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Bioconversion of lignocellulosic hydrolysate to acetic acid using *Moorella thermoacetica*

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A thesis

Submitted in partial fulfillment of the  
requirements for the degree of

Master of Science

University of Washington

2015

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Program Authorized to Offer Degree:

School of Environmental and Forest Resources

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Abstract

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**Abstract**

A systematic study of bioconversion of lignocellulosic sugars to acetic acid by *Moorella thermoacetica* strain ATCC 39073 was conducted. Four different hydrolysates obtained after steam pretreatment of lignocellulosic biomass were selected and fermented to acetic acid in batch fermentations. The bacterial strain (ATCC 39073) can effectively ferment xylose and glucose in steam exploded hydrolysates from wheat straw, forest residues, switchgrass, and sugarcane straw to acetic acid. This homoacetogen prefers xylose over glucose. The highest acetic acid yield obtained was in steam pretreated sugarcane straw hydrolysate and the lowest in forest residues hydrolysate (71% and 39% of theoretical respectively based on total sugars). This acetogen can partially consume arabinose, galactose and mannose within 72 h of fermentation. Acetic acid yield in forest residues was adversely affected by high concentration of arabinose, galactose and mannose. Glucose to xylose ratio has a direct effect on production yield. It was also observed that *M. thermoacetica* can tolerate process derived inhibitory compounds from steam explosion pretreatment (total phenolics up to 3 g/L and furans up to 0.5 g/L).

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## List of abbreviation

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°C	degree Celsius
C6	hexoses
C5	pentoses
CO	carbon monoxide
CO <sub>2</sub>	carbon dioxide
C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	glucose
CH <sub>3</sub> COOH	acetic acid
C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>	xylose
CH <sub>4</sub>	methane
CCR	carbon catabolite repression
CaCl <sub>2</sub>	calcium chloride
Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	cobalt nitrate hexahydrate
CSTR	continues stirred-tank reactor
DOE	department of energy
DP	degree of polymerization
FF	furfural
Fe(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ·6H <sub>2</sub> O	ammonium ferrous sulfate hexahydrate
g/L	gram per liter
h	hour
H <sub>2</sub>	hydrogen
HMF	5-hydroxymethylfurfural
KH <sub>2</sub> PO <sub>4</sub>	monopotassium phosphate
K <sub>2</sub> HPO <sub>4</sub>	dipotassium phosphate
M	Molar
mL	milliliter
mL/min	Milliliter per minute
µm	micrometer
MgCl <sub>2</sub> ·6H <sub>2</sub> O	Magnesium chloride hexahydrate
N <sub>2</sub>	nitrogen gas
NaHCO <sub>3</sub>	sodium bicarbonate
NaOH	sodium hydroxide
NaMoO <sub>4</sub> ·2H <sub>2</sub> O	sodium molybdate dihydrate
Na <sub>2</sub> SeO <sub>3</sub>	sodium selenite
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	ammonium sulphate
NiCl <sub>2</sub> ·6H <sub>2</sub> O	nickel chloride hexahydrate
nm	nanometer
OD	optical density
ODW	oven-dried weight
rpm	Revolutions per minute
SO <sub>2</sub>	Sulfur dioxide
USDA	United States Department of Agriculture
UV	ultraviolet light
v/v	volume per volume

## Acknowledgements

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I would like to express my deepest appreciation to my supervisor and chair Dr. Renata Bura for her support and motivation. She provided insight and continuously challenged me to become a better researcher. Her teachings on thoroughness and thoughtfulness will serve me for the rest of my life.

I would like to thank my committee members Dr. Richard Roy Gustafson, and Dr. William McKean for allowing me the opportunity to present my research to them.

It has been an honor to work with Biofuel and bioproducts laboratory group. My sincere thanks to Dr. Shannon Ewanick and Dr. Azra Vajzovic Suko for their valuable mentorship as well as friendship. I thank my colleagues and friends Dr. Elliott C. Schmitt, Dr. Rodrigo Morales Vera, Chang Dou, Erik Budsberg, Jordan Crawford, Oliver Jan and Guanqun Luo for their support.

I take this opportunity to express my gratitude to all of the Department faculty members at school of environmental and forest sciences for their constant assistance. This project would not have been possible without the help of numerous graduate and undergraduate students. I am thankful to Ternessa Cao, Erika Green, Yuka Harada, Kelsey Lewis, Adam Ropp, Jake Singer, Anna Song, Kaila Turner, Conner Ross, Victoria Yuen, Zeyu Song, Vinh Tran, Josie Nguyen, Therese Krieg, Naila Ribeiro Mori and Wilian Marcondes.

Last but not least, I would like to thank my family and friends for their continuous support throughout my life. I am grateful to my husband, Ghassem Namazi, and my parents, Mahvash Farazandeh and Abbas Ehsanipour for inspiring me to meet new challenges.

*To my parents, Mahvash and Abbas*

*To my husband, Ghassem*

*To my sister and brother, Roxana and Mohammad*

*For their endless love, support and motivation*

# Chapter 1- Introduction

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## 1.1 Bioconversion of lignocellulosic biomass to fuels and chemicals

The world's consumption of petroleum based energy and chemicals has been increasing dramatically. In order to satisfy the global energy demand, oil production from current levels of about 80 million barrels a day needs to rise significantly to 120 million by 2040 [1]. The adverse environmental impact of such a dramatic increase in energy usage is apparent. Global warming, water contamination, air pollution, earth quakes, droughts and many other environmental issues we face today is a direct result of these non-friendly environmental policies [2]. To lessen the environmental impact of fossil fuels, the global trend in recent years has been geared towards renewable resources and biorefineries. To support this endeavor, the United States Department of Energy (DOE) and Department of Agriculture (USDA) have mandated that by year 2022, 5% of heat and power energy, 20% of liquid transportation fuels, and 25% of chemicals and materials should come from biomass[3]. The mandates emphasize particular interest in utilizing lignocellulosic biomass for the production of biofuels and biochemicals [4].

Lignocellulosic biomass, a second generation of feedstock is a promising alternative for the production of chemicals and fuels. It is the most abundant renewable biomass, with a worldwide annual production of ~200 billion tons [5, 6]. Bioconversion of lignocellulose to biochemicals and biofuels is now at the forefront of biorefinery industries.

## 1.2 Lignocellulosic biomass

Lignocellulosic feedstock is mainly originated from woody biomass such as hardwood (e.g. poplar, birch, aspen), softwood (e.g. spruce, pine), agricultural residues (e.g. corn stover, wheat straw, sugarcane straw), herbaceous or energy crops (alfalfa, switchgrass), forest residues, and other wastes (e.g. municipal and industrial) [7]. Lignocellulose makes up the major structural material

of plants and is composed of a complex matrix of polysaccharides including cellulose and hemicellulose, lignin, extractives and ash [8, 9]. The chemical composition of lignocellulose depends on plant species, age and growth conditions. Table 1 presents the content of cellulose, hemicelluloses, lignin, extractives and ash which varies significantly between different plants [7, 10-12].

Table 1. Composition of different lignocellulosic feedstock

Lignocellulosic biomass	Composition (%)				
	Cellulose	Hemicellulose	Lignin	Extractives	Ash
Hardwood	40-55	24-40	18-25	1-5	1-4
Softwood	45-50	25-35	25-35	3-8	0.3-2
Agricultural residues	25-45	10-40	5-15	12-16	1-3

### 1.2.1 Cellulose

Cellulose is comprised of a long chain of glucose monomers linked to one another by  $\beta$ -1, 4 glycosidic bonds. The number of glucose units repeated in the chain is known as degree of polymerization (DP) which may be 10,000 or higher [9]. The linear structure of glucose chain enables intra and intermolecular hydrogen bonds between different layers of homo-polysaccharide. This results in elementary fibrils that contain crystalline and amorphous regions. Likewise, intermolecular hydrogen bonds contribute to the resistance of crystalline cellulose towards degradation. Cellulose is highly crystalline and is resistant to hydrolysis and biological digestion [9, 13].

### 1.2.2 Hemicellulose

Hemicellulose is an amorphous polymer which is, highly branched and water-insoluble [14]. This heteropolysaccharide consists of major building blocks including hexoses (D-glucose, D-galactose and D-mannose) and pentoses sugars (D-xylose and L-arabinose) with backbone chain composed

of xylan  $\beta$ -1, 4 linkages [7]. The degree of branching and functional groups such as acetate and methoxyl, as well as sugars in hemicelluloses varies depending on the type of plant [13]. Agricultural residues and hardwoods are rich in pentose sugar xylose (O-acetyl-4-O-methylglucuronoxylan), whereas softwoods are rich in hexose sugar mannose (O-acetylgalactoglucomanan). Acetyl groups can be cleaved off from hemicellulose and form acetic acids during pretreatment [9].

### **1.2.3 Lignin structure**

Lignin, derived from Latin lignum, is a carbon-rich renewable resource and one of the most abundant natural polymers, only second to cellulose [15]. It plays an important role in plant biology by greatly increasing the strength of the cell, thus giving it the necessary rigidity [16]. It is covalently linked to hemicellulose via ferulic acid ester linkages and holds cellulose and hemicellulose [13]. Lignin is a highly branched amorphous and complex random polymer of hydroxylated and methoxylated phenylpropane units. The biosynthesis of lignin involves free radical polymerization of phenylpropane building blocks resulting in an extremely variable structure, even within the same plant. There are three major aromatic units or monolignols: *p*-coumaryl, coniferyl, and sinapyl alcohols. These monolignols are incorporated into lignin in the form of phenylpropanoid units: *p*-hydroxyphenyl (H-lignin), guaiacyl (G-lignin), syringyl (S-lignin) [17].

### **1.2.4 Extractives and ash**

A minor component of lignocellulosic structure known as extractives contains a variety of specific chemicals. These include phenolics such as tannins, terpene alcohols, ketones as well as resin components such as fatty acids, alcohols, resin acids, phytosterols, and minerals (calcium, magnesium and potassium) [18, 19]. These compounds can be extracted using polar and non-polar

solvents. The extractives are often responsible for certain characteristics in plants including color, odor, physical and flavor properties [20].

### **1.3 Bioconversion process**

Bioconversion is the key process in a biorefinery when converting lignocellulosic biomass into low-value high-volume fuels and high-value low-volume chemicals [14]. The bioconversion process encompasses upstream and downstream processes, from feedstock preparation to product recovery. Some of these steps include i) pretreatment to overcome recalcitrant nature of lignocellulosic biomass, and unlock polymeric sugars and improve accessibility of the substrate to subsequent steps ii) enzymatic hydrolysis to depolymerize cellulose and hemicellulose, and iii) fermentation of released sugars to different biochemicals and biofuels [21]. Process design criteria significantly depends on the type of lignocellulosic biomass and desired product that will be produced.

#### **1.3.1 Pretreatment**

Pretreatment is a fractionation technique (physical, chemical, or both) meant to disrupt the structural bonds, break the lignin sheath, separate hemicelluloses from cellulose, and alter crystallinity of cellulose. There have been a lot of effort to develop efficient pretreatment methods to overcome the complex structure of lignocellulosic materials [22]. Amongst pretreatment methods, steam explosion has proven to have the ability to fractionate a wide variety of lignocellulosic biomass including woody (softwoods and hardwoods), agricultural and herbaceous residues, as well as energy crops [23] with high sugar recovery [24]. Steam explosion pretreatment can be carried out at high temperatures (160-260°C), pressure (0.69-4.83 MPa or 100-700 Psi) and residence time for several seconds to a few minutes [25]. Fractionation can be improved by sulfur dioxide impregnation (1-4% w/w substrate) [22]. To maximize hydrolyzability of solids and

hemicellulose removal in softwoods and hardwoods, it is necessary to use SO<sub>2</sub> gas as a catalyst in steam pretreatment [9]. Assessment of pretreatment condition is described as “severity factor”, a correlation between steam temperature and residence time, where t is time in seconds and T is temperature in degrees Celsius:

$$R_o = t \cdot e^{(T-100)/14.75} \quad (\text{Equation 1.1})$$

One will have to include the pH value when pretreatment is performed with acidic pH, using SO<sub>2</sub> gas. The following equation describes the combined severity (CS), where pH is measured after pretreatment.

$$CS = \log(R_o) - \text{pH} \quad (\text{Equation 1.2})$$

Depending on lignocellulosic biomass, a low severity factor may result in an incomplete fractionation of biomass, whereas a high severity factor results in a more complete deconstruction with better hemicellulose solubility and further delignification [24].

### **1.3.2 Enzymatic hydrolysis**

Enzymatic hydrolysis is a process in which biocatalysts facilitate the conversion of physio-chemically pretreated substrate to fermentable sugars. Enzymatic pretreatment of lignocellulosic biomass relies on two enzyme complexes including cellulases and hemicellulases, multi-domain proteins [26]. Thus efficient conversion of lignocellulosic biomass during enzymatic hydrolysis requires a mixture of different enzymes acting sequentially or in concert.

In a natural fermentation process, a potent cellulolytic organism such as *Trichoderma reesei* produces complex mixtures of enzymes composed of three major groups. The groups include endoglucanases (CM-cellulases), exoglucanases or cellobiohydrolases, and β-glucosidases or cellobiases. Digestibility of celluloses according to its structure occurs as below [27]:

- Endoglucanases cleave the internal  $\beta$ -1, 4-glycosidic bonds of amorphous, swollen cellulose and release glucose, cellobiose and cello-oligosaccharides.
- The exoglucanases cleave cellobiose units from the ends of the polysaccharide chains.
- $\beta$ -glucosidases cleave cellobiose to release glucose.

