1-1-2009

Acid pretreatment and fractionation of source-separated organic waste for lignocellulosic saccharification

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ACID PRETREATMENT AND FRACTIONATION OF SOURCE-SEPARATED ORGANIC WASTE FOR LIGNOCELLULOSIC SACCHARIFICATION

by

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A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of Master of Applied Science in the program of

Environmental Applied Science and Management

Toronto, Ontario, Canada, 2009

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Master of Applied Science

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Abstract

This study compared two acidic pretreatments on Source-Separated Organic (SSO) waste, preprocessed by Aufbereitungs Technology and System thermal-screw, on the basis of fermentable sugars for bioethanol production. The result showed that the SSO contained on average 27% glucan, 5.4% xylan, 1.2% arabinan, 5.7% mannan and 1.2% galactan. Dilute sulfuric acid pretreatment (at 121°C and 16.2 psi) was insufficient to solubilize cellulose and hemicellulose and did not remove much of the lignin. Cellulose-solvent and Organic Solvent-based Lignocellulose Fractionation (COSLIF) (at 50°C and atmospheric pressure) generated high glucose yield (70%). Substituting ethanol for acetone as organic solvent increased the yield to 89.5%. Fermentation using *Zymomonas mobilis 8b* with this hydrolysate confirmed the pretreatment is promising for the SSO conversion. Amenability of the SSO for biofuel production is validated. Enzymatic hydrolysis of both pretreatments using Accellerase 1500 is preferred over Celluclast 1.5L due to higher activity. Future work includes design of an appropriate batch and/or continuous bioreactor, and further understanding of *Zymomonas mobilis 8b*.

Acknowledgments

I wish to express my sincere thanks and gratitude to my supervisor, Dr. Grace Luk, for her patient guidance and helpful comments during the conduct of this research. Her support and thoroughness have inspired me and this experience will serve me in all my future endeavors. Many thanks as well to the project's industrial sponsor, Mr. Jamie Bakos of Clean 16 Environmental Technologies Corp., for the opportunity to work on this exciting project. My appreciation also extends to Ms. Balinder Rai of Ontario Centres of Excellence for her support and coordination of the project.

I would like to acknowledge that the completion of this research project is a result of a team effort. Special thanks to my teammates at Ryerson University – Grace Lin, Benjamin Percy, Mina Mirzajani, Michael Faye, Robin Luong, Valeriy Bekmuradov and Bonnie Wilkinson, for all of your time and advice, and also to Eva Cheng, an undergraduate student from McMaster University, for helping us in laboratory experiments.

I am greatly indebted to all of the faculty members and technologists at Environmental Applied Science and Management program, Civil Engineering Department, and also the staff of graduate studies of Ryerson University for the facilities and assistance provided throughout the development of my thesis. The HPLC work described in this thesis was performed with the technical guidance of Shawn McFadden, the technologist at the Ryerson University Analytical Centre located in the Chemistry and Biology Department, as well as Dan Mathers and Ying Wania, Supervisor and technician of Analytical Lab for Environmental Science Research and Training (ANALEST) at the University of Toronto.

My sincerer gratitude and special thanks to:

Ryerson University;

Dr. Maurice Yeates, Dean of Graduate Studies

Dr. Michal Bardecki, Program Director - Environmental Applied Science and Management.

Dr. Ronald Pushchak

Dr. Gideon Wolfaardt, and his PhD students Alexandru and Romeo Dumitrache

Dr. Ginette Turcotte

Dr. John Marshall

Technologists at Department of Chemistry and Biology:

Sylvia O'Sullivan, Miriam E. de Jong, Liberty Victorio-Walz, and Wei Zhang

Technical Officer - Scanning Electron Microscope Department of Mechanical and Industrial Engineering: Qiang Li

National Renewable Energy Laboratory, US Department of Energy:

Dr. Ali Mohagheghi, Senior Scientists

Dr. Eric Payan, Senior Licensing Executive

Ms. Dee Sheaffer

MBI International, a wholly-owned subsidiary of the Michigan State University Foundation:

Dr. Farzaneh Teymouri, Senior Scientists

Genecore, a Danisco Division, USA

Dr. Mian Li, Senior Applications Scientist, Biomass Application Group

Ms. Darla Green, Customer Relations Grain & Ethanol/F & HC

Thanks to the Almighty God who is Compassionate and Merciful,

This thesis is dedicated to my loving parents,

Mahvash Farazandeh and Abbas Ehsanipour,

for their endless love, support and motivation throughout my life.

