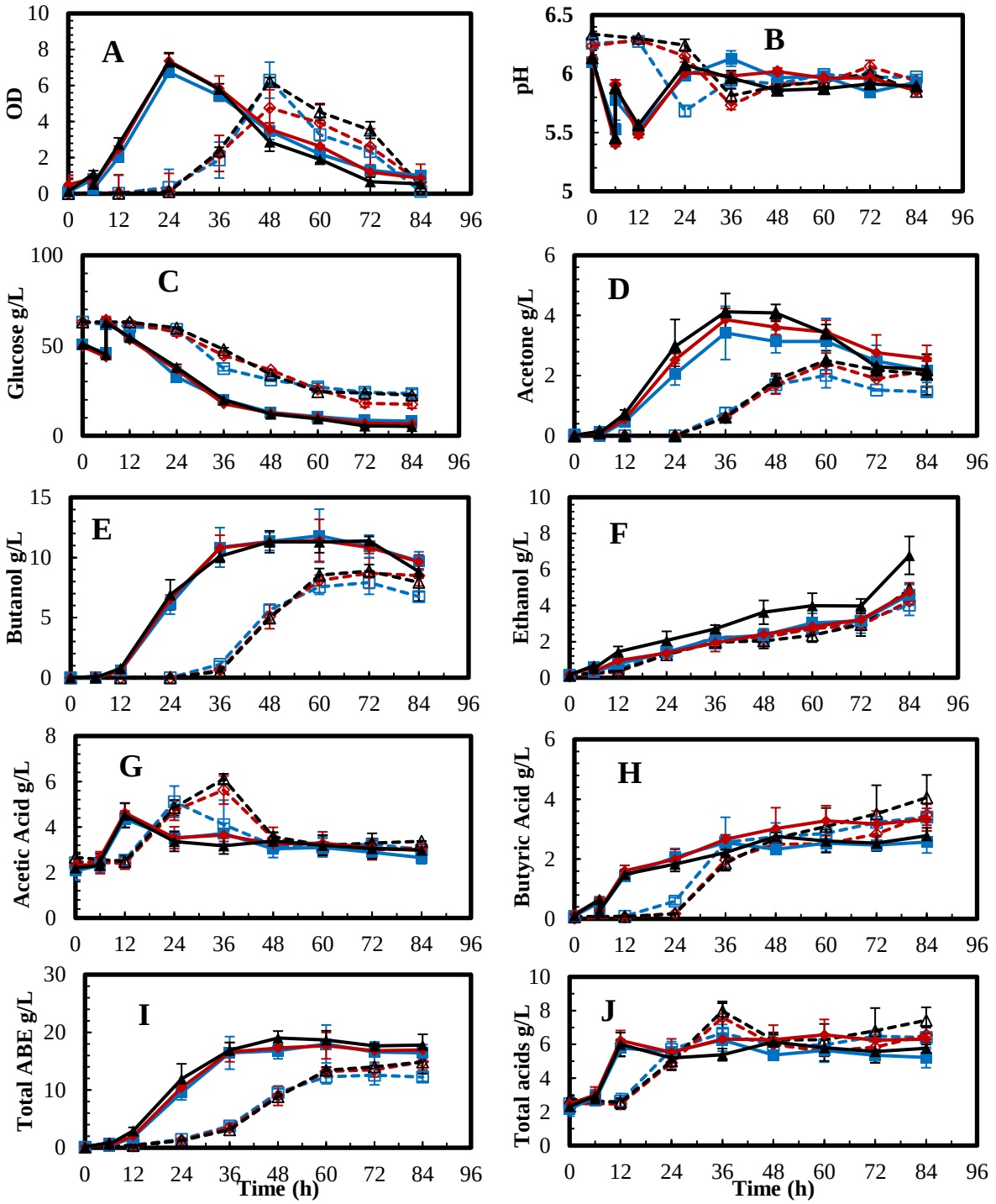


Figure 5.4: Profiles of OD, pH, glucose consumption, productions of acetone, butanol, ethanol, acetic acid and butyric acid for treatments wild type (■), AKR (◆) and SDR (▲) strains of *Clostridium beijerinckii* in non-detoxified hydrolysate media during 84 h of fermentation with and without intermittent feeding. Solid symbols and lines represent intermittent feeding technique while open symbols and dashed lines represent without intermittent feeding. Error bar represents standard deviation of three replicates (n=3).

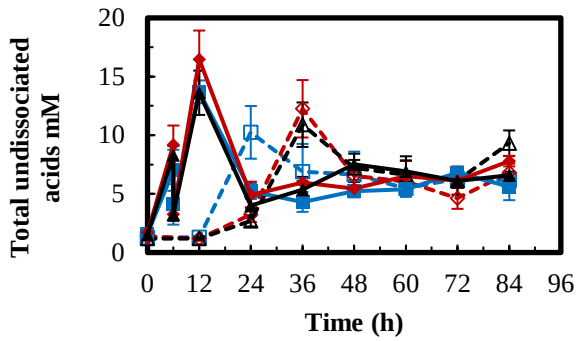


Figure 5.5: Total undissociated acids profile using wild type (■), AKR (◆) and SDR (▲) strains of *Clostridium beijerinckii* non-detoxified hydrolysate medium during 84 h of fermentation with and without intermittent feeding. Solid symbols and lines represent intermittent feeding technique while open symbols and dashed lines represent without intermittent feeding. Error bar represents standard deviation of three replicates (n=3).

CHAPTER VI

CONCLUSIONS

- Hydrothermolysis pretreated Alamo switchgrass yielded 55% glucan, 2% xylan and 35% lignin. Compared to the composition analysis of the raw switchgrass, glucan level after pretreatment increased by 57% which shows that the pretreatment method delignified the switchgrass and exposed more glucan while about 94% of the xylan was lost.
- About 67 g/L of glucose was produced after enzymatic hydrolysis of the structural carbohydrates for 60 h.
- Due to pretreatment, microbial inhibitory compounds were generated in the hydrolysate, which can inhibit the activities of fermenting microbes at certain concentrations.
- Inhibitor analysis showed that small concentrations of furan and phenolic compounds were present in the non-detoxified hydrolysate medium. The concentrations of furfural and HMF in the non-detoxified hydrolysate were about 63 and 4 mg/L, respectively, while the concentrations of vanillin, syringaldehyde, and p-coumaric acid were 7.8, 6.5 and 3 mg/L, respectively. These microbial inhibitory compounds were within the tolerance range for the studied wild type, AKR and SDR strains of *C. beijerinckii*.
- Inhibitor removal up to 80% for some phenolic compounds was achieved by detoxification of hydrolysate using activated carbon.