### 1.3.3 Fermentation

Fermentation is a metabolic process that harnesses a microorganism's ability to digest lignocellulosic sugars (pentoses and hexoses) to produce products such as ethanol, butanol, acetone, acetic acid, butyric acid, H<sub>2</sub> gas, and CH<sub>4</sub> gas [28]. Numerous microbial strains (bacteria and fungi) have been utilized in fermentation of lignocellulosic substrates [29]. Some of the well utilized microorganisms include *Zymomonas mobilis* [30], *Saccharomyces cerevisiae*, *Clostridium thermocellum* as ethanologenic [31]; *Moorella thermoacetica*, *Clostridium ljungdahlii* as acetogenic [32]; *Clostridium tyrobutyricum JM*, a hydrogen producing [33]; and *Clostridium acetobutylicum* for production of acetone, ethanol, and butanol [34].

The versatility of microorganisms plays a significant role in fermentation processes employed in biorefineries [29]. In all fermentations a high level of production yield is desired which depends on specific microbial traits including high tolerance to substrate concentration, and end product as well as inhibitor compounds. Furthermore, it is required to have a broad substrate utilization (C<sub>6</sub> and C<sub>5</sub> sugars), with minimal byproduct formation [35].

### 1.3.4 Inhibitors

A drawback during pretreatment is formation of chemical compounds that may inhibit microbial activity or increase the lag phase of microbial growth during a fermentation process. Inhibitors (toxic substances) may also decrease the production yield in a fermentation process [35]. The

composition of toxic compounds depends on type of lignocellulosic biomass and the severity of pretreatment method [36]. Inhibitors are categorized in three different groups i) weak acids: acetic acid, levulinic acid, glucuronic acid, furoic acid and humic acid, ii) furan derivatives: hydroxymethylfurfural (HMF), furfural (FF), and iii) phenolic compounds: hydroxybenzoic acid, vanillic acid, and ferulic acid [37]. Acetic acid forms through acetyl cleavage in hemicellulose, furfural through degradation of pentose monomers, and HMF from degradation of hexoses. FF and HMF can also be further degraded to levulinic and humic acids. Furthermore, degradation of lignin results in phenolic derivatives [37-40]. The concentration of these chemical compounds varies according to type of lignocellulosic biomass and severity of pretreatment conditions [35, 41]. These fermentation inhibitors are building blocks for other chemicals such as furfural [42] for tetrahydrofurfuryl alcohol [43] and levulinic acid for valerolactone [42]. The US Department of Energy (DOE) has created a list of value added chemicals that may be extracted from lignocellulosic biomass, thus giving credibility to lignocellulosic biorefinery [44]. Acetic acid is a value added chemical that can also be produced through a fermentation process.

#### **1.4 Acetic acid**

The current global interest in renewable resources and biorefineries has provided momentum and new opportunities in the area of manufacturing biochemicals. In order for current biorefineries to be competitive with legacy based fossil fuel technologies, a more efficient process for the production of bio-based chemicals needs to be developed. One such bio-based chemical is acetic acid, an important carboxylic acid with a broad spectrum of industrial and household applications including polymers, paints, foods and pharmaceuticals. Acetic acid is a major building block for chemicals such as polyvinyl and cellulose acetate which is further used in the production of fibers and fabrics [45].

Currently, more than 65% of global acetic acid manufacturing process is based on catalytic carbonylation of methanol utilizing rhodium complex catalyst [45, 46]. Other procedures involve oxidation of acetaldehyde, liquid phase oxidation of n-butane or naphtha, and chemical conversion of syngas produced through gasification of coal [47]. The United States acetic acid production capacity is 788 MM gal/year with spot market price at \$2.5 per gallon [47, 48]. In comparison, bioethanol production capacity is 15,135 MM gal/year [49] with a price of \$1.8 per gallon [50]. Hence, production of acetic acid, a high-value commodity through bioconversion of lignocellulosic biomass is economically viable [51]. Acetogenic bacteria has shown to be a promising candidate for the production of acetic acid [52].

### **1.5 *Moorella thermoacetica***

Since 1923, over a hundred acetogenic species representing twenty two genera have been isolated from a variety of habitats [32]. Among acetogens, *Moorella thermoacetica* (a gram-positive thermophilic, anaerobic bacterium, previously known as *clostridium thermoacetica*) is a homoacetogen in which acetate is the major product of its metabolic pathway [53]. This acetogen is robust with a wide range of growth temperatures (45-65°C) and pH levels (5.7-7.7). The optimum growth temperature range is 55-60°C and pH level of 6.8 [54]. This bacteria has a diverse and unique metabolic capability. It is able to produce acetic acid or acetate by metabolizing certain sugars including xylose, fructose and glucose. Xylose is the preferred sugar and consumed first, followed by fructose and glucose [55-57].

Metabolic pathway of *Moorella thermoacetica* has been extensively studied by Wood, Ljungdahl, Daniel and Drake. Basically, glucose or fructose is oxidized to pyruvate through Embden-Meyehof-Parnas glycolytic pathway (glycolysis) and is further metabolized to two molecules of acetate and two molecules of carbon dioxide (CO<sub>2</sub>). This acetogen is also able to synthesize acetate

from carbon dioxide through acetyl-CoA Wood–Ljungdahl pathway [54, 58]. As a result, syngases may be utilized as a substrate (chemolithoautotrophic growth on H<sub>2</sub>/CO<sub>2</sub> or CO/CO<sub>2</sub>) [52-54]. This acetogen is capable of fixing CO<sub>2</sub> in which one molecule of acetate is synthesized from carbon dioxide through Acetyl-CoA pathway (Wood–Ljungdahl pathway) reactions [54, 58]. Conversion of glucose to acetic acid is presented in the following stoichiometry reactions. Sources of CO<sub>2</sub> can be from outside of cells (exogenous CO<sub>2</sub> used to maintain anaerobic condition) or through decarboxylation of pyruvate (inside of cells) [53].



Unlike glucose, the pathway for conversion of xylose to acetate in *M. thermoacetica* has not been well investigated [54]. However, Pierce and coworkers, 2008, emphasized that *M. thermoacetica* encodes xylose to acetate through pentose phosphate pathway in which xylose converts to xylitol. Xylitol is subsequently oxidized to xylulose followed by another conversion to glyceraldehyde 3-phosphate. At this point it merges with glycolysis pathway [59]. Stoichiometric conversion of xylose to acetic acid is summarized in the following reaction [54].



A typical acetic acid production in a batch fermentation using synthetic glucose (20 g/L) is around 13 g/L [51]. The highest acetic acid production reported was at 31 g/L using CO/CO<sub>2</sub> gas mixture [60]. On average, bacterial tolerance of acetate is between 30-50 g/L in a batch fermentation [61]. Previous efforts regarding production of acetic acid utilizing *M. thermoacetica* have by and large concentrated on industrially suitable culture media and fermentation setups using synthetic sugars.

### **1.5.1 Cultivation Media**

*M. thermoacetica* has complex nutrient requirements in which a typical cultivation medium includes carbon source, trace metals, salts, reducers, yeast extract and buffer solutions [52]. One of the major issues has been the development of a cost effective growth medium using different substitutions for yeast extract [62-65]. Corn steep liquor (CSL) pretreated with dolime (calcium and magnesium oxide, CaO-MgO) and vitamin supplementation has shown to be a promising replacement since final acetate concentration was improved by 110% (from 19 to 40 g/L in a fed-batch fermentation) [65].

### **1.5.2 Fermentation setups**

Various fermentation setups have also been evaluated to optimize acetate production [66-69]. Results from fed-batch fermentation and continuous stirred-tank reactor (CSTR), using cell recycle membrane system were shown to have a higher concentration of acetate (34-38 g/L) than one-step CSTR (25.5 g/L) [65].

### **1.5.3 Extracted sugars**

A number of commercial and extracted sugars have also been evaluated for the production of acetic acid. Brownell and coworker utilized extracted xylose from debarked poplar wood chips (Soxhlet extraction using benzene–ethanol) and commercial oat spelt xylan (average acetate yield of 70%) [70]. The commercial glucose from  $\alpha$ -cellulose and pulp mill sludge with high enzyme loading at 50 IFPU/g glucan were also utilized. Overall acetate yield was 60% and 85% respectively. The authors stipulated that acetate production was limited by toxic materials from the sludge [71].

## **1.6 Research objectives**

To the best of the author's knowledge, sugars obtained from bioconversion of lignocellulosic biomass have not been investigated for acetic acid production. For the first time this paper presents an original study about bioconversion of lignocellulosic hydrolysate to acetic acid utilizing *M. thermoacetica*. The objective of this research is to examine the ability of *M. thermoacetica* to ferment monomeric sugars in four different steam exploded hydrolysates including wheat straw, forest residues, switchgrass, and sugarcane straw.

## **Chapter 2 Fermentation of lignocellulosic sugars to acetic acid by *Moorella thermoacetica***

*To be submitted for publication in an international journal.*

---

### **Abstract**

A systematic study of bioconversion of lignocellulosic sugars to acetic acid by *Moorella thermoacetica* strain ATCC 39073 was conducted. Four different water-soluble fractions (hydrolysates) obtained after steam pretreatment of lignocellulosic biomass were selected and fermented to acetic acid in batch fermentations. The bacterial strain (ATCC 39073) can effectively ferment xylose and glucose in hydrolysates from wheat straw, forest residues, switchgrass, and sugarcane straw to acetic acid. This homoacetogen prefers xylose over glucose. The highest acetic acid yield was obtained from sugarcane straw hydrolysate with 71% of theoretical based on total sugars. The lowest acetic acid yield was observed in forest residues hydrolysate with 39% of theoretical based on total sugars. This acetogen consumes arabinose, galactose and mannose within 72 h of fermentation in the mixture of lignocellulosic sugars. Acetic acid yield in forest residues was adversely affected by high concentration of arabinose, galactose and mannose. It was also observed that *M. thermoacetica* can tolerate process derived inhibitory compounds from steam explosion pretreatment (total phenolics up to 3 g/L and furans up to 0.5 g/L).

### **2.1 Introduction**

The current global interest in renewable resources and biorefineries has provided momentum and new opportunities in the area of manufacturing biochemicals. In order for current biorefineries to be competitive with legacy based fossil fuel technologies, a more efficient process for the production of bio-based chemicals needs to be developed. One such bio-based chemical is acetic acid, an important carboxylic acid with a broad spectrum of industrial and household applications including polymers, paints, foods and pharmaceuticals. Acetic acid is a major building block for chemicals such as polyvinyl and cellulose acetate which is further used in the production of fibers and fabrics [1].

Currently, more than 65% of global acetic acid manufacturing process is based on methanol carbonylation utilizing rhodium complex (rhodium iodides) catalyst [1, 2]. Other procedures involve oxidation of acetaldehyde, liquid phase oxidation of n-butane or naphtha, and chemical

conversion of syngas produced through gasification of coal [3]. The United States acetic acid production is 788 MM gal/year with spot market price at \$2.5 per gallon [3, 4]. In comparison, bioethanol production is 15,135 MM gal/year [5] with a price of \$1.8 per gallon [6]. Hence, production of acetic acid, a high-value commodity through bioconversion of lignocellulosic biomass is economically viable [7]. Acetogenic bacteria has shown to be a promising candidate for the production of acetic acid [8].

Amongst acetogens, *Moorella thermoacetica* (a gram-positive thermophilic, anaerobic bacterium, previously known as *Clostridium thermoacetica*) is a homoacetogen in which acetate is the major product of its metabolic pathway [9]. *M. thermoacetica* is a robust and versatile heterotroph with optimum growth temperature range of (55-60) °C, and pH level of 6.8 [10]. This bacteria has a diverse and unique metabolic capability. It is able to produce acetic acid / acetate by metabolizing certain sugars including xylose, fructose and glucose. Xylose is the preferred sugar and consumed first, followed by fructose and glucose [11-13]. The conversion of glucose and fructose is through Embden-Meyehof-Parnas glycolytic pathway [10, 14] while xylose is through pentose phosphate pathway [15]. Furthermore, *M. thermoacetica* is able to synthesize acetate from carbon dioxide through acetyl-CoA Wood–Ljungdahl pathway [10, 14]. As a result, syngases may be utilized as a substrate (chemolithoautotrophic growth on H<sub>2</sub>/CO<sub>2</sub> or CO/CO<sub>2</sub>) [8-10]. Typical acetic acid production in a batch fermentation using synthetic glucose (20 g/L) is around 13 g/L [7]. The highest acetic acid production reported was at 31 g/L using CO/CO<sub>2</sub> gas mixture [16]. On average, bacterial tolerance of acetate is between 30-50 g/L in a batch fermentation [17].

Previous efforts regarding production of acetic acid utilizing *M. thermoacetica* have by and large concentrated on developing industrially suitable culture media and fermentation setups using synthetic sugars. *M. thermoacetica* has complex nutrient requirements in which a typical

cultivation medium includes carbon source, trace metals, salts, reducers, yeast extract and buffer solution [8]. One of the major issues has been the development of a cost effective growth medium using different substitutions for yeast extract [18-21]. Corn steep liquor (CSL) pretreated with dolime (calcium and magnesium oxide, CaO-MgO) and vitamin supplementation has shown to be a promising replacement since final acetate concentration was improved by 110% (from 19 to 40 g/L in a fed-batch fermentation) [21]. Various fermentation setups have also been evaluated to optimize acetate production [22-25]. Results from fed-batch fermentation and continuous stirred-tank reactor (CSTR), using cell recycle membrane system were shown to have a higher concentration of acetate (34-38 g/L) than one-step CSTR (25.5 g/L) [21].