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List of Abbreviations

AFEX	Ammonia Fiber Explosion
AIL	Acid Insoluble Lignin
ASL	Acid Soluble Lignin
ASTM	American Society for Testing and Materials
ATS	Automation and Tooling System (Aufbereitungs Technology and System)
Ave.	Average
CCME	Canadian Council of Ministers of the Environment
CBP	Consolidated Bioprocessing
(CM)-cellulases	Carboxymethylcellulase
CMC	Carboxymethyl Cellulose
Con.	Concentration
COSLIF	Cellulose-solvent and Organic Solvent-based Lignocellulose Fractionation
DA	Dilute Acid
DDW	Distilled Deionized Water
DOE	Department of Energy
DP	Degree of Polymerization
DSA	Dilute Sulfuric Acid
EG	Endo-glucanase
FP	Filter Paper
g	Gram
gdcw	Gram dry cell weight
g/L	Gram per Liter
HMF	Hydroxymethylfurfural
hr	Hour
Kg	Kilogram
kW	Kilowatt
min	Minute
mL	Milliliter
MSW	Municipal Solid Waste
NREL	National Renewable Energy Laboratory
ODW	Oven Dry Weight
OFMSW	Organic Fraction of Municipal Solid Waste
PASC	Phosphoric Acid Swollen Cellulose
pNPG	*p*-nitrophenyl-ß-D-glucopyranoside
psi	Pound per square inch

Con't

rpm	Rotation per minute
SEM	Scanning Electron Microscope
SHF	Separate (or Sequential) Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
SSO	Source-Separated Organic
SWM	Solid Waste Management
TAPPI	Technical Association of the Pulp and Paper Industry
TKN	Total Kjeldahl Nitrogen
TS	Total Solid
UNDP	United Nations Development Program
v	Volume
VFA	Volatile Fatty Acid
VOC	Volatile Organic Compound
VS	Volatile Solid
v/v	Volume per volume
W	Weight
w/v	Weight per volume
w/w	Weight per weight
µV.Sec	Microvolt second

Introduction

1.1 Background

Since the late 1970s, bioethanol industry has been broadly expanded by worldwide recognition for environmental, economic, and renewable attributes. The emphasis of ethanol fuel promotion included not only energy security but also environmental benefits. Today, bioethanol is by far the largest and most immediately available alternative to petroleum and diesel, decreasing the adverse environmental effects and production dependency typical in fossil fuel consumption (Wyman, 2001; von Blottnitz & Curran, 2007). It is a renewable energy with the following benefits (Pembina Institute, 2001):

- Emitting less toxic emissions into the air, land, and water
- Protecting global climates
- Having an unlimited feedstock supply
- Creating job opportunities
- Guaranteeing energy price stability due to the availability of resources
- Helping to manage energy costs
- Geopolitical stability

A glimpse at the current media reports on the past and today's status of bioethanol indicates industrial ushering in a new energy era. Numerous efforts corresponding to rapidly growing demand on bioethanol production have been made to produce ethanol from various feedstocks including: 1) major energy crops (food crops such as corn, wheat, rice, potato, and sugarcane; non food crops such as switchgrass, willow, and poplar), 2) forestry and agricultural waste leftovers (*i.e.*, woodchips from tree stumps and foliage, wheat straw, rice hull, molasses, corn stover, corn cob), and 3) wasted crops defined as crop lost in distribution (Kim & Dale, 2004).