- The AKR and SDR strains consumed 30% more glucose in the detoxified hydrolysate medium compared to the P2 glucose medium while about 17 g/L total ABE was achieved by the SDR strain in the detoxified hydrolysate.
- ABE fermentation in the non-detoxified medium with and without intermittent feeding lasted for 84 h. However, an extended lag phase for about 24 h was observed in the non-detoxified hydrolysate without intermittent feeding while a maximum optical density of about 6 was observed after 48 h of fermentation without intermittent feeding. This occurred due to small concentrations of phenolic compound present in the non-detoxified hydrolysate. As a result, about 14, 18 and 16 g/L total ABE was produced by the wild type, AKR and SDR strains, respectively, without intermittent feeding.
- ABE fermentation in non-detoxified hydrolysate with intermittent feeding yielded a maximum OD of about 7 after 24 h of fermentation. Also, intermittent feeding strategy improved glucose consumption by 20% when compared without intermittent feeding while total ABE concentrations of about 19, 21 and 20 g/L were produced by the wild type, AKR and SDR strains, respectively.
- The inhibitor tolerant strains (SDR and AKR) eliminated the need for detoxification of hydrolysate. The use of wild type, AKR and SDR strains and intermittent feeding strategy improved ABE fermentation by 28, 26 and 17%, respectively, using non-detoxified hydrolysate when compared to the detoxified hydrolysate medium.
- Fermentation results with the intermittent feeding experiments showed no significant differences between the three strains in terms of inhibitor tolerance.

CHAPTER VII

FUTURE WORK

Due to the successful fermentation using intermittent feeding strategy using non-detoxified hydrolysate, different feeding ratios like 20% / 80%, 40% / 60%, 50% /50%, 60% / 40%, 70% / 30% should be tested to find the optimal feeding ratio for ABE fermentation.

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APPENDICES

APPENDIX A

A.1 Particle Size Distribution of Alamo Switchgrass

Milling switchgrass results in different grass particle sizes. Particle size distribution analysis provides the information of the grass particle sizes present before and after milling. Table A.1 shows the particle size distribution of the switchgrass used for ABE fermentation in 2015. From Figure A.1, the distribution is somewhat symmetrical while 2.0 – 2.36 mm sizes appear to be the peak size (about 28% of the whole sample). This was closely followed by 1.00 - 2.00 mm sizes (21% of the whole sample).

Table A.2 shows the particle size distribution of SG biomass for four different boxes 1, 2, 4, and 5 that was recently milled. The highest standard deviation was 4.3% for switchgrass sizes 1.0 – 2.0 mm. The standard deviation of the particle size across the four boxes is less than 5%. This means that switchgrass biomass in boxes 1, 2, 4, and 5 can be mixed together or used interchangeably. Table A.2 shows the particle size distribution of the biomass used for this study. Switchgrass was collected from box 5 throughout this study.

Table A.1: Particle size distribution for the 2015 batch SG biomass

Sieve No.	Mesh (mm)	Percentage by weight (%)	Weight (g)
½ inch	>12.50	0.09	0.09
¼ inch	6.30 – 12.50	0.62	0.63
No. 6	3.35 – 6.30	3.14	3.20
No. 8	2.36 – 3.35	11.33	11.53
No. 10	2.00 – 2.36	27.71	28.21
No. 18	1.00 – 2.00	20.76	21.13
No. 30	0.59 – 1.00	13.81	14.05
No. 40	0.43 – 0.59	6.88	7.01
No. 100	0.15 – 0.43	11.23	11.43
	>0.15	4.43	4.51
Total		100	101.79

Table A.2: Particle size distribution of the newly milled SG biomass (Box 1, 2, 4 and 5)

	Weight (g)				Percentage (% weight)				Average weight (g)	Average weight (%)	SD (% weight)
Mesh size (mm)	# 1	# 2	# 4	# 5	#1	#2	#4	#5			
>2.00	0.04	0.002	0.0017	0.042	0.066	0.0032	0.0023	0.092	0.02	0.041	0.05
1.00 – 2.00	3.31	5.58	11.57	5.37	5.48	8.88	15.56	11.72	6.46	10.41	4.28
0.59 – 1.00	15.50	20.31	23.42	15.60	25.67	32.31	31.51	34.06	18.71	30.89	3.64
0.15 – 0.59	28.75	27.76	31.00	19.98	47.61	44.16	41.72	43.63	26.87	44.28	2.46
0.08 – 0.15	5.96	3.84	3.80	2.58	9.87	6.11	5.11	5.62	4.04	6.68	2.17
<0.08	6.83	5.37	4.53	2.23	11.30	8.54	6.10	4.87	4.74	7.70	2.84
Total	60.41	62.87	74.31	45.80	100	100	100	100			