A number of commercial and solvent extracted sugars have also been evaluated for the production of acetic acid. Brownell and coworker utilized extracted xylose from debarked poplar wood chips (Soxhlet extraction using benzene–ethanol) and commercial oat spelt xylan (average acetate yield of 70%) [26]. The commercial glucose from  $\alpha$ -cellulose and pulp mill sludge were also utilized and the overall acetate yield was 60% and 85%, respectively. The authors stipulated that acetate production was limited by toxic materials from the sludge [27].

To the best of our knowledge, this is the first report on the bioconversion of lignocellulosic sugars to acetic acid by *M. thermoacetica*. The objective of this work is to examine *M. thermoacetica* in fermentation of four different types of hydrolysates obtained after steam pretreatment of lignocellulosic biomass including wheat straw, forest residues, switchgrass, and sugarcane straw. The ultimate goal of our research is to establish bioconversion process of lignocellulosic biomass to acetic acid using both hexose and pentose fractions after steam pretreatment for production of acetic acid.

## **2.2 Materials and methods**

### **2.2.1 *Moorella Thermoacetica* strain (ATCC 39073)**

*Moorella thermoacetica* (ATCC 39073) was acquired from the American Type Culture Collection (Manassas, VA) grown in a growth medium containing g/L of the following compounds: yeast extract, 10.0; glucose, 10.0; xylose, 10.0; L-Cysteine Hydrochloride Monohydrate 1.0; KH<sub>2</sub>PO<sub>4</sub> 7.0; NaHCO<sub>3</sub> 16.0; K<sub>2</sub>HPO<sub>4</sub> 5.5; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.0; MgCl<sub>2</sub>-6H<sub>2</sub>O, 0.33; CaCl<sub>2</sub> 0.05; NaMoO<sub>4</sub>-2H<sub>2</sub>O 0.0025; Fe(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-6H<sub>2</sub>O 0.04; Co(NO<sub>3</sub>)<sub>2</sub>-6H<sub>2</sub>O 0.03; Na<sub>2</sub>SeO<sub>3</sub> 0.0002; NiCl<sub>2</sub>-6H<sub>2</sub>O 0.0002. The pH level of the medium was adjusted to 7.3 using 5M NaOH [7, 11].

### **2.2.2 Culture media conditions**

Cells were grown to a high cell density through three sequential passagings, from 50 mL to 200 mL in stoppered and crimp-sealed Wheaton serum bottles. Prior to inoculation, Wheaton serum bottles containing growth medium were sparged with filter-sterilized 100% CO<sub>2</sub> and inoculated with 10% by volume serum bottles incubated at 58°C for 36 h in dark condition (incubator in Bactron II anaerobic chamber, Sheldon manufacturing, Inc.). The third passage (200 mL) was used as the inoculum for fermentation. After 36 h of growth, cell cultures were harvested and centrifuged at 4000 rpm (Beckman GS-15R, Germany) for 5 minutes. They were subsequently decanted to yield cell pellets with a concentration of 7 g/L dry cell weight (DCW). The concentration of dry cell weight was measured based on optical density using a UV spectrophotometer (Shimadzu UV-1700, Columbia, MD) via standard curves relating 600nm absorbance to DCW per liter.

## **2.3 Water-soluble fraction (hydrolysate)**

The water-soluble fractions (hydrolysates) used in this study were produced from wheat straw, forest residues, switchgrass, and sugarcane straw. Each lignocellulosic biomass was soaked to

saturation in deionized (DI) water overnight prior to SO<sub>2</sub>-catalyzed steam explosion. The detailed procedure of steam explosion experiments has been described previously by Ewanick [28]. Briefly, samples of 200g oven-dried weight (ODW) lignocellulosic biomass were impregnated overnight with anhydrous 3% (w/w) SO<sub>2</sub>. The samples were subsequently loaded in a steam gun and kept for five minutes at a temperature of 195°C, except for sugarcane straw which was treated at 195°C (Figure 1). The water-soluble fraction (hydrolysate) from steam explosion of each lignocellulosic biomass was recovered by filtration and kept at 4°C until use. The initial concentration of sugars present in the hydrolysates was measured.

## **2.4 Overliming**

Prior to fermentation, each hydrolysate was conditioned by an overliming (OL) process in which the pH level was increased from 1.9 to 10.0 with Ca(OH)<sub>2</sub> (industrial and chemical purposes, Graymont, USA). The pH level was next readjusted to 5.0 with additional sulfuric acid. The overliming process was carried out based on procedure described by national renewable energy laboratory (NREL) [29].

## **2.5 Fermentation**

Four different lignocellulosic hydrolysates as well as their corresponding synthetic sugar models were fermented. Figure 1 presents a simplified scheme of fermentation experiments. All fermentation experiments were performed in duplicates using a 1.3 L Bioflo 115 (New Brunswick Scientific Co., Inc., Edison, NJ) bioreactor with 500 mL of working volume under anaerobic conditions using filter-sterilized 100% CO<sub>2</sub> gas sparged into the bottom of the fermenter vessel continuously prior to inoculation. Sparging was maintained at headspace after inoculation and throughout fermentation. Inoculation was performed with 10% v/v of three-stage seeded bacterial cells grown on glucose and xylose in a 1:1 ratio. All fermentations were maintained at 58°C with

continuous agitation (175 rpm), CO<sub>2</sub> gas flow rate of 1 standard liter per minute (SLPM) and monitored for 72 h. A solution of 5 M NaOH was added to sustain pH value of 6.8 throughout fermentation. Samples were taken at the time of inoculation and at specific intervals thereafter. One milliliter of aliquots was immediately centrifuged (10,000 rpm) for 5 minutes at 4°C to separate microbial cell and supernatant. The supernatant was filtered by using a 0.2 µm syringe filter and then stored at -20°C until analysis. The bacterial growth was monitored during fermentations. Optical density (OD) of cell was measured at 600nm by spectrophotometer (Shimadzu UV-1700, Columbia, MD). Conversion of OD to dry cell mass was constructed based on NREL protocol (N. Dowe and J. McMillan, 2001).

### **2.5.1 Hydrolysate fermentation**

Prior to fermentation, all overlimed hydrolysates were supplemented with nutrients used in growth medium and initial pH level was adjusted to 7.3. Xylose concentration in all hydrolysates was spiked to 20 g/L in order to establish a baseline. All hydrolysates were filter sterilized using a 500 mL rapid flow bottle top with 0.2 µm sterile filter.

### **2.5.2 Synthetic sugars fermentation**

Synthetic sugars fermentation was performed as an appropriate control for each type of hydrolysate. The media was prepared similarly to growth medium except for sugars content. Glucose, arabinose, galactose and mannose were added based on their corresponding concentration measured in each one of overlimed hydrolysates. For all controls, xylose concentration was kept at 20 g/L. The fermentation of all synthetic sugars models were performed with the same manner as fermentation experiments of hydrolysates.

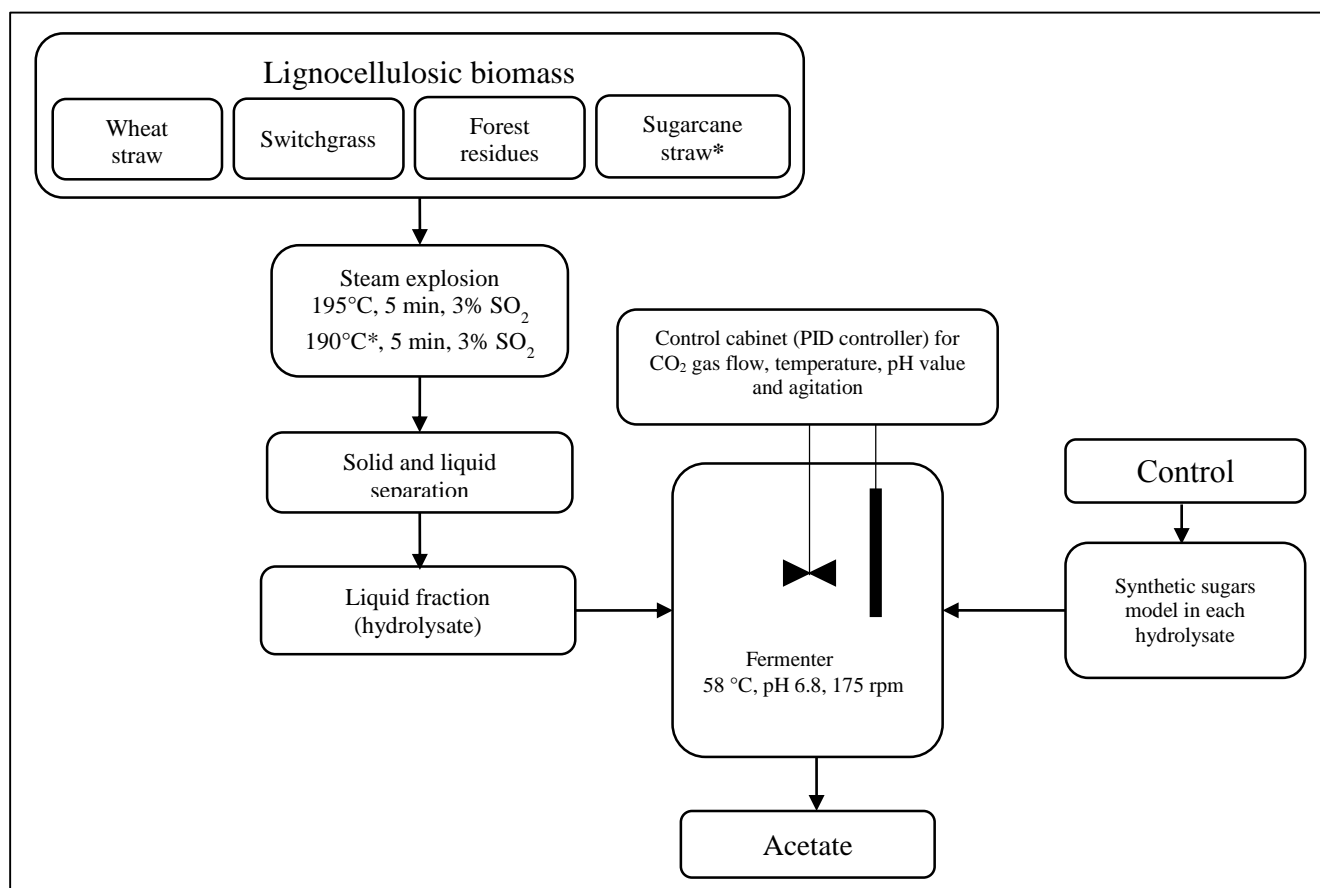


Figure 1. Schematic diagram of fermentation experiments. All lignocellulosic biomass were steam exploded at 195°C, except for sugarcane straw. Sugarcane straw was steam exploded at 190°C.

## 2.6 HPLC analysis

### 2.6.1 Monomeric sugars

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) was measured on a Dionex (Sunnyvale, CA) high-performance liquid chromatography (HPLC, ICS-3000) system equipped with AS (auto sampler), ED (electrochemical detector), dual pumps and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1.0 mL/min was used as eluent, post column addition of 0.2 M NaOH at a flow rate of 0.5 mL/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. Ten microliters of each sample was injected after filtration through a 0.2  $\mu$ m

syringe filter (Restek Corp., Bellefonte, PA, USA). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose and mannose to encompass the same range of concentration as the samples. Fucose was added to all samples as an internal standard.

### **2.6.2 Acetate, hydroxymethylfurfural and furfural analysis**

Acetic acid, hydroxymethylfurfural and furfural were measured using refractive index detection on a Shimadzu Prominence LC. The separation of these compounds was achieved by an anion exchange column [REZEX RHM-Mono saccharide H<sup>+</sup>(8%); Phenomenex, Inc., Torrance, CA, USA] with an isocratic mobile phase that consisted of 5mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL/min. The column oven was maintained at a constant temperature of 63°C. Ten microliters of each sample was injected after being appropriately diluted in deionized water and filtered through a 0.2 µm syringe filter. The standards were prepared and used to quantify the unknown samples.

### **2.7 Phenolic measurement**

Total phenolic was measured based on colorimetric reaction using Folin-Ciocalteu (FC) method. Gallic acid was utilized as an equivalent to the phenolic compounds. Gallic acid (0.500 g) was dissolved in 10 mL of ethanol and diluted to 100 mL in volumetric flask using distilled/deionized water. A set of standards (different concentration of gallic acid) was prepared to create a calibration curve. Sodium carbonate solution was used for colorimetric reaction. Procedure was followed as described by A. L. Waterhouse, 2012 [30].

### **2.8 Results and discussion**

In order to examine the ability of *M. thermoacetica* to ferment lignocellulosic sugars to acetic acid, fermentation of four different types of lignocellulosic hydrolysates were conducted. Hydrolysates obtained from steam pretreated wheat straw, forest residues, switchgrass, and sugarcane straw were used as medium for production of acetic acid by *M. thermoacetica* (ATCC 39073). The

synthetic sugars models (controls) consisted of synthetic sugars at the same concentration as measured in hydrolysates (described in “Methods” section). A typical fermentation profile for sugar consumption, acetic acid production, and cell growth for each hydrolysate is shown in Figure 2, plots A, B, C and D.