While the conflicting views on bioethanol feedstocks have raised food versus fuel argument, (Rosillo-Calle & Hall, 1987; Ziegler, 2007). This dilemma has been quickly embedding echo on scientific perspective, causing two major concerns including (Kim & Dale, 2004):

1) Biomass availability and critical need for proper substitution for energy crops and agricultural waste which have food value, trying to eradicate the competition between food and fuel
2) Fast and cost-effective bioethanol technology appropriate for conversion of biomass feedstock to fermentable sugars – focusing on pretreatment, a priority area

In a growing atmosphere of environmental stewardships and partnerships in Solid Waste Management (SWM) and 4R's (reduce, reuse, recycle, and recover resources) initiatives, waste-to- energy concept has given a new horizon to bioethanol feedstock supply by introducing lignocellulosic waste and Organic Fraction of Municipal Solid Waste (OFMSW). It is a locally available, inexpensive energy resource and a major contribution to sustainable development (Zsigraiova et al, 2009). In addition, trash conversion into bioethanol helps to:

- Streamline the problem with bioethanol feedstock and resolve the conflicts between human food and industrial use of crops,
- Decrease waste piling up in landfills (landfill reduction),
- Reduce greenhouse gas emission, such as methane (CH_4) and carbon dioxide (CO_2).

One of the forms of the OFMSW is Source-Separated Organic (SSO) waste, a processed organic food waste with ATS thermal screw machine and blended with 10 to30% woodchips. The ATS thermal screw is a unit process with combination of shearing action and high pressure between

the plates of the screw causing a transformation of the SSO to very fibrous and more homogenous material. The SSO is subcategory of lignocellulosic waste feedstock (Vartek Ltd., 2005).

The heterogeneous and recalcitrant nature of the SSO is the major bottleneck and hampers the efficient release of locked polysaccharides from the SSO feedstock to fermentable sugars. Likewise, effective pretreatment of this kind of lignocellulosic waste governs related- operational systems in an ethanol biorefinery (major role of pretreatment is discussed in greater details in chapter 2). Pretreatment requires to be evaluated through enzymatic hydrolysis followed by fermentation.

Enzymatic hydrolysis depends on the function of cellulases and hemicellulases. This hydrolysis step, a saccharification process, represents one of the largest costs in converting lignocellulosic biomass to fuel ethanol. Currently, different types of commercial enzyme complexes are available in the market. It is believed that yield of fermentable sugars and production costs can be significantly reduced by applying enzymes which are improved by modern biotechnology and bioprocess engineering.

This research discusses pretreatment and saccharification of the SSO waste, through comparative study on two different acidic pretreatments including dilute sulfuric acid (H_2SO_4) pretreatment and lignocellulosic fractionation by cellulose-solvent (phosphoric acid, H_3PO_4) and organic-solvent, (acetone, $OC(CH_3)_2$ and/or ethanol, C_2H_6O). Then enzymatic hydrolysis behaviours on the basis of glucose and xylose yields are investigated to compare the performance of two commercial enzyme complexes. Evaluation is finalized by influence of pretreatment on fermentation process with selected strain of bacteria (characterization and experimental models

developed for dilute acid pretreatment and fractionation of SSO substrate are presented in chapter 3 followed by results and discussion in chapter 4).

This thesis is part of collaborative research project and corresponds to the last stage of phase 3, pretreatment and saccharification of SSO. Detailed history of the research projects, including phases, subtopics and stages, timeline, facilitators and partners are presented in appendix VI. The SSO waste has never been investigated as a substrate for ethanol production and that is the point differentiates this study from others. Scientific sources used in this study are available from literature including previous study reports on cellulosic ethanol production, scientific articles, papers, recently registered patent on this technology, industrial reports, handbooks (i.e., bioethanol production and utilization, biofuel technology, compost engineering), and analytical procedures presented by the National Renewable Energy Laboratory (NREL) of the U.S. department of energy.