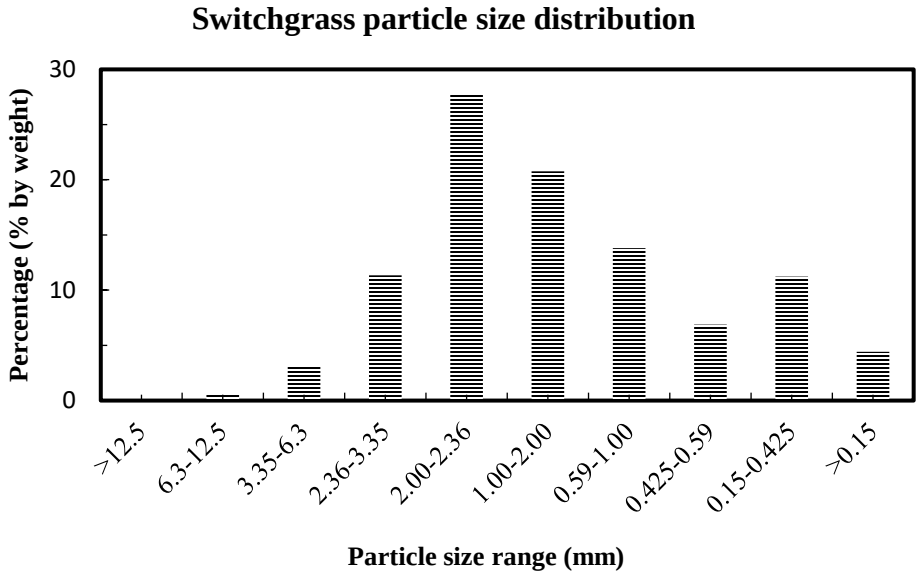

Figure A.1: Particle size distribution for the 2015 batch SG biomass

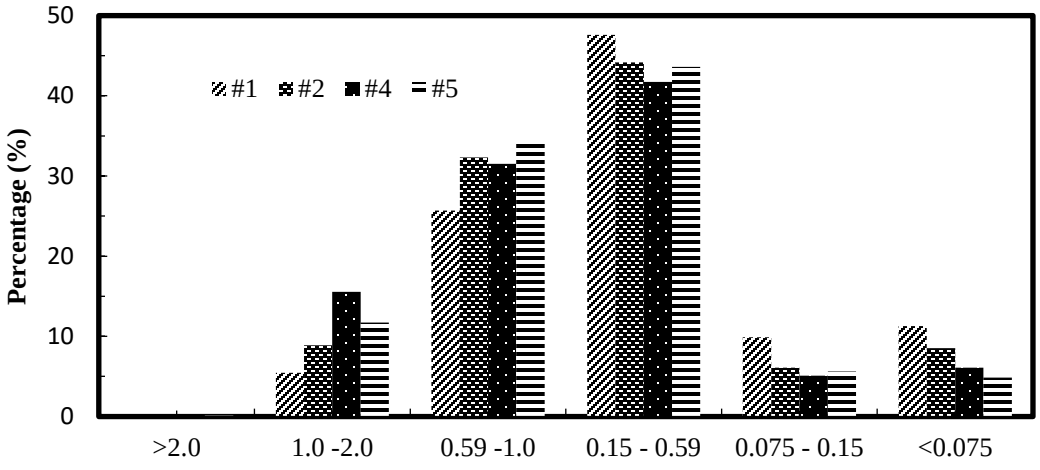

Figure A.2: Particle Size distribution for the newly milled SG biomass.

A.2 Sample calculations involving switchgrass pretreatment

Experimental data for sub-batches 20, 21, 22, 23, 24 and 25 washed pretreated solids

A.2.1 Pretreatment Calculation for the washed solids (Batch 20)

Actual Switchgrass loaded: 65.53 g

Dry mass of grass loaded (DSL) = $\left(1 - \frac{MC}{100}\right) \times 65.53\ g$

Dry mass of grass loaded (DSL) = $\left(1 - \frac{7.61}{100}\right) \times 65.53\ g = 60.54\ g$

DI water to be added: 534.47 g

Weight of prehyrolysate: 424.1 g

Weight of pretreated solids (before washing): 148.48g

Percentage Solids Recovered (% RS)

% RS = $\frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water}$

% RS = $\frac{(148.48+424.1) \times 100}{65.53\ g + 534.47} = 95.43\%$

Percentage Solids in Unwashed Solids (% SUS)

% Solids in Pretreated Solids (%SPS) = $\left(1 - \frac{A-B}{A-C}\right) \times 100$

Where:

A is the mass of pretreated solids and aluminum pan = 9.7200 g

B is the mass of oven dry pretreated grass and aluminum pan = 3.8240 g

C is the mass of aluminum pan = 1.3375 g

% SUS =$(1 - \frac{9.7200-3.8240}{9.7200-1.3375})$ * 100 = 29.66 %

Pretreated Solids Obtained on Dry Basis

Pretreated Solids Obtained on Dry Basis (DS) = mass of washed solids * % SUS

=148.48 * 29.66 % = 44.04 g

Percentage Dissolved Solids

Percentage dissolved solids = $\left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$

% dissolved solids = $\left[1 - \frac{44.04}{60.54 * 0.9543}\right] * 100$ = 23.77 %

A.2.2 Pretreatment Calculation for the washed solids (Batch 21)

Actual Switchgrass loaded: 65.53 g

Dry mass of grass loaded (DSL) = $\left(1 - \frac{MC}{100}\right) \times 65.53\ g$

Dry mass of grass loaded (DSL) = $\left(1 - \frac{7.61}{100}\right) \times 65.53\ g = 60.54\ g$

DI water to be added: 534.47 g

Weight of prehyrolysate: 423.0 g

Weight of pretreated solids (before washing): 151.78g

Percentage Solids Recovered (% RS)

% RS = $\frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water}$

% RS = $\frac{(151.78+423) \times 100}{65.53\ g + 534.47} = 95.80\%$

Percentage Solids in washed Solids (% SUS)

% Solids in Pretreated Solids (%SPS) = $\left(1 - \frac{A-B}{A-C}\right) \times 100$

Where:

A is the mass of pretreated solids and aluminum pan = 9.0336 g

B is the mass of oven dry pretreated grass and aluminum pan = 3.5640 g

C is the mass of aluminum pan = 1.3356 g

% SUS =$(1 - \frac{9.0336-3.5640}{9.0336-1.3356})$ * 100 = 28.95 %

Pretreated Solids Obtained on Dry Basis

Pretreated Solids Obtained on Dry Basis (DS) = mass of washed solids * % SUS

=151.78 * 28.95 % = 43.94 g

Percentage Dissolved Solids

Percentage dissolved solids = $\left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$

% dissolved solids = $\left[1 - \frac{43.94}{60.54*0.9580}\right] * 100$ = 24.24 %

A.2.3 Pretreatment Calculation for the washed solids (Batch 22)

Actual Switchgrass loaded: 65.53 g

Dry mass of grass loaded (DSL) = $\left(1 - \frac{MC}{100}\right) \times 65.53\ g$

Dry mass of grass loaded (DSL) = $\left(1 - \frac{7.61}{100}\right) \times 65.53\ g = 60.54\ g$

DI water to be added: 534.47 g

Weight of prehyrolysate: 422.4 g

Weight of pretreated solids (before washing): 149.28g

Percentage Solids Recovered (% RS)

$$\% \text{ RS} = \frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water}$$

$$\% \text{ RS} = \frac{(149.28+422.4)\times 100}{65.53\ g + 534.47} = 95.28\%$$

Percentage Solids in Unwashed Solids (% SPS)

$$\% \text{ Solids in Pretreated Solids (\%SPS)} = \left(1 - \frac{A-B}{A-C}\right) \times 100$$

Where:

A is the mass of pretreated solids and aluminum pan = 9.3062 g

B is the mass of oven dry pretreated grass and aluminum pan = 3.6485 g

C is the mass of aluminum pan = 1.3330 g

$$\% \text{ SPS} = \left(1 - \frac{9.3062-3.6485}{9.3062-1.3330}\right) * 100 = 29.04\ \%$$