In general, it was shown that xylose was the initial sugar which was completely metabolized by *M. thermoacetica* in all hydrolysates. Glucose started to be metabolized after more than half of xylose was consumed. A minimal amount of arabinose, galactose and mannose was gradually consumed in a period of 72 h of fermentation. The fastest xylose consumption (35 h) was observed in sugarcane straw and switchgrass hydrolysates while the slowest (50 h) was observed in forest residues hydrolysate (Figure 2, plots B, D). Xylose uptake commenced by *M. thermoacetica* in all hydrolysates without any lag phase, except for wheat straw hydrolysate which had a lag phase of 12 h. Glucose was completely consumed within a range of 40 h (fastest in sugarcane straw hydrolysate) to 70 h (slowest in forest residues hydrolysate). Low concentrations of minor sugars including arabinose, galactose and mannose were detected in all hydrolysates except for forest residues which had a considerable amount of these sugars (Table 1). The consumption of these sugars was incomplete and the following was observed: the lowest consumption of arabinose in switchgrass (37%), galactose and mannose in sugarcane straw (18% and 28% respectively), and the highest consumption of arabinose and galactose in wheat straw (62% and 49% respectively), mannose in switchgrass (66%).

In fermentation of controls, a similar trend in sugars consumption was observed (graphs for controls not shown). Xylose uptake in each control was the same as its corresponding hydrolysate except for wheat straw and forest residues controls in which it was consumed slower (60 h and 65 h respectively). The pattern for glucose consumption was also comparable to hydrolysates.

However, glucose in control for forest residues was not completely utilized over the fermentation period of 72 h (1 g/L remained, plots not shown). The incomplete consumption of arabinose, galactose and mannose in controls were different from hydrolysates. The lowest arabinose and galactose consumption was observed in forest residues control (42% and 13% respectively) and mannose in controls for switchgrass (12%). The highest consumption of these sugars was observed as follows: arabinose in control for sugarcane straw (54%), galactose in control for switchgrass (36%), and mannose in control for sugarcane straw (55%).

The highest cell biomass was observed in wheat straw hydrolysate (23 g/L) while the lowest was observed in sugarcane straw hydrolysate (21.4 g/L) (Table 1; Figure 2). In synthetic sugar models, wheat straw control had the highest cell concentration (24.5 g/L) while forest residues control had the lowest (19 g/L). In general, cell concentration was higher (0.2-1.5 g/L) in each control compared to its hydrolysate except for synthetic sugar model for forest residue (Table 1).

Acetic acid production yield was calculated based on total initial sugars (Table 1). The highest acetic acid yield was observed in steam pretreated sugarcane straw hydrolysate with 71% of theoretical yield (17.2 g/L) while the lowest yield was observed in forest residues hydrolysate with 38% of theoretical (18.2 g/L). Acetic acid yield was higher for all hydrolysates compared to their corresponding controls except for forest residues control (39%) and switchgrass control (55%). The conversion of sugars to acetic acid in fermentation of hydrolysates were as good as or higher than their corresponding controls.

It was observed that *M. thermoacetica* was not able to consume arabinose, galactose and mannose in a fermentation of single sugar which resulted in a cluster of dead cells (Table 2). The effects of minor sugars on acetic acid yield was investigated in two separate flask fermentations using mixed sugars with equal concentrations (Table 3). In one fermentation, inoculant was pre-grown on the

same mixture of sugars while in the second fermentation inoculant was pre-grown on a 1:1 ratio of glucose to xylose. Table 3 presents that *M thermoacetica* could completely metabolize xylose and glucose while the consumption of other sugars was incomplete (<40%) which resulted in a very low acetic acid yield.

There are not any references regarding fermentation of mixed lignocellulosic sugars by *M. thermoacetica*. In a study by Balasubramanian and coworkers, fermentation of mixed synthetic sugars (4.5 g/L xylose, 5.5 g/L glucose, 5 g/L fructose, 5.5 g/L arabinose, 4.5 g/L galactose and 5 g/L mannose) using *M. thermoacetica*'s inoculant with a very low cell concentration (~2%, v/v) was investigated[13]. The consumption of minor sugars for a fermentation period of 135 h was estimated as follows: arabinose 36%, galactose 11%, and mannose 20%. Additionally, acetic acid yield based on total sugars was 45% [13]. The authors utilized an average concentration of 5 g/L for each sugar and inoculant with ~2% (v/v) pre-grown on glucose and subsequent transfer in xylose. By contrast, in our experiments the consumption of arabinose, galactose and mannose was less than 50%. This difference in partial consumption of minor sugars can be attributed to the following: i) presence of fructose in their fermentation medium, ii) the initial concentrations of sugars and iii) inoculant's cell concentration. It is worth noting that the presence of these minor sugars lowered the acetic acid yield which is comparable to fermentation results of forest residues hydrolysate.

Another possible explanation for low acetic acid yield could be the glucose to xylose ratio. Table 4 presents pH-controlled batch fermentation results from the mixtures of glucose and xylose with different concentrations. The concentration of glucose and xylose in fermentation number (no.) 1 corresponds to wheat straw, switchgrass and sugarcane straw hydrolysates. Glucose and xylose concentration in fermentation no. 3 is similar to forest residues hydrolysates. In this set of

experiments, glucose and xylose consumption as well as acetic acid yield were monitored over a period of 50 h, 75 h and 96 h of fermentation. In a 50 h period of fermentation, the highest acetic acid yield based on total sugars (75% of theoretical) was observed in mixtures with high concentration of glucose to xylose (20 g/L to 3 g/L and 20g/L to 9 g/L). In these mixtures, xylose and glucose were completely metabolized except for fermentation no. 4 which had a residual glucose less than 1 g/L. By contrast, low acetic acid yield was observed in fermentation no. 3 and 4 (50% and 59% of theoretical respectively) with considerable residual of glucose and xylose (Table 4, fermentation no. 1 and 3). After 75 h of fermentation, media with high glucose concentration (Table 4, fermentation no. 2 and 4) presented higher acetic acid yield (75% and 77% of theoretical respectively) than fermentation no. 1 and 3 with high xylose concentration (67% and 70% of theoretical respectively). A Similar trend in acetic acid yield was also observed after 96 h. It was noted that glucose to xylose ratio had a significant influence on acetic acid yield and sugars uptake.

In fermentation of hydrolysates and their controls, as well as mixtures of glucose and xylose, glucose uptake was clearly repressed by xylose which emphasizes the presence of carbon catabolite repression (CCR) mechanism in *M. thermoacetica*. The CCR mechanism in *M. thermoacetica* appeared to influence acetic acid yield. Glucose to xylose ratio as well as the presence of arabinose, galactose and mannose intensify CCR behavior in *M. thermoacetica*. Specifically, in forest residues hydrolysate, acetate yield was adversely affected by high concentration and incomplete consumption of minor sugars as well as glucose to xylose ratio (Figure 2, plot B; Table 1). Furthermore, bacterial pre-growth on a 1:1 ratio of glucose to xylose may have a direct influence on CCR and subsequently on acetate yield. Brumm observed a 20%

lower acetate yield in fermentation of xylose utilizing culture which was grown on an equivalent amount of fructose or glucose compared to culture pre-grown on xylose [12].

Based on our research results, in order to obtain an optimum acetic acid yield, it is imperative to have lignocellulosic hydrolysate with a high xylose content and zero or a very minimal amount of other lignocellulosic sugars. Hence, steam explosion pretreatment design is a key process that needs to be customized to extract xylose and glucose in a step-wise manner. In the initial step, with low severity factor, collect as much xylose in liquid stream (hydrolysate) as possible. In the second step, steam explode the solid stream to collect the remaining xylose which is in water-soluble stream. The choice of lignocellulosic feedstock also improves acetic acid yield. Hardwoods and agricultural residues have a great potential for the production of acetic acid. Softwoods would not be a good option due to their high content of galactoglucomannan [31].

*M. thermoacetica* tolerated process derived inhibitory compounds from steam explosion pretreatment including hydroxymethylfurfural (HMF) and furfural (FF) (up to 0.28 g/L and 0.9 g/L, respectively, data are not shown) and total phenolics of up to 3 g/L (Table 1). This bacterial strain did not metabolize phenolic compounds during fermentations. However, it did consume HMF and FF in switchgrass (0.44 g/L and 0.1 g/L respectively) and sugarcane straw (0.01 g/L of each) hydrolysates in the first 12 h of fermentation (Table 1). The consumption of HMF and FF may also intensify CCR mechanism. Total phenolics and furans did not inhibit microbial growth or acetic acid production. This characteristic has also been noted in a prior study utilizing yeast for production of xylitol and ethanol from lignocellulosic hydrolysate. This study used a mixture of hybrid poplar and Douglas fir collected after steam explosion pretreatments [32].

## 2.9 Conclusion

It was presented for the first time that *M. thermoacetica* (ATCC 39073) can effectively ferment xylose and glucose content in water-soluble fraction of steam exploded lignocellulosic biomass including wheat straw, forest residues, switchgrass, and sugarcane straw to acetic acid. In all fermentations xylose was the major and the first sugar being consumed. Xylose consumption was faster in switchgrass and sugarcane straw hydrolysates (35 h) than wheat straw and forest residues hydrolysates (40 and 45 h respectively). Xylose repressed glucose uptake in which glucose was metabolized when the majority of xylose was consumed. *M. thermoacetica* consumed less than 50% of arabinose, galactose and mannose within 72 h of fermentation in a mixture of sugars. The highest cell biomass was observed in wheat straw hydrolysate (23 g/L) and the lowest in sugarcane straw hydrolysate (21.4 g/L). The highest acetic acid production based on total sugars was obtained from sugarcane straw with 71% of theoretical yield while it was 64% in its control. The lowest acetic acid yield based on total sugars was observed in forest residues with 39% of theoretical. The incomplete consumption of arabinose, galactose and mannose adversely affected acetic acid yield. *M. thermoacetica* demonstrated that it tolerates process derived inhibitory compounds from steam explosion pretreatment including HMF (0.44 g/L), FF (0.1 g/L) and total phenolics (3 g/L). The variations observed in acetic acid yields, repressed glucose uptake by xylose, and incomplete consumption of minor sugars confirmed CCR behavior in *M. thermoacetica*.

## Acknowledgements

This research was supported by U. S. Department of Agriculture with funding from Agriculture and Food Research Initiative (USDA AFRI grant 2011-68005-30407). We would like to thank the University of Washington-Denman Professorship funds in Bioresource Engineering as well as

Biofuels and Bioproducts Laboratory Research Group. Our special thanks to Dr. Shannon Ewanick, Dr. Rodrigo Morales, and Naila Ribeiro Mori for providing hydrolysates.

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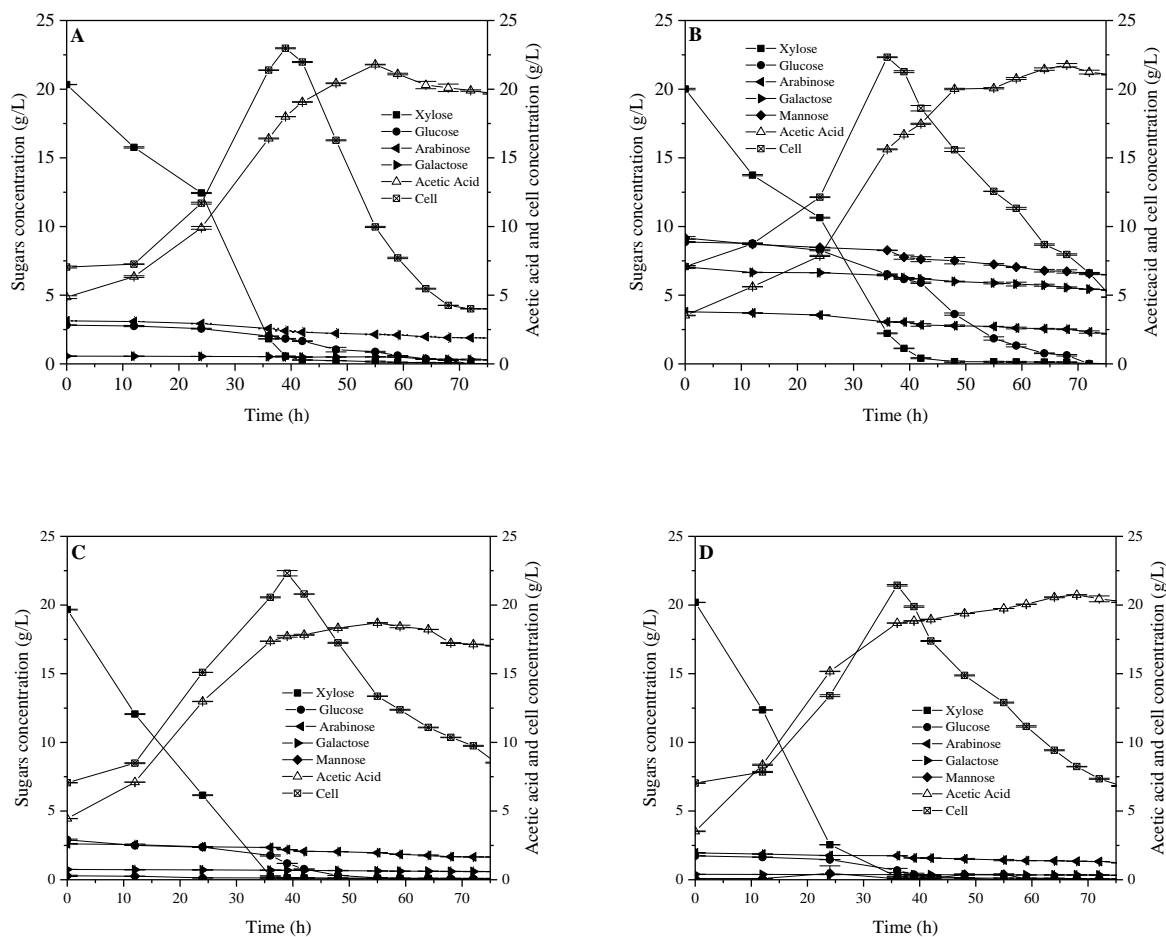


Figure 2. Sugars consumption, acetic acid production and cell concentration during fermentation of A) wheat straw, B) forest residues, C) switchgrass, and D) sugarcane straw hydrolysates using *M. thermoacetica*. The error bars indicate standard deviation.