1.2 Study Objectives

This study is intended to compare the efficiency of two acidic pretreatments at different conditions on processed SSO waste by ATS thermal screw on the basis of fermentable sugars for ethanol production with the following tasks:

- To present a thorough literature review on ethanol biorefineries with emphasis on biomass feedstock and pretreatment technology,
- To determine the compositional analysis of SSO sample for pretreatment design,
- To carry out an experimental investigation on dilute sulfuric acid pretreatment,
- To carry out an experimental investigation on cellulose solvent (phosphoric acid) and organic solvent (acetone and/or ethanol) based lignocellulose fractionation,

- To investigate enzymatic hydrolysis behavior after each acidic pretreatment on the basis of
    - fermentable sugar yield,
    - performance of two commercial enzyme complexes,
- To evaluate the influence of pretreatment on fermentation process with selected strain of bacteria.

It is foreseen that the results obtained in this study will improve the overall understanding about SSO characteristics and pretreatment, which correspond to the projected goal. It will also provide important information for the next step of further investigation on batch and continuous culture design.

Literature Review

2.1 Ethanol Biorefineries

Over the past two decades, bioethanol technology has been improved by means of an increasing engineering knowledge base, comprehension of new and varied feedstocks, and integrated energy balance data during all processes (Wyman, 2001). However, no single process design offers the most cost-effective way to produce ethanol from biomass. Important parameters involved with cost estimation data comprised of the type and cost of biomass as a raw material, the utilization process and energy demand, and also the capital cost of the plant. The design of the plant as well as its individual steps used in processes need to be based on an accurate and reliable data (Galbe et al., 2007; Rutz & Janssen, 2008).

Many hybrid techniques have been developed for ethanol biorefineries from different fields for example microbiology, bioengineering, and biomass engineering. Currently, there are two platforms (or bases) for ethanol biorefineries and Figure 2-1 presents a general view on these conversion technologies including (Fernando et al., 2006):

1) Thermochemical method, a gasification dominant technique with syngas platform:

It i**s** a unique technique using syngas (a mixture of hydrogen and carbon monoxide) derived from biomass and waste products (mostly lignocellulosic waste material) to produce bioethanol (Younesi et al., 2005). As shown in Figure 2-1, there are two main steps for creating syngas: i) pyrolysis of carbon-based waste products, and ii) gasification, (Rutz & Janssen, 2008; Wagner, 2007). On the basis of selecting a catalyst, there are two optional systems for syngas conversion to ethanol including chemical compound and microorganism such as *Clostridium ljungdahlii* capable of fermenting the synthesis gas to ethanol and acetate (Demirbas, 2005).