Pretreated Solids Obtained on Dry Basis

Pretreated Solids Obtained on Dry Basis (DS) = mass of washed solids * % SUS

=149.28 * 29.04 % = 43.35 g

Percentage Dissolved Solids

Percentage dissolved solids = $\left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$

% dissolved solids = $\left[1 - \frac{43.35}{60.54 * 0.9528}\right] * 100$ = 24.84 %

A.2.4 Pretreatment Calculation for the washed solids (Batch 23)

Actual Switchgrass loaded: 65.53 g

Dry mass of grass loaded (DSL) = $\left(1 - \frac{MC}{100}\right) \times 65.53\ g$

Dry mass of grass loaded (DSL) = $\left(1 - \frac{7.61}{100}\right) \times 65.53\ g = 60.54\ g$

DI water to be added: 534.47 g

Weight of prehyrolysate: 422.0 g

Weight of pretreated solids (before washing): 151.68 g

Percentage Solids Recovered (% RS)

% RS = $\frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water}$

% RS = $\frac{(151.68 + 422.0) \times 100}{65.53\ g + 534.47} = 95.61$

Percentage Solids in washed Solids (% SPS)

% Solids in Pretreated Solids (%SPS) = $\left(1 - \frac{A-B}{A-C}\right) \times 100$

Where:

A is the mass of pretreated solids and aluminum pan = 9.4633 g

B is the mass of oven dry pretreated grass and aluminum pan = 3.7086 g

C is the mass of aluminum pan = 1.3485 g

% SPS =$(1 - \frac{9.4633-3.7086}{9.4633-1.3485})$ * 100 = 29.08 %

Pretreated Solids Obtained on Dry Basis

Pretreated Solids Obtained on Dry Basis (DS) = mass of washed solids * % SPS

=151.68 * 29.08 % = 44.11 g

Percentage Dissolved Solids

Percentage dissolved solids = $\left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$

% dissolved solids = $\left[1 - \frac{44.11}{60.54*0.9561}\right] * 100$ = 23.79 %

A.2.5 Pretreatment Calculation for the washed solids (Batch 24)

Actual Switchgrass loaded: 65.53 g

Dry mass of grass loaded (DSL) = $\left(1 - \frac{MC}{100}\right) \times 65.53\ g$

Dry mass of grass loaded (DSL) = $\left(1 - \frac{7.61}{100}\right) \times 65.53\ g = 60.54\ g$

DI water to be added: 534.47 g

Weight of prehyrolysate: 421.6 g

Weight of pretreated solids (before washing): 147.38 g

Percentage Solids Recovered (% RS)

$$\% \text{ RS} = \frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water}$$

$$\% \text{ RS} = \frac{(147.38+421.6)\times 100}{65.53\ g + 534.47} = 94.83\%$$

Percentage Solids in Unwashed Solids (% SPS)

$$\% \text{ Solids in Pretreated Solids (\%SPS)} = \left(1 - \frac{A-B}{A-C}\right) \times 100$$

Where:

A is the mass of pretreated solids and aluminum pan = 9.5042 g

B is the mass of oven dry pretreated grass and aluminum pan = 3.7885 g

C is the mass of aluminum pan = 1.3329 g

$$\% \text{ SPS} = \left(1 - \frac{9.5042-3.7885}{9.5042-1.3329}\right) * 100 = 30.05\ \%$$

Pretreated Solids Obtained on Dry Basis

Pretreated Solids Obtained on Dry Basis (DS) = mass of washed solids * % SPS

=147.38 * 30.05 % = 44.29 g

Percentage Dissolved Solids

$$\text{Percentage dissolved solids} = \left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$$

$$\% \text{ dissolved solids} = \left[1 - \frac{44.29}{60.54*0.9483}\right] * 100 = 22.85\ \%$$

A.2.6 Pretreatment Calculation for the washed solids (Batch 25)

Actual Switchgrass loaded: 65.53 g

Dry mass of grass loaded (DSL) = $\left(1 - \frac{MC}{100}\right) \times 65.53\ g$

Dry mass of grass loaded (DSL) = $\left(1 - \frac{7.61}{100}\right) \times 65.53\ g = 60.54\ g$

DI water to be added: 534.47 g

Weight of prehyrolysate: 423.8 g

Weight of pretreated solids (before washing): 149.18 g

Percentage Solids Recovered (% RS)

% RS = $\frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water}$

% RS = $\frac{(149.18+423.8) \times 100}{65.53\ g + 534.47} = 95.50\%$

Percentage Solids in Unwashed Solids (% SPS)

% Solids in Pretreated Solids (%SPS) = $\left(1 - \frac{A-B}{A-C}\right) \times 100$

Where:

A is the mass of pretreated solids and aluminum pan = 9.6081 g

B is the mass of oven dry pretreated grass and aluminum pan = 3.8862 g

C is the mass of aluminum pan = 1.3342 g

% SPS =$(1 - \frac{9.6081-3.8862}{9.6081-1.3342})$ * 100 = 30.84 %

Pretreated Solids Obtained on Dry Basis

Pretreated Solids Obtained on Dry Basis (DS) = mass of washed solids * % SPS

=149.18 * 30.84 % = 46.01 g

Percentage Dissolved Solids

Percentage dissolved solids = $\left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$

% dissolved solids = $\left[1 - \frac{46.01}{60.54 * 0.9550}\right] * 100$ = 20.41 %

Table A.3: Data for pretreated batches 20 – 25.

Sample	% Recovered Solids	% SPS	Solid Obtained DB[b] (g)
ASG[a]- Batch 20	95.60	30.59	45.66
ASG[a]- Batch 21	96.03	30.58	44.55
ASG[a]- Batch 22	95.28	29.04	43.35
ASG[a]- Batch 23	95.61	29.08	44.11
ASG[a]- Batch 24	94.83	30.05	44.29
ASG[a]- Batch 25	95.50	30.84	46.01

a: Alamo switchgrass
b: Dry basis

Table A.4: Data for pretreated batches 26-28.

Sample	% Recovered Solids	% SPS	Solid Obtained DB[b] (g)
ASG[a]- Batch 26	96.60	32.69	44.36
ASG[a]- Batch 27	95.53	31.39	44.25
ASG[a]- Batch 28	95.18	29.74	43.85

a: Alamo switchgrass
b: Dry basis

Table A.5: Compositional Analysis Results for Batches 26 -28.