Table 2. Summary of process variables during pH-controlled batch fermentation of hydrolysates and controls

Hydrolysate (H), Synthetic (S)	Furfural (g/L), STD	HMF (g/L), STD	Total phenolics (g/L), STD	Initial sugars <sup>5</sup> (g/L), STD					Max Cell Concentration <sup>6</sup> (g/L), STD	Acid produced (g/L), STD	Yield (%) , STD
				Xyl	Glu	Ara	Gala	Man			
WS <sup>1</sup> – H	0.01(0.01)	0.01(0.01)	2.77 (0.01)	20.34 (0.01)	2.82 (0.01)	3.13 (0.01)	0.56 (0.02)	0.00	23.01 (0.03)	16.90 (0.01)	63.06 (0.53)
WS – S	0.00	0.00	0.00	20.02 (0.07)	3.02 (0.04)	2.61 (0.05)	0.50 (0.05)	0.00	24.50 (0.05)	14.20 (0.01)	54.38 (0.15)
FR <sup>2</sup> – H	0.01(0.01)	0.01(0.01)	2.52 (0.01)	20.01 (0.05)	8.87 (0.03)	3.79 (0.02)	7.06 (0.10)	9.17 (0.09)	22.30 (0.28)	18.22 (0.14)	37.59 (0.04)
FR – S	0.00	0.00	0.00	19.70 (0.07)	9.12 (0.06)	3.89 (0.02)	6.94 (0.06)	9.33 (0.01)	19.30 (0.01)	19.01 (0.02)	38.82 (0.06)
SG <sup>3</sup> – H	0.44 (0.01)	0.067 (0.01)	2.92 (0.01)	19.67 (0.03)	2.91 (0.04)	2.62 (0.03)	0.75 (0.01)	0.29 (0.04)	22.30 (0.2)	14.11 (0.01)	53.79 (0.04)
SG – S	0.00	0.00	0.00	20.46 (0.02)	2.11 (0.01)	1.63 (0.01)	0.39 (0.01)	0.04 (0.01)	22.40 (0.3)	13.54 (0.01)	55.02 (0.35)
SCS <sup>4</sup> – H	0.01(0.01)	0.01(0.01)	2.80 (0.1)	20.19 (0.23)	1.74 (0.01)	1.96 (0.03)	0.39 (0.01)	0.09 (0.01)	21.40 (0.01)	17.20 (0.02)	70.66 (0.66)
SCS – S	0.00	0.00	0.00	20.05 (0.01)	1.77 (0.01)	1.59 (0.01)	0.31 (0.01)	0.01 (0.01)	22.60 (0.01)	15.20 (0.01)	64.12 (0.01)

<sup>1</sup>WS, wheat straw; <sup>2</sup>FS, forest residues; <sup>3</sup>SG, switchgrass; <sup>4</sup>SCS, sugarcane straw; <sup>5</sup>Xyl, xylose; Glu, glucose, Ara, arabinose; Man, mannose <sup>6</sup>max cell concentration calculated based on dry cell

Table 3. Summary of process variables during flask fermentation of single sugar to acetic acid, comparing arabinose, galactose and mannose to glucose during 50 h fermentation

Sugar	Concentration (g/L)	Acetic acid yield (%)
Glucose	22 (0.08)	66 (0.02)
Arabinose	20 (0.08)	0 (0.01)
Galactose	20 (0.06)	0 (0.01)
Mannose	20 (0.08)	0 (0.01)

Table 4. Summary of process variables during flask fermentation of mixed sugars, monitoring consumed sugars and produced acetic acid

Fermentation no.	Initial sugar (g/L) / Consumed (%)					Cell growth / initial cell concentration (g/L)	Max cell concentration (g/L), STD	Acid produced (g/L), STD	Yield (%) based on total sugars, STD
	Ara*	Gal*	Glu*	Xyl*	Man*				
	STD	STD	STD	STD	STD				
1	3.40 / 39.70	3.50 / 32.90	3.10 / 97.70	3.20 / 100	3.80 / 36.80	mixture of five sugars / 0.2	6.60 (0.01)	4.70 (0.01)	27.00 (0.02)
	0.02 / 0.01	0.01 / 0.01	0.01 / 0.01	0.01 / 0.01	0.01 / 0.01				
2	4.20 / 25.70	4.30 / 14.50	4.10 / 100	3.60 / 100	3.00 / 29.70	1:1 / 7	13.20 (0.01)	6.00 (0.01)	32.00 (0.01)
	0.01 / 0.01	0.01 / 0.01	0.01 / 0.01	0.01 / 0.01	0.02 / 0.01				

\*Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose

Table 5. Summary of process variables during pH-controlled batch fermentation of glucose and xylose mixture, with different concentration, over 50 h , 75 h and 96 h of fermentation

Fermentation no.	Initial sugars (g/L)		Total sugars (g/L)	Max cell conc.* (g/L)	50 h			75 h			96 h					
	Glucose	Xylose			Acid produced (g/L)	Residual sugars (g/L)		Yield (%)	Acid produced (g/L)	Residual sugars (g/L)		Yield (%)	Acid produced (g/L)	Residual sugars (g/L)		Yield (%)
						Glucose	Xylose			Glucose	Xylose			Glucose	Xylose	
1	3.2	19.54	22.7	20.8	13.6	2.8	2.1	59.9	15.8	0	0	69.4	15.8	0	0	69.4
2	20.6	3.1	23.7	20	17.6	0	0	74.6	17.6	0	0	74.6	17.6	0	0	74.6
3	8.7	19.4	28.1	24.5	14.0	6.5	1.3	50.0	18.7	3	0	66.7	20.6	0	0	73.3
4	19.4	9.5	28.9	21.9	21.7	0.8	0	75.1	22.3	0	0	77.1	22.3	0	0	77.1

\*Conc., concentration; the reported results are the average of triplicate studies with a standard deviation of  $\leq 0.02$

## Chapter 3 – Conclusion and future work

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### 3.1 Conclusion

It was presented for the first time that *M. thermoacetica* (ATCC 39073) can effectively ferment xylose and glucose content in water-soluble fraction of steam exploded lignocellulosic biomass including wheat straw, forest residues, switchgrass, and sugarcane straw to acetic acid. In all fermentations xylose was the major and the first sugar being consumed. Xylose consumption was faster in switchgrass and sugarcane straw hydrolysates (35 h) than wheat straw and forest residues hydrolysates (40 and 45 h respectively). Xylose repressed glucose uptake in which glucose was metabolized when the majority of xylose was consumed. *M. thermoacetica* consumed less than 50% of arabinose, galactose and mannose within 72 h of fermentation in a mixture of sugars. The highest cell biomass was observed in wheat straw hydrolysate (23 g/L) and the lowest in sugarcane straw hydrolysate (21.4 g/L). The highest acetic acid production based on total sugars was obtained from sugarcane straw with 71% of theoretical yield while it was 64% in its control. The lowest acetic acid yield based on total sugars was observed in forest residues with 39% of theoretical. The incomplete consumption of arabinose, galactose and mannose adversely affected acetic acid yield. *M. thermoacetica* demonstrated that it tolerates process derived inhibitory compounds from steam explosion pretreatment including HMF (0.44 g/L), FF (0.1 g/L) and total phenolics (3 g/L). The variations observed in acetic acid yields, repressed glucose uptake by xylose, and incomplete consumption of minor sugars confirmed CCR behavior in *M. thermoacetica*.

## **3.2 Future work**

This research has focused on bioconversion of lignocellulosic biomass to acetate for the production of acetic acid. The ultimate goal is to establish a viable bioconversion process that is technically and economically feasible in an industrial setting. Several areas are recommended for future investigations.

### **Optimization of fermentation**

One area that will improve acetic acid yield is fermentation condition. In this study, all fermentations were conducted by utilizing CO<sub>2</sub> gas and mechanical agitation at 175 RPM. Additionally, inoculant was grown in a 1:1 ratio of synthetic glucose and xylose. Further optimizations are essential in the areas of medium composition, stirring and utilization of N<sub>2</sub> gas instead of CO<sub>2</sub>. Usage of inoculant pre-grown on hydrolysate, lower agitation rate and N<sub>2</sub> gas will provide additional information on how the fermentation process may be optimized.

### **Detoxifying hydrolysates**

There are various detoxification techniques to remove fermentation inhibitors (e.g. furans and phenolics) from lignocellulosic hydrolysates prior to fermentation. In this study, overliming method was utilized to detoxify lignocellulosic hydrolysates. Negative side effects of overliming include sugar loss in hydrolysate and presence of excess salts (Ca<sup>2+</sup> and Na<sup>+</sup>) on microbial growth. Detoxification of lignocellulosic hydrolysates by activated charcoal is recommended. This is due to the fact that adsorption of activated charcoal is widely used to remove inhibitors such as furan and phenolic compounds without significant sugar loss. Thus, higher acetate yield and a more efficient inhibitors removal may be achieved by hydrolysate treated with activated charcoal.

## **Continuous fermentation process**

Various fermentation setups utilizing CSTR and synthetic sugar substrates were investigated in order to improve productivity in acetic acid fermentation. However, challenges around process design utilizing lignocellulosic hydrolysate in continuous fermentation still remains. One of the major difficulties is the accumulation of acetic acid product and inhibitory compounds which hinders the fermentation process and lowers the yield. In order to address this issue, reverse electro-enhanced dialysis can be applied to improve the production yield.

### **3.3 Significance**

Lignocellulosic acetic acid is a value added and promising sugar-derived chemical that serves as a major economic driver for a biorefinery. The ultimate goal is to develop poplar-based biofuels and biochemicals through a sustainable and economical process. This study has provided significant insight regarding the bioconversion of lignocellulosic biomass to acetic acid utilizing *Moorella thermoacetica*. The following details the significance of the main findings:

- The research has demonstrated for the first time the ability of *M. thermoacetica* to ferment monomeric sugars in lignocellulosic hydrolysates obtained after steam explosion pretreatment to acetate.
- *M. thermoacetica* partially consumes arabinose, galactose and mannose in a mixture of sugars.
- In order to obtain an optimum acetic acid yield, it is imperative to have lignocellulosic hydrolysate with the highest xylose content and the least or no amount of other lignocellulosic sugars.

- Steam explosion pretreatment design is a key process that needs to be customized and applied in two or three steps. First, with low severity factor, collect as much xylose in liquid stream (hydrolysate) as possible. The next step is to provide a solid stream with glucose content only. Liquid and solid phases need to be refined to separate arabinose, galactose and mannose.
- Choice of lignocellulosic feedstock also improves acetate yield. Hardwoods and agricultural residues have a great potential for the production of acetic acid. Softwoods would not be a good option due to their high content of galactoglucomannan.

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## **Appendices**

Additional fermentations using synthetic media were conducted at different conditions in triplicates. Except for sugars content, ingredients used in growth and fermentation media were the same as described in chapter 2, materials and methods page 24. The following is a general description of how fermentations were prepared.

**Inoculants preparation:**

- Pre-grown on 1:1 ratio of xylose and glucose for 36 hr at 58°C without agitation, incubated in dark condition (incubator in Bactron II anaerobic chamber, Sheldon manufacturing, Inc.).
- Low dry cell concentration included 0.2 to 4 g/L of bacterial cell (without harvesting cell by centrifugation, culture was transferred by 10% (v/v) to fermenter).
- High dry cell concentration included 7 g/L of bacterial cell (cells were harvested and centrifuged at 4000 rpm for 5 minutes, decanted to yield cell pellets concentration of 7 g/L).

**Flask fermentations conditions:**

- Synthetic fermentation media prepared with pH level at 7.5.
- Serum vials with 100 mL working volume filled with synthetic fermentation medium and sparged with filter-sterilized 100% CO<sub>2</sub>.
- Inoculation performed with 10% (v/v) with varied concentration of dry cell (low and high).
- Fermentation performed with agitation at 175 rpm (shaking incubator) or no agitation (static incubator), temperature at 58°C for a period of 48 to 50 h (longer in some cases from 119 to 168 h).