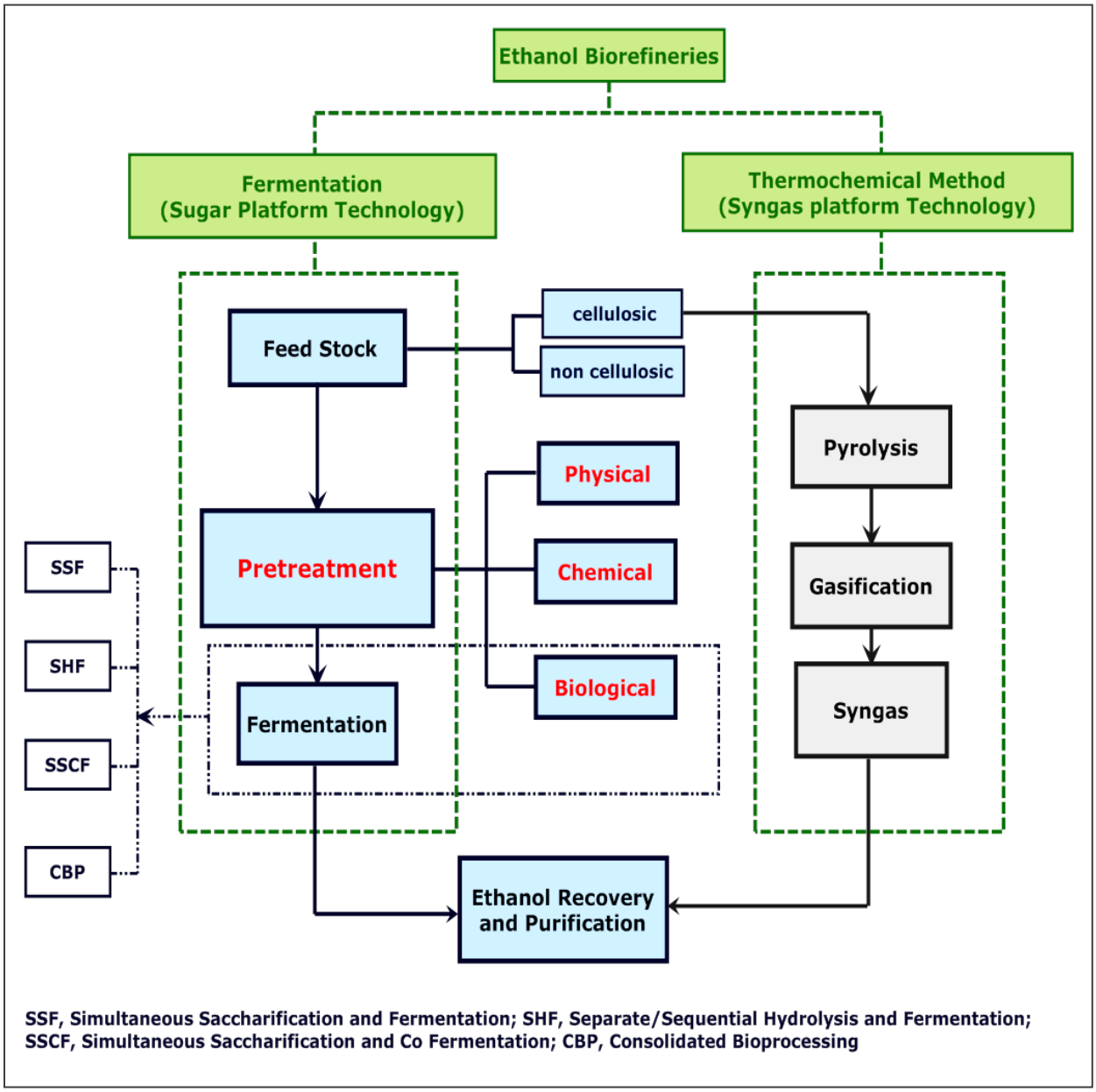

Figure 2-1 General scheme of ethanol biorefineries

2) Fermentation, a biological dominant technique with sugar platform:

Technically, the state of art fermentation system involves two major conversions that include feedstock to monomeric sugar, then sugars to alcohol through the following steps (Rutz & Janssen, 2008**;** ENERS Energy Concept, 2008; Zeikus, 1980) :

1. Feedstock supply which relies on harvesting, reception, storage.
2. Pretreatment, a crucial step which depends on type of the biomass, and includes physical, chemical and biological.
3. Microbial fermentation which is monomeric sugar conversion to ethanol.
4. Distillation which is separation of ethanol.

In addition, where enzymatic hydrolysis, a biological pretreatment, is applied, different levels of process integration are possible, including (Hamelinck et al., 2005):

- Separate (or Sequential) Hydrolysis and Fermentation (SHF),
- Simultaneous Saccharification and Fermentation (SSF),
- Simultaneous Saccharification and Co-Fermentation (SSCF), and
- Consolidated Bioprocessing (CBP) system.

2.2 Bioethanol Feedstocks

Bioethanol feedstock supply sectors can be classified into woody biomass, agriculture, industry and waste as shown in Table 2-1(Sims, 2004; EUBIA, 2007). On the basis of natural composition of bioethanol feedstock, the above classification is categorized in three major groups: 1) starchy biomass (i.e. corn, rice, and tubers like cassava), 2) sugar-based biomass (i.e. sugar beet, and sugar cane); and 3) lignocellulosic material (i.e. wheat straw, rice hull, corn stover). Sugar and starch based feedstocks are often referred to as “first generation” of bioethanol feedstocks. Lignocellulosic feedstocks are known as “second generation” due to the recent advanced technologies which have provided the opportunity to use non edible feedstocks for producing bioethanol (Rutz & Janssen, 2008; ENERS Energy Concept, 2008).