Compound	Batch 26+28	Batch 27
Glucan (%)	54.88 ± 2.88	54.48 ± 0.19
Xylan (%)	2.86 ± 0.14	3.30 ± 0.81
Galactan (%)	0.24 ± 0.03	-
Lignin (%)	36.92 ± 3.91	35.86 ± 3.84

Table A.5 shows glucan content of 54 % for both the mixed batches 26 and 28 and single batch 27. Also, the xylan content is about 3 % while the lignin content is about 36 %. These composition analysis results is close to previously achieved batches which contains about 52-54 % glucan content, and about 35 % lignin. Furthermore, this result compares well with previously reported glucan content (Liu et al., 2015a) with about 3% difference and less than 1% difference on the glucan side. With these results, the three batches of pretreated switchgrass will be mixed prior to enzymatic hydrolysis.

APPENDIX B

B.1 Preliminary Experiments to Investigate Intermittent Feeding Strategy for (ABE) Fermentation from Non-detoxified Switchgrass Hydrolysate using Novel Strains of *Clostridium beijerinckii*

Table B.1: The composition of Non-detoxified hydrolysate, P2 buffer stock solution, vitamins and minerals stock solutions for the preliminary study based on 10 mL working volume

Components	AKR Non-detox	SDR Non-detox	WT Non-detox
Stocks solutions	mL/10mL		
Non-detoxified hydrolysate[a]	8.9	8.9	8.9
P2 buffer stock solution	0.1	0.1	0.1
Vitamin	0.1	0.1	0.1
Mineral	0.1	0.1	0.1
50 g/L YE	0.2	0.2	0.2
Inoculum[b]	0.6	0.6	0.6

[a]Dilute the hydrolysate with sterilized DI water to obtain a final glucose concentration in the hydrolysate with all added stock solutions to 60 g/L.
[b]Values based on 6% inoculation rate

Table B.2: The composition of glucose P2 medium, P2 buffer stock solution, vitamins and minerals stock solutions for the preliminary study based on 10 mL working volume

Components	AKR Glucose P2 Medium	SDR Glucose P2 Medium	WT Glucose P2 Medium
Stocks solutions	mL/10mL		
Glucose P2 medium	9.1	9.1	9.1
P2 buffer stock solution	0.1	0.1	0.1
Vitamin	0.1	0.1	0.1
Mineral	0.1	0.1	0.1
Inoculum[a]	0.6	0.6	0.6

[a] Values based on 6% inoculation rate

Table B.3: Composition of Glucose (P2) medium, TGY Medium and Stock Solutions for *Clostridium beijerinckii*

Medium Component	Formula	Amount g/L
Glucose P2 medium		
Glucose	$C_6H_{12}O_6$	63.83
Yeast Extract	-	1
TGY Medium		
Tryptone	-	30
Glucose	$C_6H_{12}O_6$	20
Yeast Extract	-	10
L-Cysteine	$C_3H_7NO_2S$	1
P2 buffer stock solution (100X)		
Potassium phosphate monobasic	KH_2PO_4	50
Potassium phosphate dibasic	K_2HPO_4	50
Ammonium Acetate	$NH_4CH_3CO_2$	220
Vitamins		
p-(4)-aminobenzoic acid	$C_7H_7NO_2$	0.1
Thiamine	$C_{12}H_{17}N_4OS^+$	0.1
Biotin	$C_{10}H_{16}N_2O_3S$	0.01
Minerals stock solution		
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	20
Manganese sulfate heptahydrate	$MnSO_4 \cdot 7H_2O$	1
Ferrous sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	1
Sodium Chloride	NaCl	1

Figures B.1 and B.2 shows the volume for the non-detoxified hydrolysates, P2 glucose medium, P2 buffer, vitamins and mineral stocks in the fermentation medium with 10 mL working volume. During enzymatic hydrolysis, no furfural and HMF was detected in the non-detoxified hydrolysate. However, previous studies detected furfural (4.86 g/L) and HMF (0.2 g/L) in the non-detoxified hydrolysate (Liu et al., 2015a)

This study investigated the optimal intermittent feeding time. Two time intervals of 6 h and 10 h of intermittent feeding were tested. At time 0 h, 30 % of the total non-detoxified hydrolysate and P2 glucose medium was inoculated with 0.6 mL of actively growing inoculum which has been prepared in the TGY medium prior to the experiment. After 6 hours, the remaining 70% of the non-detoxified hydrolysate and the P2 glucose medium was transferred into the test tubes with the 6-hour study while 70 % of the non-detoxified hydrolysate and the P2 glucose medium was transferred into the test tubes with the 10-hour study after 10 hours from time 0 hours.