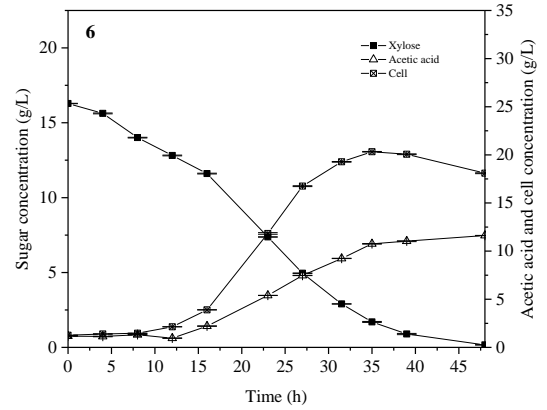
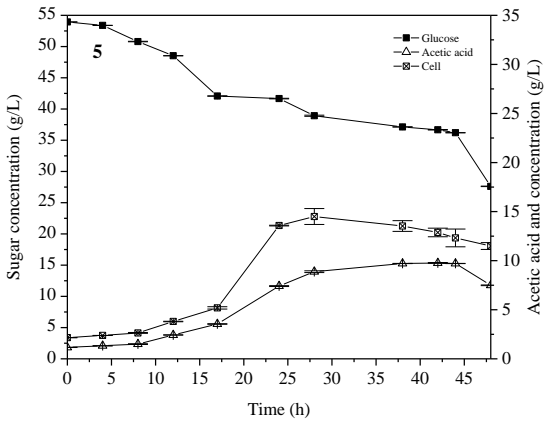
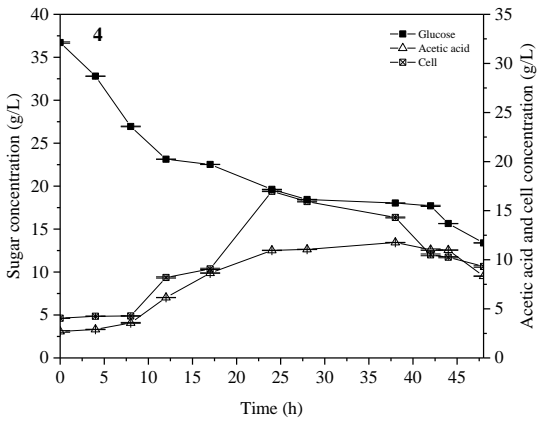
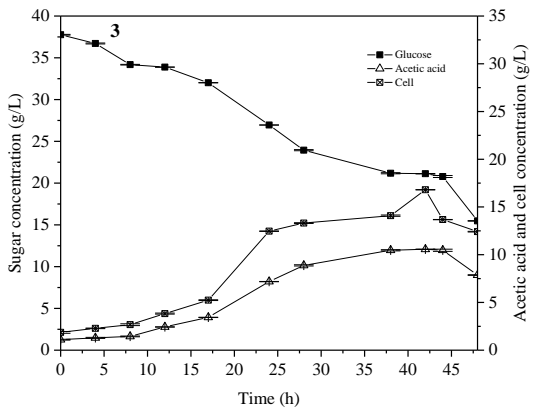
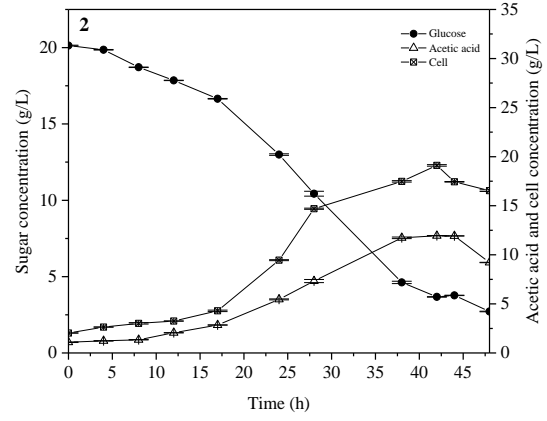
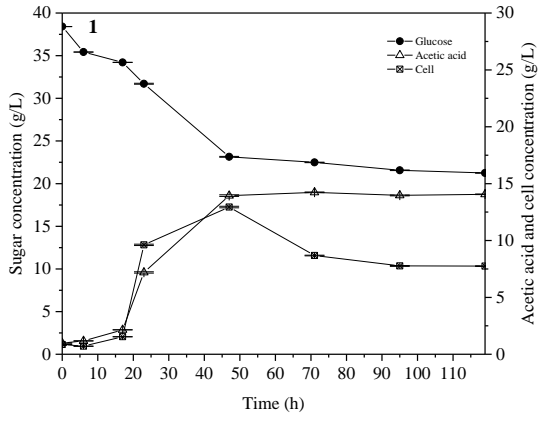
**pH-controlled batch fermentations in fermenter 1.3L Bioflo 115 (New Brunswick Scientific Co., Inc., Edison, NJ) bioreactor:**

- Synthetic fermentation media prepared with pH level at 7.5.
- Fermenter with 500 mL working volume filled with synthetic fermentation medium.
- Anaerobic conditions using filter-sterilized 100% CO<sub>2</sub> gas sparged into the bottom of the fermenter vessel continuously prior to inoculation.
- Anaerobic condition maintained by sparging 100% CO<sub>2</sub> gas at headspace after inoculation and throughout fermentation.
- Inoculation performed with 10% (v/v) and dry cell concentration of 7 g/L.
- Fermentations performed with agitation at 175 rpm, temperature at 58°C, pH level controlled at 6.8 for a period of 72 h or longer (up to 168 h).

**1 Flask fermentations utilizing inoculant with low cell concentration (0.2 – 4 g/L)**

Preliminary fermentations were conducted to characterize *M. thermoacetica* with the following specifications:

- Inoculants pre-grown on glucose, xylose, 1:1 ratio of glucose to xylose, mixture of sugars (xylose, glucose, arabinose, galactose and mannose).
- Flask fermentations kept in static incubator (without agitation) and in a dark setting.



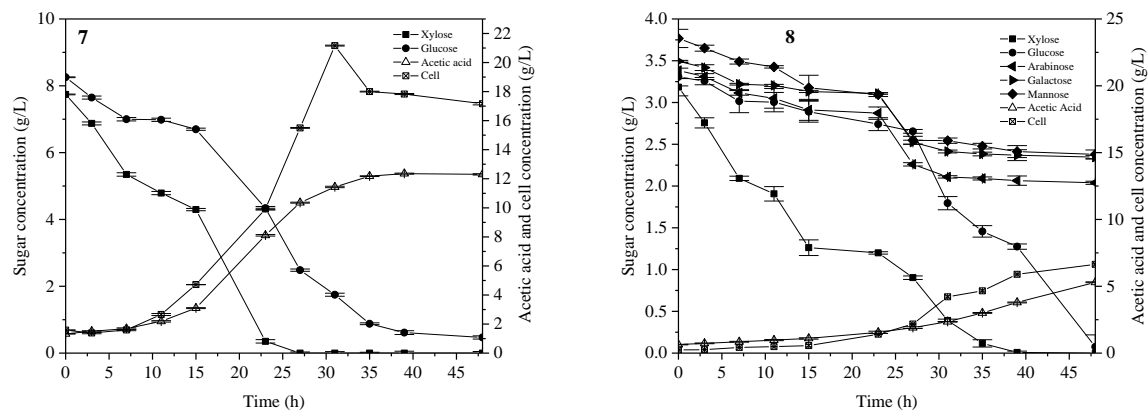


Figure 2. Sugars consumption, acetic acid production and cell concentration during fermentation of synthetic sugars, including single sugar (plots 1, 2, 3, 4, 5, 6), double sugars (plot 7) and mixture of sugars (plot 8)

Table 6 Summary of process variables, from preliminary flask fermentations, utilizing inoculant with low cell content

Graph	Initial sugars (g/L), STD		Fermentation time (h)	Inoculant pre-growth /cell conc. <sup>4</sup> (g/L)	Max Cell Concentration <sup>5</sup> (g/L), STD	Acid produced (g/L), STD	Yield (%), STD		pH @ 25 °C	
	Glucose	Xylose					Based on total sugars	Before	After	
1	38.41 (0.01)	-	119	glucose / 1	12.94 (0.06)	13.25 (0.01)	34.49 (0.01)	7.5	5.3	
1	38.41 (0.01)	-	48	glucose / 1	12.94 (0.06)	12.39 (0.01)	32.28 (0.01)	7.5	5.3	
2	20.13 (0.02)	-	48	glucose / 2	19.11 (0.08)	11.94 (0.02)	53.89 (0.01)	7.5	5.3	
3	37.76 (0.08)	-	48	glucose / 2	16.80 (0.02)	9.47 (0.02)	25.01 (0.02)	7.5	5.5	
4	36.71 (0.08) <sup>1</sup>	-	48	glucose / 4	16.97 (0.04)	9.06 (0.01)	24.68 (0.01)	7.5	5.5	
5	53.93 (0.01)	-	48	glucose / 2	14.50 (0.01)	8.60 (0.04)	15.95 (0.02)	7.5	5.6	
-	-	19.75 (0.02)	48	glucose / 2	2.40 (0.02)	< 1 (0.01)	< 4 (0.01)	7.5	6.7	
6	-	16.26 (0.01)	48	xylose / 1.5	20.33 (0.03)	10.44 (0.01)	64.20 (0.01)	7.5	5.3	
7	8.25 (0.01)	7.74 (0.01)	48	1:1 / 1.5	21.17 (0.03)	11.03 (0.04)	68.94 (0.01)	7.5	5.3	
8	Mixture of sugars <sup>2</sup>		48	mixture <sup>3</sup> / 0.2	6.64 (0.04)	4.66 (0.03)	27.22 (0.02)	7.5	6.5	

<sup>1</sup> Inoculation was 20% (v/v);

<sup>2</sup> Mixture of glucose 3.31 g/L ( $\pm 0.10$ ), xylose 3.18 g/L ( $\pm 0.10$ ), arabinose 3.38 g/L ( $\pm 0.10$ ), galactose 3.49 g/L ( $\pm 0.01$ ), and mannose 3.77 g/L ( $\pm 0.10$ );

<sup>3</sup> Inoculant was pre-grown on mixture of glucose, xylose, arabinose, galactose, and mannose

<sup>4</sup> Cell concentration.

<sup>5</sup> Maximum cell concentration calculated based on dry cell

## Conclusions:

- Acetate production in a fermentation period of 48 h was inhibited by low pH level (< 5.7) and an increase in glucose substrate. *M. thermoacetica* grows in a pH range of 5.7 to 7.7. Acetate concentration was decreased from 12 g/L to 8.6 g/L as concentration of glucose

was increased from 20 g/L to 60 g/L within 48 h of fermentation. A similar trend was reported by Balasubramanian and his coworkers based on the effect of initial sugar concentration on acetate production over a batch fermentation period of 120 h. It was also confirmed that as unconsumed sugar increased in medium, acetate production yield was decreased [57].

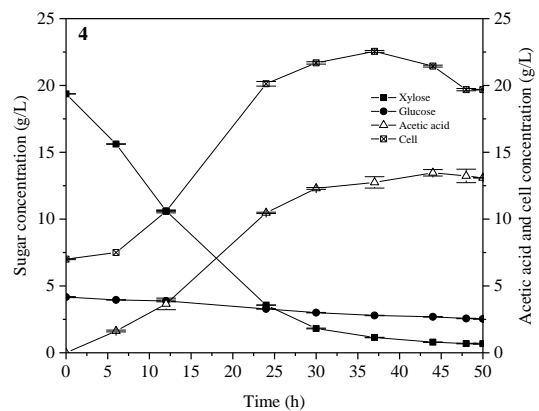
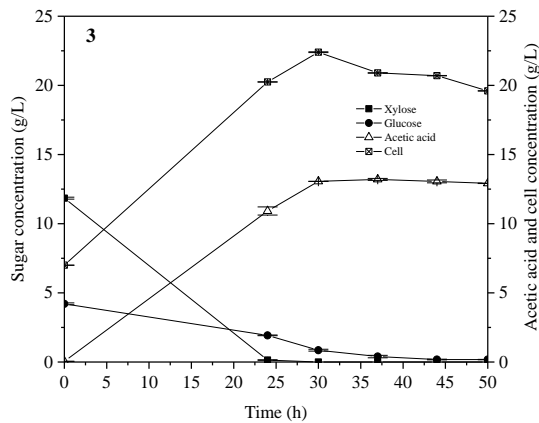
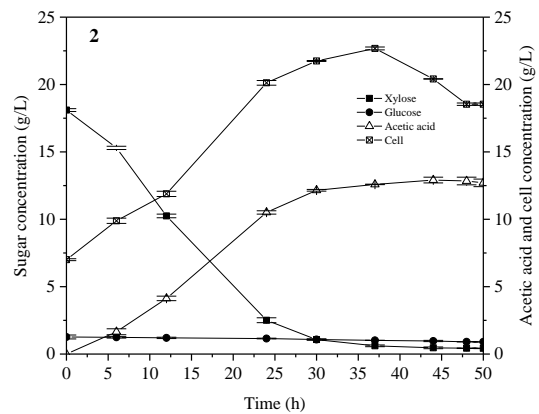
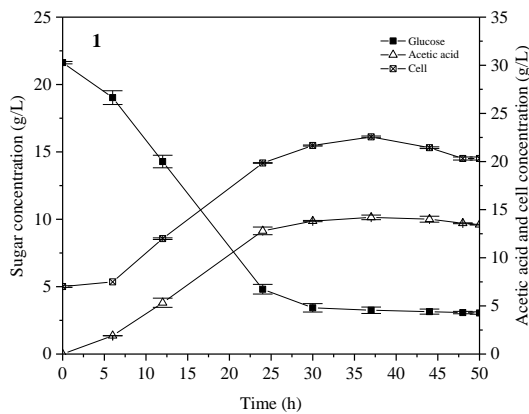
- In fermentation of 40 g/L of glucose, higher cell concentration caused faster sugars uptake at the beginning of fermentation (24 h). Figure 1, plots 3 and 4 indicate cell concentration of 4 g/L consumed glucose at a more rapid rate when compared with cell concentration of 2 g/L (1.125 g/L.h and 0.8 g/L.h respectively).
- Microbial cells adaptation to xylose and glucose was required to improve acetate yield (xylose was not metabolized with inoculant pre-grown on glucose only).
- Presence of arabinose, galactose and mannose increased duration of sugars uptake and had negative influence on acetate yield based on total sugars. Figure 1, plots 7 and 8 present fermentations with average sugar concentration of 16.5 g/L. In a 1:1 ratio of two sugars, xylose was consumed in 27 h while in 1:1 ratio of five sugars it took 48h. This was an early indicator of CCR mechanism in *M. thermoacetica*.
- Cell concentration of inoculant pre-grown on a mixture of five lignocellulosic sugars was very low (0.2 g/L) while it was higher when grown on single sugar or a mixture of xylose and glucose (2 and 1.5 g/L respectively). This was also indicator of CCR mechanism in *M. thermoacetica*.
- *M. thermoacetica* metabolized arabinose, galactose and mannose (23% of total sugars) at the presence of glucose and xylose with the lowest maximum cell concentration (6.6 g/L).