Table 2-1 Classification of bioethanol feedstocks (Sims, 2004; EUBIA, 2007)

Supply sector	Type	Example
Woody Biomass	Dedicated forestry	Short rotation plantations (e.g. willow, poplar, eucalyptus)
	Forestry by-products	Wood blocks, wood chips from thinnings,
	Wood process residue	Bark, sawdust, shavings, wood chips and off-cuts
	Recovered wood fuels	Recovered wood fuels from activities such as land clearance & municipal green waste
Agriculture	Dry lignocellulosic energy crops	Herbaceous crops (e.g. miscanthus, reed canarygrass, giant reed)
	Energy crops short rotation and annuals	Oil seeds for methylesters (e.g. rape seed, sunflower)
		Sugar crops for ethanol (e.g. sugar cane, sweet sorghum)
		Starch crops for ethanol (e.g. maize, wheat)
	Agricultural residues	Straw, prunings from vineyards and fruit trees
	Livestock waste	Wet and dry manure (cattle, pigs, horses and poultry as well as human)
	Agro- industrial by-products	Bagasse, rice husks
	Water vegetation	Algae, water hyacinths, seaweeds
Industry	Industrial residue	Industrial waste wood, sawdust from sawmills
		Fibrous vegetable waste from paper industries
Waste	Dry lignocellulosic	Residues from parks and gardens (e.g. prunings, grass)
	Contaminated waste	Demolition wood
		Organic fraction of municipal solid waste
		Biodegradable landfill waste, landfill gas
		Sewage sludge

In practice, local considerations should be brought to attention. The choice of raw material and bioethanol technology depend on what grows best under the prevailing climate conditions, landscape and soil composition, as well as on the sugar content and ease of processing of the various plants available (EUBIA, 2008; BIOCAP Canada, 2008; Rutz & Janssen, 2008). For

example, the most bioethanol produced from sugar cane is in South America (Brazil), and corn in North America (USA). Table 2-2 presents locations of the most common bioethanol feedstocks (von Blottnitz & Curran, 2007).

Table 2-2 Locations of common bioethanol feedstocks (von Blottnitz & Curran, 2007)

<table>
<tr><th>Sugar Crops</th><th>Starch Crops</th><th>Lignocellulosic Biomass (Cellulosic Crops)</th><th>Waste Biomass Forestry, municipal, and agricultural wastes</th></tr>
<tr><td rowspan="2">Sugar cane in Europe, North America, South America, India, Australia, and South Africa</td><td>Corn in Europe, and North America</td><td rowspan="4">Europe, Australia, North America, Philippines (Bagass[2] in India)</td><td rowspan="4">Europe, North America, South America, India, Australia, South Africa</td></tr>
<tr><td>Cassava in China</td></tr>
<tr><td>Sugar beet in Europe, and Australia</td><td>Wheat in Europe, North America, and Australia</td></tr>
<tr><td>Molasses[1] in India and South Africa</td><td>Potato in Europe</td></tr>
<tr><td colspan="4">Molasses[1]: Thick syrup by-product from processing of the sugarcane or sugar beet,
Bagasse[2]: Biomass remaining after processing sugarcane or sorghum stalks</td></tr>
</table>

Currently, lignocellulosic feedstock as the most abundant biomass has attracted considerable attention (Kuhad & Singh, 1993) and is often a major or the sole components of different waste streams from agriculture and forestry to municipalities' waste (Taherzadeh & Karimi, 2008). Lignocellulosic waste represents huge amounts of unutilized renewable resource and depending on its origin has been divided into three groups (Rutz & Janssen, 2008):

1. Primary waste, originated from harvesting of food crops (e.g., straw, corn stalks and leaves), and residues from forestry (e.g. wood thinning from commercial forestry).
2. Secondary waste, from beverage and food industry, (e.g., nut shells, sugar cane, bagasse, and saw dust).
3. Tertiary waste, a large variety of different waste fractions (e.g., OFMSW).