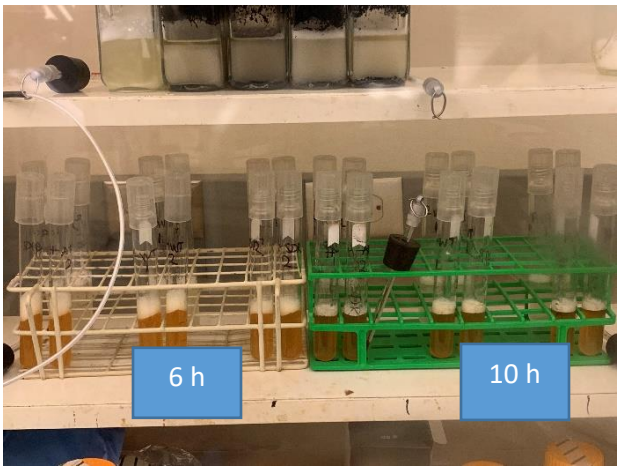

Figure B.1: ABE fermentation after 12 hours

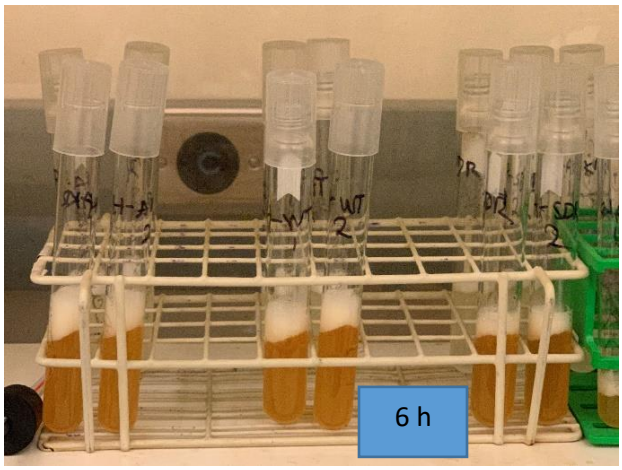

Figure B.2: ABE Fermentation in the 6 h Intermittent feeding after 12 hours

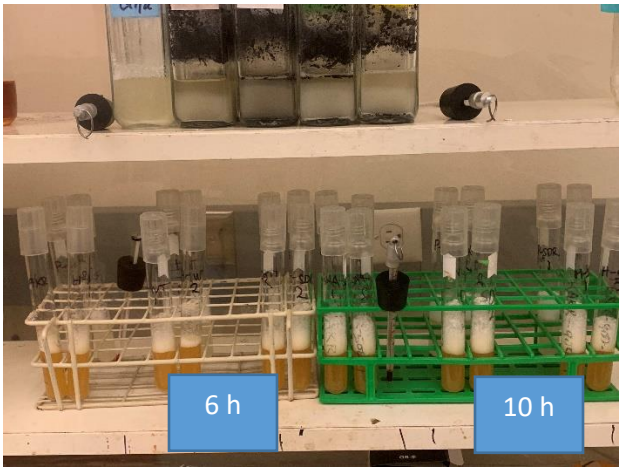

Figure B.3: ABE fermentation after 36 hours in both the 6 hour (left) and 10 hours (right) intermittent feeding after 36 hours**.**

Table B.4: Solvent, and organic acid production and glucose consumption results in the P2 glucose medium

Treatment	P2-AKR-6h	P2-WT-6h	P2-SDR-6h	P2-AKR-10h	P2-WT-10h	P2-SDR-10h
Maximum OD	4.8 ± 1.13	4.25 ± 0.21	5.4 ± 0.14	5.3 ± 1.56	5.85 ± 0.64	5.58 ± 0.49
Glucose consumed, g/L	23.2 ± 0	28.4 ± 0[D]	22.5 ± 0	47.9 ± 0	46.2 ± 0	44 ± 0
Final acetone g/L	1.5 ± 0.05	1.62 ± 0.2	1.86 ± 0.03	3.19 ± 0.29	2.85 ± 1.00	3.42 ± 0.48
Final ethanol, g/L	3.31 ± 1.25	2.98 ± 0.17	1.38 ± 0.19	2.32 ± 0.51	1.87 ± 0.97	1.93 ± 0.41
Final butanol, g/L	4.89 ± 0.04	5.97 ± 0.29	4.74 ± 0.45	9.81 ± 1.14	11.02 ± 0.6	10.42 ± 0
Final total ABE, g/L	9.7 ± 1.27	10.57 ± 0.7	7.98 ± 0.28	15.32 ± 0.74	15.74 ± 2.5	15.77 ± 0.89
ABE yield g/g	0.42 ± 0.01	0.37± 0.01	0.35 ± 0.01	0.32 ± 0.01	0.34 ± 0.01	0.36 ± 0.03
Final acetic acid, g/L	1.32 ± 0.19	1.28 ± 0.08	1.12 ± 0.06	0.76 ± 0.39	0.58 ± 0.11	0.62 ± 0.02
Final butyric acid, g/L	1.81 ± 0.07	1.44 ± 0.17	0.94 ± 0.01	0.4 ± 0.19	0.27 ± 0.01	0.36 ± 0.06
Final total acids, g/L	3.13 ± 0.13	2.72 ± 0.25	2.06 ± 0.05	1.16 ± 0.59	0.85 ± 0.09	0.98 ± 0.08

Table B.5: Solvent, organic acid production and glucose consumption results in the non-detoxified hydrolysate medium

Treatment	H-AKR-6h	H-WT-6h	H-SDR-6h	H-AKR-10h	H-WT-10h	H-SDR-10h
Maximum OD	8.05 ± 1.34	7.80 ± 0.56	9.40 ± 1.13	10.20 ± 1.06	11.92 ± 2.01	11.70 ± 0.85
Glucose consumed, g/L	55.30 ± 0.00	52.00 ± 0.00	54.00 ± 0.00	58.30 ± 0.00	60.00 ± 0.00	65.00 ± 0.00
Final acetone, g/L	2.73 ± 0.21	2.73 ± 0.21	3.73 ± 0.30	3.51 ± 0.58	3.44 ± 0.11	4.53 ± 0.38
Final ethanol, g/L	4.10 ± 0.28	3.16 ± 1.07	3.03 ± 0.32	2.80 ± 0.22	2.33 ± 0.21	2.53 ± 0.18
Final butanol, g/L	11.15 ± 0.52	11.07 ± 0.70	11.67 ± 0.24	12.21 ± 0.75	12.41 ± 0.50	13.10 ± 0.14
Final total ABE, g/L	17.98 ± 0.45	16.98 ± 0.60	18.43 ± 0.21	18.52 ± 1.55	18.18 ± 0.80	20.16 ± 0.34
ABE yield, g/g	0.33 ± 0.03	0.33 ± 0.02	0.34 ± 0.01	0.32 ± 0.04	0.30 ± 0.01	0.31 ± 0.01
Final acetic acid, g/L	2.46 ± 0.22	2.73 ± 0.35	2.85 ± 0.12	2.49 ± 0.01	2.64 ± 0.68	2.22 ± 0.14
Final butyric acid, g/L	3.36 ± 0.02	2.77 ± 0.01	1.91 ± 0.04	1.33 ± 0.24	1.94 ± 0.06	0.86 ± 0.08
Final total acids, g/L	5.82 ± 0.17	5.50 ± 0.34	4.76 ± 0.17	3.82 ± 0.23	4.58 ± 0.62	3.08 ± 0.06