- Based on the results of this experiment, it was decided to utilize inoculant pre-grown on a 1:1 ratio of glucose to xylose and with a higher dry cell concentration of 7 g/L for future fermentations.

## 2 Flask fermentations utilizing inoculant with high cell concentration (7 g/L)

The following fermentations were conducted to evaluate the effects of different glucose to xylose ratios on acetate production:

- Inoculants were pre-grown on a 1:1 ratio of glucose to xylose.
- Inoculation was performed with 10% (v/v) and dry cell concentration of 7 g/L.
- All flask fermentations were kept in a shaking incubator at 175 rpm for 50 h.



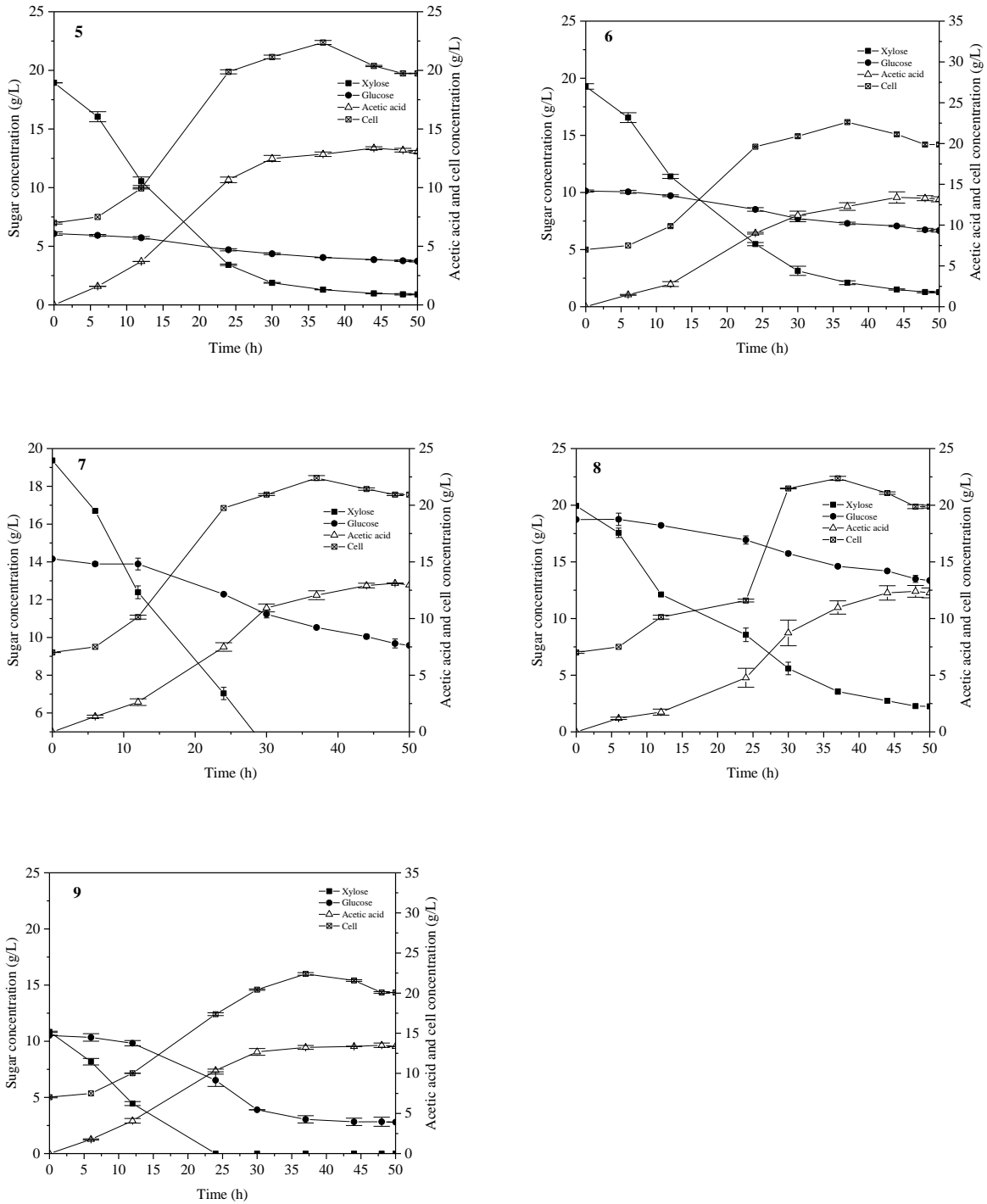


Figure 3. Sugars consumption, acetic acid production and cell concentration during fermentation of synthetic sugars including single sugar (plots 1, 2), mixture of different ratio of xylose and glucose (plots 3, 4, 5, 6, 7, 8, 9)

Table 7. Summary of process variables during fermentation of synthetic glucose and xylose utilizing inoculant with high dry cell concentration (7 g/L)

Graph	Initial sugars (g/L)		Sugars ratio (G:X) <sup>2</sup>	Fermentation Time (h)	Total sugars (g/L), STD	Max Cell conc. <sup>4</sup> (g/L), STD	Acid produced (g/L), STD	Residual sugars (g/L)		Yield (%), STD
	Glucose	Xylose						Glucose	Xylose	
Fig2-1	21.63 (0.08)	0	-	50	21.63 (0.08)	22.56 (0.08)	14.20 (0.20)	3.04 (0.08)	0	65.65 (0.02)
Fig2-2	1.27 (0.10) <sup>1</sup>	18.12 (0.10)	1:14	50	19.37 (0.02)	22.68 (0.08)	12.91 (0.20)	0.90 (0.05)	0.43 (0.01)	66.64 (0.03)
Fig2-3	4.21 (0.06)	11.85 (0.06)	1:3	50	16.05 (0.06)	22.40 (0.03)	13.20 (0.07)	0.18 (0.05)	0	82.24 (0.02)
Fig2-4	4.17 (0.04)	19.37 (0.01)	1:5	50	23.54 (0.01)	22.55 (0.07)	13.46 (0.24)	2.54 (0.05)	0.68 (0.02)	57.18 (0.02)
Fig 5-2 <sup>3</sup>	3.20 (0.01)	19.54 (0.01)	1:6	75	22.74 (0.05)	20.77 (0.02)	15.78 (0.01)	0	0	69.41 (0.01)
Fig 5-2 <sup>3</sup>	3.20 (0.01)	19.54 (0.01)	1:6	50	22.74 (0.05)	20.53 (0.02)	13.61 (0.01)	2.84 (0.01)	2.14 (0.01)	59.85 (0.01)
Fig 5-1 <sup>3</sup>	20.56 (0.01)	3.09 (0.01)	7:1	75	23.65 (0.01)	20.01 (0.01)	17.63 (0.02)	0	0	74.55 (0.01)
Fig 5-1 <sup>3</sup>	20.56 (0.01)	3.09 (0.01)	7:1	50	23.65 (0.01)	20.01 (0.01)	17.63 (0.01)	0	0	74.55 (0.01)
Fig2-5	6.08 (0.10)	18.94 (0.02)	1:3	50	25.02 (0.02)	22.37 (0.08)	13.36 (0.10)	3.73 (0.04)	0.90 (0.02)	53.40 (0.02)
Fig2-6	10.12 (0.10)	19.28 (0.10)	1:2	50	29.40 (0.1)	22.65 (0.10)	13.38 (0.1)	6.66 (0.10)	1.27 (0.10)	45.51 (0.10)
Fig 5-4 <sup>3</sup>	8.73 (0.01)	19.36 (0.01)	1:2	75	28.08 (0.01)	24.45 (0.01)	18.72 (0.01)	2.97 (0.01)	0	66.67 (0.01)
Fig 5-4 <sup>3</sup>	8.73 (0.01)	19.36 (0.01)	1:2	50	28.08 (0.01)	24.45 (0.01)	14.03 (0.01)	6.53 (0.01)	1.25 (0.01)	49.96 (0.01)
Fig 5-3 <sup>3</sup>	19.42 (0;01)	9.47 (0.01)	2:1	75	28.89 (0.01)	21.92 (0.01)	22.27 (0.01)	0	0	77.07 (0.02)
Fig 5-3 <sup>3</sup>	19.42 (0.01)	9.47 (0.01)	2:1	50	28.89 (0.01)	21.92 (0.01)	21.70 (0.01)	0.84 (0.01)	0	75.11 (0.02)
Fig2-7	14.16 (0.06)	19.37 (0.01)	1:4	50	33.52 (0.01)	22.40 (0.20)	13.10 (0.03)	9.58 (0.03)	1.78 (0.02)	39.08 (0.01)
Fig2-8	18.74 (0.04)	19.94 (0.01)	1:1	50	38.67 (0.01)	22.38 (0.10)	12.40 (0.05)	13.35 (0.02)	2.25 (0.04)	32.07 (0.02)
Fig2-9	10.52 (0.01)	10.84 (0.06)	1:1	50	21.35 (0.05)	22.38 (0.08)	13.50 (0.03)	2.80 (0.01)	0	63.23 (0.01)

<sup>1</sup> Presence of glucose is due to inoculant growing on 1:1 ratio of glucose and xylose

<sup>2</sup> Glucose to xylose ratio

<sup>3</sup> Highlighted rows: batch fermentations conducted in pH- controlled fermenter

<sup>4</sup> Maximum cell concentration, calculated based on dry cell

## Conclusions:

- In a 50 h fermentation period, sugar ratios did not have any effect on cell growth.
- Acetate yield based on total sugars was negatively affected by an increase in unconsumed sugars over a 50 h fermentation period. As an example, using a 1:3 glucose to xylose ratio, fermentation with 16 g/L of initial sugars resulted in a 29% higher acetate yield (based on total sugars) than another fermentation with 25 g/L of total sugars (Figure 2: plots 3 and 5).
- Sugars ratio had a significant influence on acetic acid yield based on total sugars. Higher glucose to xylose ratios of 2:1 and 7:1 (Figure 5: plots 1 and 3) resulted in a higher acetic acid yield (25% and 15% respectively over 50 h fermentation) than similar ratios of xylose to glucose (Figure 5: plots 2 and 4). This is an indicator of CCR mechanism in *M. thermoacetica*.
- Results from pH-controlled batch fermentations during a 50 h fermentation period are comparable with their corresponding flask fermentations:
  - i. Figure 5, plot 2 to Figure 2, plot 4 (acetic acid yield based on total sugars 60% and 57 % respectively).
  - ii. Figure 5, plot 4 to Figure 2, plot 6 (acetic acid yield based on total sugars 50% and 45.5 % respectively).

### **2.1 Flask fermentations - comparison of arabinose, galactose and mannose to glucose**

The following fermentations were conducted to compare fermentation of arabinose, galactose and mannose with glucose:

- Inoculants were pre-grown on a 1:1 ratio of glucose to xylose.

- Inoculation was performed with 10% (v/v) and dry cell concentration of 7 g/L.
- All flask fermentations were kept in a shaking incubator at 175 rpm for 50 h.

Table 8. Summary of process variables during flask fermentation of single sugar-comparing arabinose, galactose and mannose with glucose

Type of sugar	Initial sugar (g/L), STD	Max Cell conc. <sup>1</sup> (g/L), STD	Acid produced (g/L), STD	Acetic acid yield based on total sugar (%), STD
Glucose	21.63 (0.08)	22.56 (0.08)	14.20 (0.20)	65.64 (0.02)
Arabinose	20 (0.01)	0	0	0
Galactose	20 (0.01)	0	0	0
Mannose	20 (0.01)	0	0	0

<sup>1</sup> Maximum cell concentration, calculated based on dry cell

Conclusions:

- In a flask fermentation of single sugar (20g/L), *M. thermoacetica* was not able to metabolize arabinose, galactose and mannose. Clusters of dead cells were formed during fermentation of these three synthetic sugars.