Waste of reusable materials is accumulated due to the throw-away philosophy and is causing major problems in Solid Waste Management (SWM) (Taherzadeh & Karimi, 2008). In this respect, the 4Rs strategy helps to curb the destruction of ecosystem, and reduce pollution. Municipal solid waste management (MSWM) systems are becoming more complex due to the move from landfill-based to resource recovery-based solutions rooted in the setting of international and national targets to divert waste from landfill and increase recycling and recovery rates (Woodruff et al., 2004).

2.3 Feedstock Selection

According to the lignocellulosic waste classification, Municipal solid waste (MSW) is one of the feasible sources for bioethanol feedstock. Generally, it includes three categories: 1) urban and residential, 2) industrial, commercial and institutional, and 3) demolition, landscaping and construction. The composition depends on what is thrown away and consists of several fractions which can be simplified into two main groups: i) Organic waste (e.g., food, paper, cardboard, plastics, textiles, rubber, leather, yard wastes, wood), and ii) inorganic waste (e.g., glass, tin cans, aluminum and other metals). Furthermore, the MSW is a function of several variables such as seasonal climate, geographical location, and the degree of recycling (Woodruff et al., 2004; BIOCAP Canada, 2008; Bradley, 2006).

In theory, anything that is organic can be used as a feedstock. However, different organic fractions respond differently to biological degradation when they are discharged into the environment or in a landfill (BIOCAP Canada, 2008). One of the forms of the OFMSW, a tertiary lignocellulosic waste mentioned in section 2.2, is SSO waste, a processed organic food waste with ATS thermal screw machine and blended with 10 to 30% woodchips.

The ATS thermal screw is a unit process with combination of shearing action and high pressure between the plates of the screw causing a transformation of the SSO to very fibrous and more homogenous material (Vartek Ltd., 2005) in such a way that the feedstock is exposed to crushing, mixing, homogenizing, granulating, cell decomposition, compacting, heat generation, and moisture reduction, all in one processing step. These functions are carried out by screws that transport the biomass or waste material from an intake hopper through several drying chambers (Jones et al., 1991).

Briefly, heat generates through friction caused by the forward pressure and turning action of the screws. The temperature rises inside the machine causing the moisture removal and heating of the feedstock which results in melting with some materials such as plastic. The normal operating temperature runs between 105 and 125°C with wet biomass. The wet waste material moves through the machine, continually blended and dried. It progressively loses moisture as it moves from one chamber to the next. The back pressure on the screws builds up to 290 bar (4500 psi) during briquetting (Jones et al., 1991).

The results of the ATS thermal screw process vary as the specific gravity and friction characteristics differ with loads of the waste material. General experience with municipal garbage, which is predominantly made of light materials, such as lignocellulosic matter, organics and plastics, is as follows (Jones et al., 1991):

- Feedstock: mixed garbage (with 50-40% moisture) predominately including papers, plastics, foods, with miscellaneous solid objects such as cans, rocks etc.
- Garbage moisture: on average 30%
- Driving power: 150 kilowatt (kW)

- Discharge output: briquetting 900 kg/hr, granulating (average) 1800 kg/hr
- Screw speed: 81 rpm
- Finished product: dense briquette or flaked material with an average moisture content of 15% (moisture content depends on defined condition for final product)
- Volume reduction: average 7:1 (this range can be as high as 10:1)

Typically, SSO waste is a recalcitrant and heterogeneous substrate. Type of the woodchips, which can be any kind of woody or agricultural waste (as presented in Table 2-1 on page 9) alters the compositional analysis of SSO. Furthermore, the composition may vary depending on several local factors: 1) sorting criteria specified by the municipality for use by the households; 2) efficiency of the citizens in sorting properly; 3) collection system including the types of collection bags used in the kitchen (paper, plastic) and local storage bins (containers, paper sacks), and finally 4) pretreatment (disc screen, screw separator, magnetic separator, etc.) prior to the biological treatment (la Cour Jansen et al., 2004). SSO components (excluding plastic, rubber and leather particles and pieces) are as follows (Tchobanoglous et al.,1993):