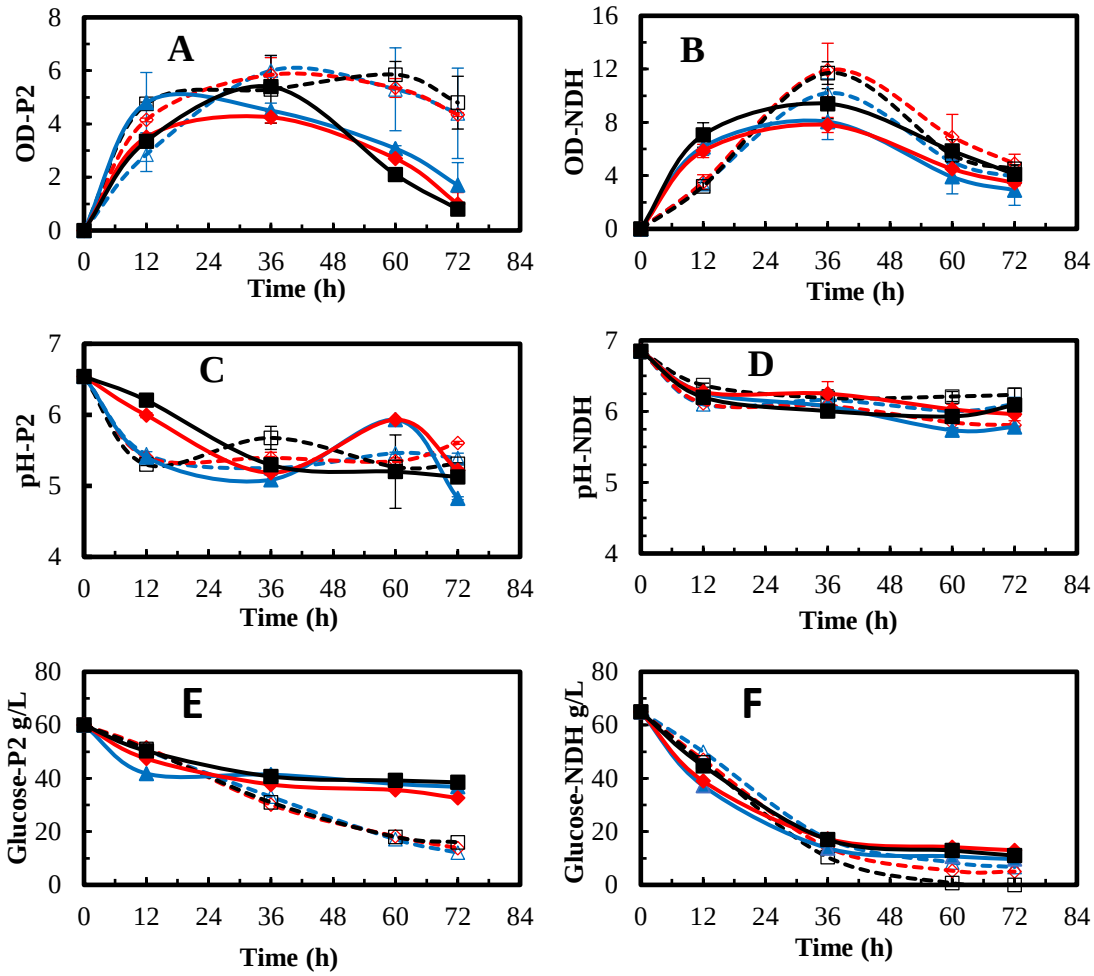

Figure B.4: (A) Optical density for P2 glucose medium, (B) optical density for non-detoxified hydrolysate, (C) pH for P2 glucose medium (D) pH for non-detoxified hydrolysate (E) glucose consumption for P2 glucose medium (F) glucose consumption profiles for ABE fermentation in P2 glucose medium and non-detoxified hydrolysate for optimal intermittent feeding time determination using *Clostridium beijerinckii* wild type (◆), AKR (■) and SDR (▲) strains of. Solid lines and symbols represent the 6 h feeding time while the open symbols and dashed lines represent the 10 h feeding time.