## 2.2 Flask fermentations of synthetic sugars - comparison of CO<sub>2</sub> to N<sub>2</sub> gases

Fermentations with the following specifications were conducted to evaluate the effects of anaerobic gases (CO<sub>2</sub> and N<sub>2</sub>) on acetate production:

- Prior to fermentation, flasks were sparged with filter-sterilized 100% CO<sub>2</sub> and 100% N<sub>2</sub>.
- Inoculants were pre-grown on a 1:1 ratio of xylose to glucose.
- Inoculation was performed with 10% (v/v) and dry cell concentration of 7 g/L.
- All flask fermentations were kept in a static incubator and a dark setting space for 159 h.

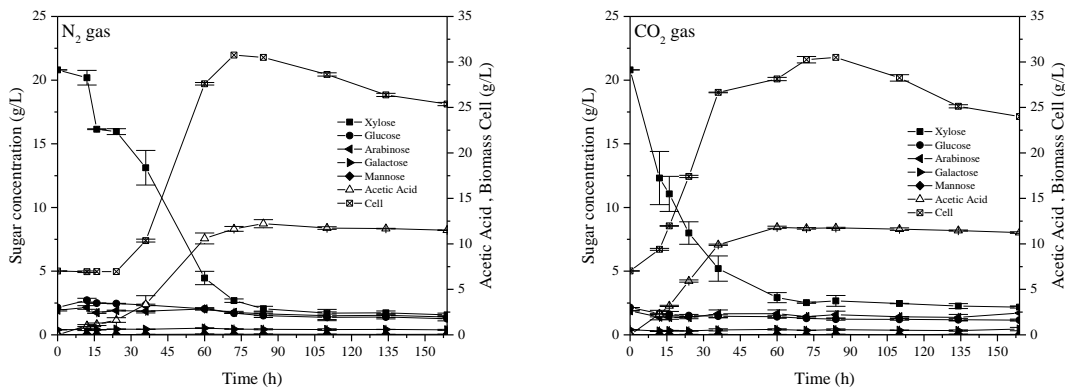


Figure 4. Sugars consumption and acetic acid production during flask fermentation utilizing synthetic sugars model for sugarcane straw hydrolysate comparison of N<sub>2</sub> and CO<sub>2</sub> gases

Table 9. Summary of process variables during flask fermentation-comparison of N<sub>2</sub> and CO<sub>2</sub> gases

Flask fermentation	Initial sugar (g/L), STD	Max Cell Concentration <sup>1</sup> (g/L), STD	Acid produced (g/L), STD	Yield (%) , STD	
				Based on total sugar	Based on consumed sugar
Under N <sub>2</sub>	25.30(0.04)	30.75 (0.03)	12.21 (0.40)	48.29 (0.1)	59.13 (0.50)
Under CO <sub>2</sub>	25.30(0.04)	30.50 (0.05)	11.76 (0.07)	46.56 (0.21)	59.27 (0.40)

<sup>1</sup> Maximum cell concentration, calculated based on dry cell

Conclusion:

- Results from flask fermentations confirmed that N<sub>2</sub> gas can be a good substitution for CO<sub>2</sub> gas in order to maintain anaerobic condition.

### 2.3 Fermentation of CO<sub>2</sub> gas as carbon source instead of sugars

Fermentations with the following specifications were conducted to evaluate the effect of CO<sub>2</sub> as a carbon source:

- Fermentation medium was prepared without sugar.
- Inoculants were pre-grown on a 1:1 ratio of glucose to xylose.
- Inoculation was performed with 10% (v/v) and dry cell concentration of 7 g/L.

- Fermentation was conducted in fermenter with agitation at 175 rpm, temperature at 58°C, pH level controlled at 6.8 for a period of 168 h.

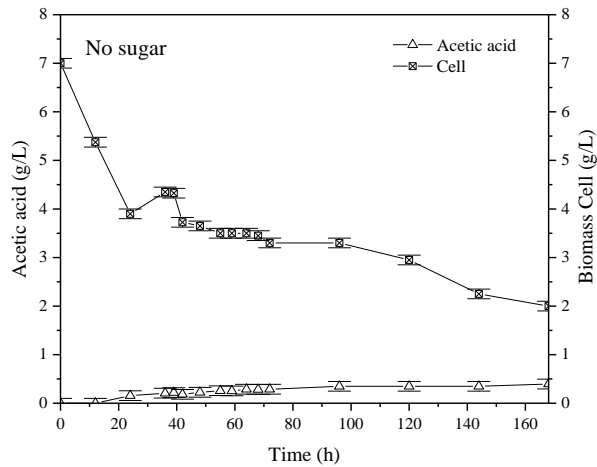


Figure 5. Acetic acid production during flask fermentation of synthetic medium without sugar

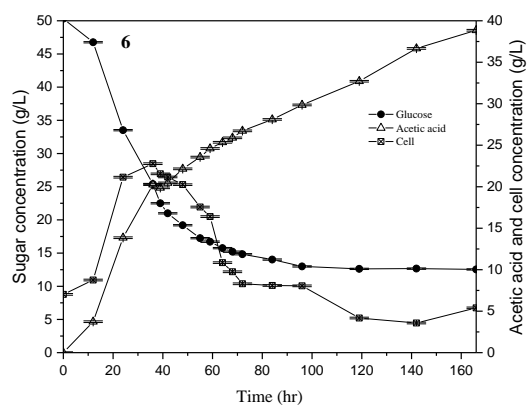
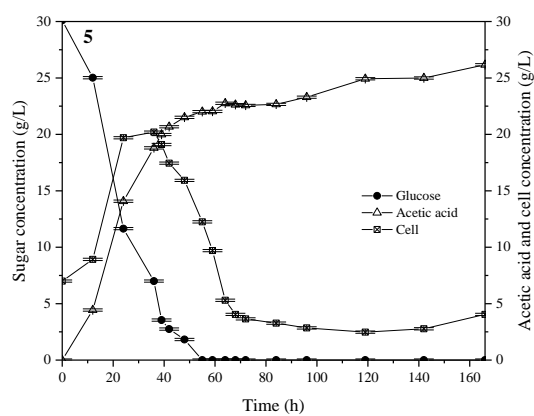
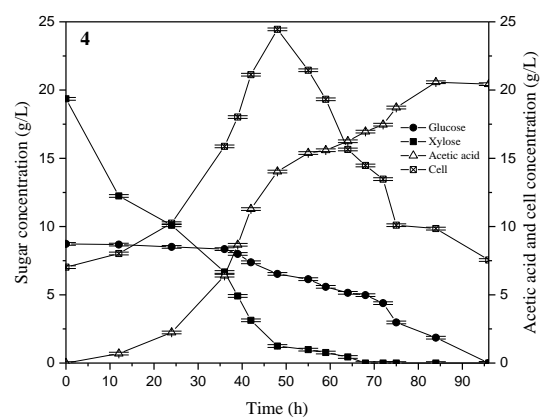
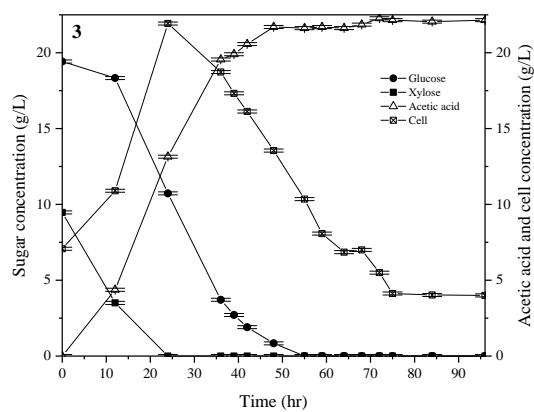
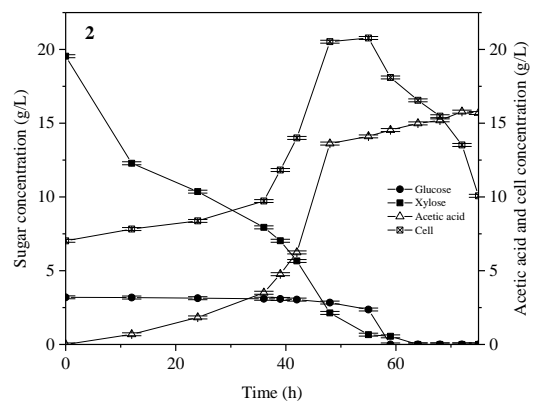
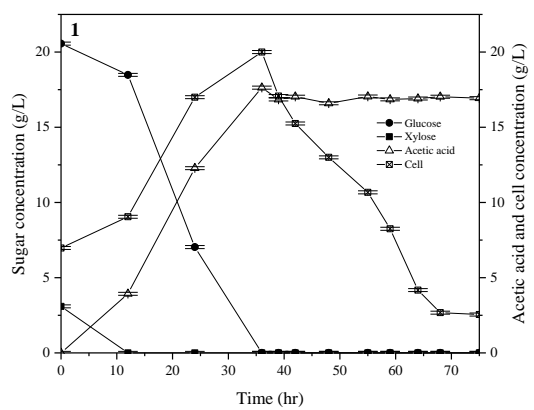
Conclusion:

- *M. thermoacetica* metabolized CO<sub>2</sub> gas in which maximum acetic acid production was 0.4 g/L within 168 h of fermentation.

### 3 pH-controlled batch fermentations of glucose and xylose

Effect of different ratios of glucose to xylose was investigated. Fermentations were conducted with the following specifications:

- Inoculants were pre-grown on a 1:1 ratio of glucose to xylose.
- Inoculations were performed with 10% (v/v) and dry cell concentration of 7 g/L.
- Fermentations were conducted in fermenter with agitation at 175 rpm, temperature at 58°C, pH level controlled at 6.8 for different periods of time (75h, 96h, and 166 h).



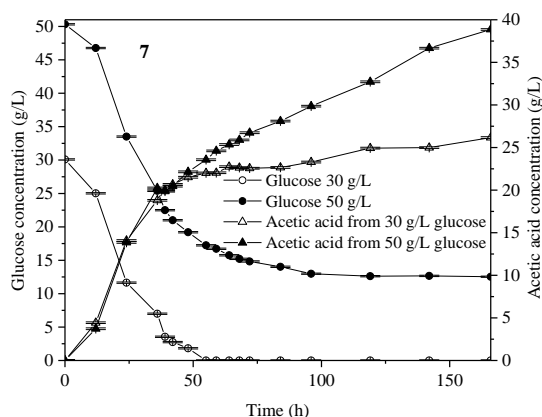


Figure 6. Sugars consumption and acetic acid production during batch fermentation of synthetic glucose and xylose in pH-controlled fermenter including various ratio of glucose to xylose (plots 1, 2, 3, 4), and different glucose concentration (plots 5, 6), effect of glucose concentration on sugar uptake and acetate product (plot 7)

Table 10. Summary of process variables during fermentation of synthetic glucose and xylose in pH-controlled fermenter

Graph	Initial sugars (g/L)		Sugars ratio (G:X) <sup>1</sup>	Fermentation time (h)	Total sugars (g/L), STD	Max Cell Concentration <sup>2</sup> (g/L), STD	Acid produced (g/L), STD	Yield (%), STD
	Glucose	xylose						Based on total sugars
1	20.56	3.09	7:1	75	23.65 (0.01)	20.01 (0.01)	17.63 (0.02)	74.55 (0.01)
2	3.20	19.54	1:6	75	22.74 (0.05)	20.77 (0.02)	15.78 (0.01)	69.41 (0.01)
3	19.42	9.47	2:1	96	28.89 (0.01)	21.92 (0.01)	22.27 (0.01)	77.07 (0.01)
3	19.42	9.47	2:1	75	28.89 (0.01)	21.92 (0.01)	22.27 (0.01)	77.07 (0.01)
4	8.73	19.36	1:2	96	28.08 (0.01)	24.45 (0.01)	20.58 (0.01)	73.26 (0.01)
4	8.73	19.36	1:2	75	28.08 (0.01)	24.45 (0.01)	18.72 (0.01)	66.67 (0.01)
5	30.1	0	-	166	30.1 (0.01)	20.20 (0.01)	26.17 (0.01)	86.94 (0.01)
5	30.1	0	-	75	30.1 (0.01)	20.20 (0.01)	22.57 (0.01)	74.98 (0.01)
6	50.31	0	-	166	50.31 (0.01)	22.78 (0.01)	38.86 (0.01)	74.55 (0.01)
6	50.31	0	-	75	50.31 (0.01)	22.78 (0.01)	26.74 (0.01)	53.15 (0.01)

<sup>1</sup>Glucose to xylose ratio; <sup>2</sup> Maximum cell concentration, calculated based on dry cell

#### Conclusions:

- The ratio of glucose to xylose had a direct effect on the duration of sugars consumption and acetic acid production yield based on total sugars.

- Total sugar consumption was considerably quicker with higher glucose to xylose ratio (7:1 and 2:1 sugars ratios took 35 and 55 h respectively) than lower glucose to xylose ratio (1:6 and 1:2 sugars ratios, took 65 and 96 h respectively).
- In a fermentation period of 166 h, initial glucose concentration of 30 g/L resulted in 12.4% higher acetate yield based on total sugar when compared to an initial glucose of 50 g/L. However, produced acetate was 13 g/L lower. Figure 5, plot 7 shows this is due to the unconsumed glucose in fermentation with 50 g/L of glucose (after 100 h of fermentation, 12.5 g/L of glucose not touched by the bacterial strain). Lower glucose concentration should be employed to avoid inhibition of substrate consumption.
- In a 60 h fermentation period, glucose uptake is similar between initial sugar concentrations of 30 g/L and 50 g/L (Figure 5, plot 7)