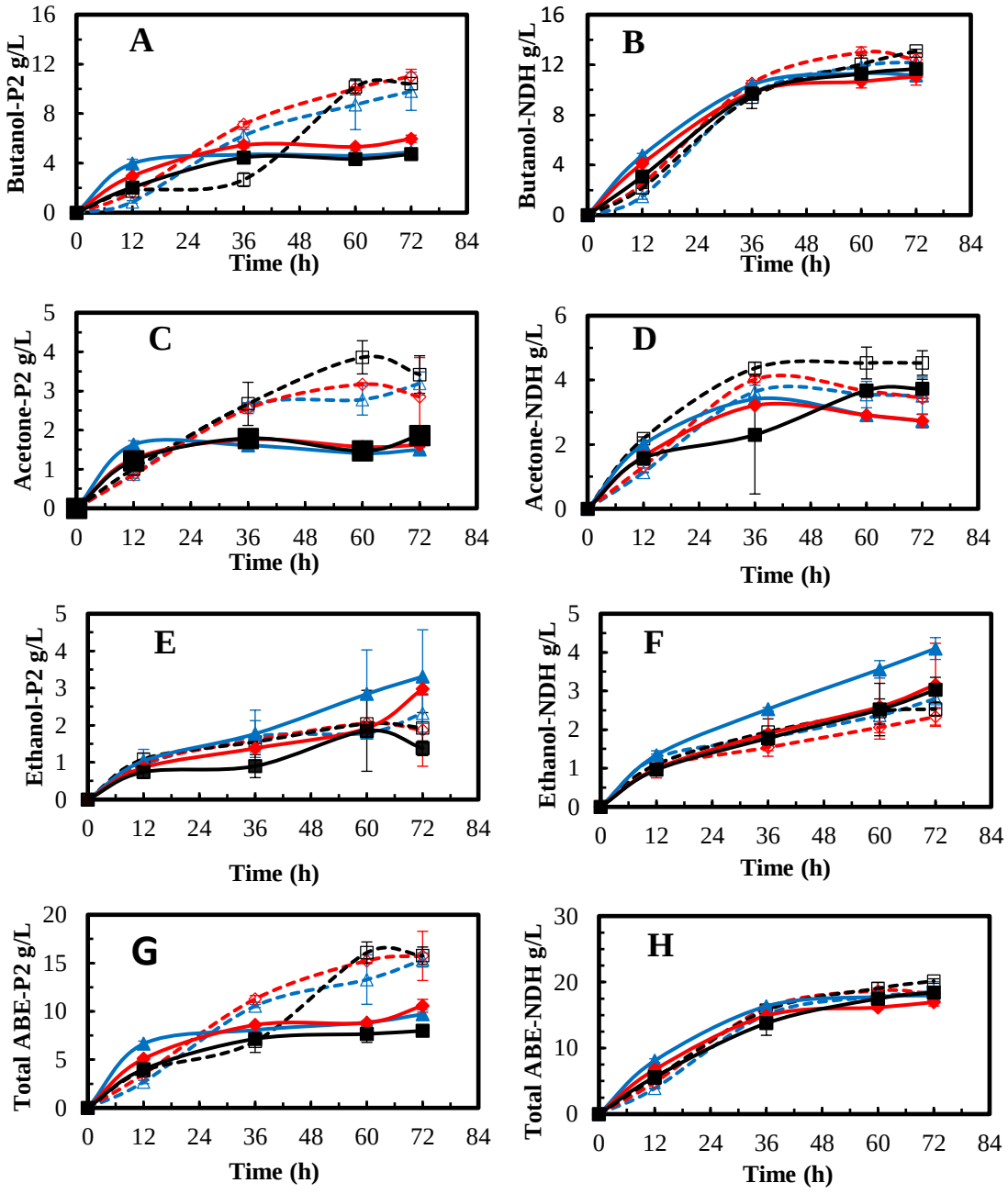

Figure B.5: (A) Butanol for P2 glucose medium, (B) butanol for non-detoxified hydrolysate, (C) acetone for P2 glucose medium (D) acetone for non-detoxified hydrolysate (E) ethanol for P2 glucose medium (F) ethanol for non-detoxified hydrolysate (G) total ABE for P2 glucose, (H) total ABE for non-detoxified hydrolysate profiles for ABE fermentation in P2 glucose medium and non-detoxified hydrolysate for optimal intermittent feeding time determination using *Clostridium beijerinckii* wild type (◆), AKR (■) and SDR (▲) strains. Solid lines and symbols represent the 6 h feeding time while the open symbols and dashed lines represents the 10 h feeding time.

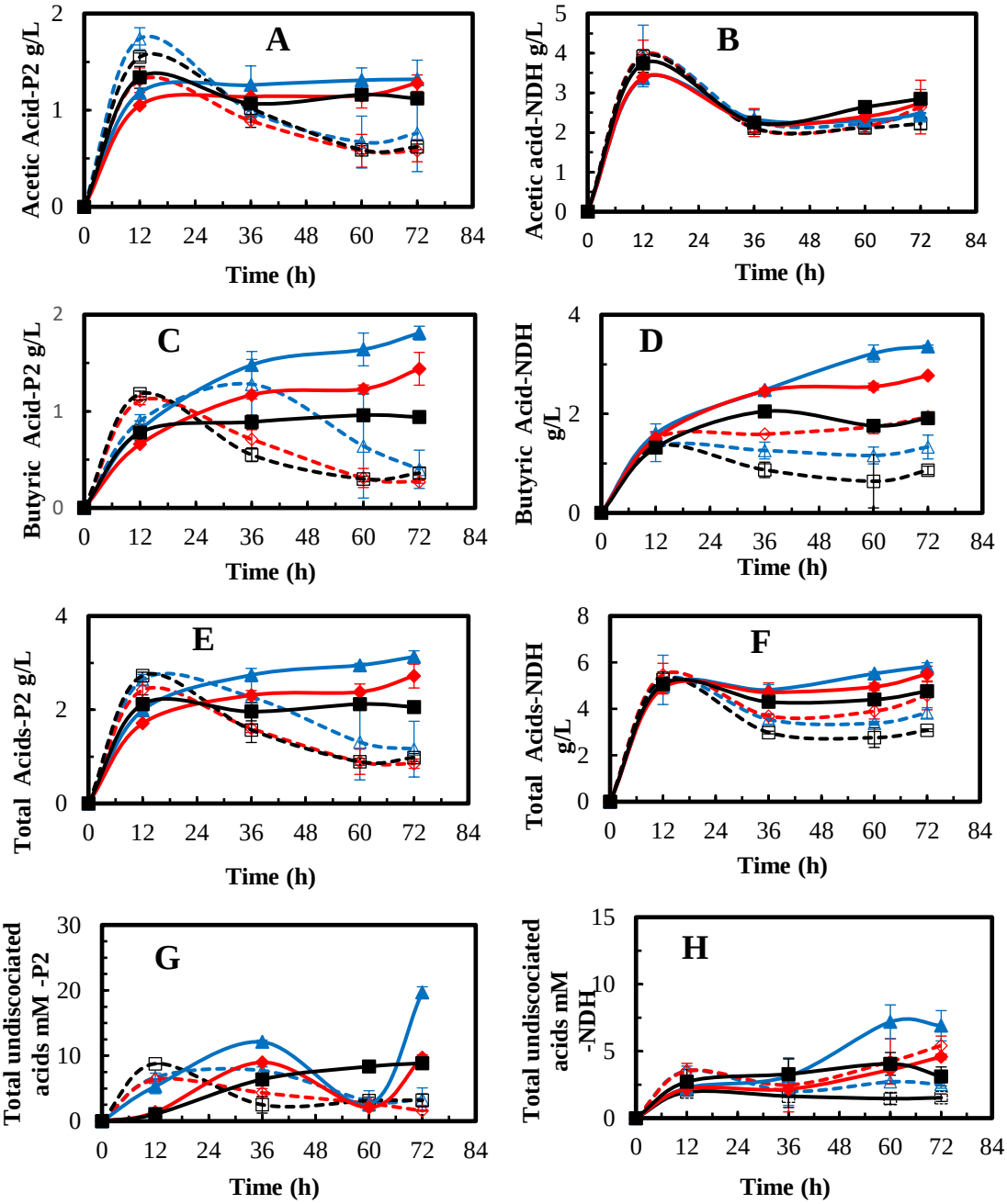

Figure B.6: (A) Acetic acid for P2 glucose medium, (B) acetic acid for non-detoxified hydrolysate, (C) butyric acid for P2 glucose medium (D) butyric acid for non-detoxified hydrolysate (E) total acids for P2 glucose medium (F) total acids for non-detoxified hydrolysate (G) total undissociated acid for P2 glucose, (H) total undissociated acid for non-detoxified hydrolysate profiles for ABE fermentation in P2 glucose medium and non-detoxified hydrolysate for optimal intermittent feeding time determination using *Clostridium beijerinckii* wild type (◆), AKR (■) and SDR (▲) strains. Solid lines and symbols represent the 6 h feeding time while the open symbols and dashed lines represent the 10 h feeding time.

The results from the figures above shows that the 10 h intermittent feeding time led to more butanol production from the SDR strain (13.1 g/L) after 72 hours as opposed to the 11.7 g/L produced by the SDR strain in the 6-hour intermittent feeding time. Furthermore, the maximum OD of 12 was reached by the wild type strain during the 10 h intermittent feeding at after 36 hours of fermentation while the maximum OD of about 10 was reached by the SDR strain during the 6 h intermittent feeding. In conclusion, the 6-hour intermittent feeding time is preferred because better yield was achieved.

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