1. Water-soluble constituent (e.g., sugars, starches, amino acids, and various organic acids)
2. Lignocellulose including:
    - Cellulose: a condensation product of six carbon sugar glucose, such as paper, wood, and yard waste (it is about 60% of the dry weight of a typical MSW stream)
    - Hemicellulose: a condensation product of five and six carbon sugars
    - Lignin: a polymeric material containing rings with methoxyl groups ($-OCH_3$), the exact chemical nature of which is still not known (present in some paper products such as newsprint, and fiberboard)

3. Fats, oils, and waxes which are esters of alcohol and long chain fatty acids
4. Proteins, which are composed of amino acids' chains

2.4 Benefits of SSO Waste Feedstock

Waste-to-energy strategies and facilities are part of the solution of the worldwide solid waste disposal problem (Woodruff et al., 2004). Hence, diverting organic fraction of MSW to ethanol would provide a unique solution not only to growing dilemmas over MSW disposal, but also food versus fuel argument. It also contributes, albeit marginally, toward diversifying energy sources (Wyman, 1999; Kalogo et al., 2007). Moreover, producing ethanol from MSW can contribute to reducing greenhouse gas (GHG) emissions and dependency on non-renewable petroleum (Kalogo et al., 2007).

Kalogo et al., (2007), presented that MSW-to-bioethanol performs better than corn-ethanol and gasoline because the net life cycle energy used in producing ethanol from MSW is less than the energy used for producing corn-ethanol. In addition, the MSW-to-bioethanol reduces net GHG emissions by 65% compared to gasoline, and by 58% when compared to corn-ethanol. Furthermore, converting MSW into ethanol instead of landfilling will result in significant fossil energy savings. In 2005, the bioethanol that could potentially be produced from MSW was estimated to be 7.7 to 13.7 billion L. While in the same year, 530 billion L of motor gasoline was consumed in the United States. This indicates that MSW-to-ethanol is likely to perform a relatively minor role in fuel market. However, it can augment the diversity of the domestic energy resource base (Kalogo et al., 2007).

Conclusively, SSO waste conversion to bioethanol is a safe solution to battle the deteriorating impact of fossil fuel consumption and waste pile-up in landfills. SSO waste is a promising substrate for bioethanol industry with significant benefits such as:

- Avoiding depletion of farm lands that should be growing food or supporting food-based production instead of feeding an energy crop-based ethanol plant
- Inexpensive
- Reducing generated waste
- Lessening the inherent problem with landfills (potential threat to human health as well as a threat to our environment: e.g. smell; rapidly filling up and taking space; tendency to pollute leachate which can end up in groundwater; GHG emissions including methane sulfur, and carbon dioxide)
- Good alternative fuel in terms of GHG emissions

2.5 Pretreatment Technology

Pretreatment is a technical procedure to convert lignocellulosic biomass into a form that enzymatic hydrolysis is effective. It is a critical unit affecting the design and economic viability in bioethanol refineries (Figure 2-1, page7). So far, recalcitrant lignocellulosic biomass is the main challenge in the pretreatment process (da Costa Sousa et al., 2009).

Lignocelluloses feedstock consists of cellulose, hemicelluloses, and lignin. Cellulose is a long chain of glucose monomers linked to one another by β-1, 4 glycosidic bonds. It is highly crystalline and resistant to hydrolysis and biological digestion. Also, hydrogen bonds between different layers of these polysaccharides, matrix of glucose chains, contribute to the resistance of crystalline cellulose towards degradation. Hemicellulose consists of six-carbon (hexoses, e.g., glucose) and five-carbon sugars (pentoses, e.g., mannose, xylose, arabinose, galactose) with D-

xylose as its major component. The degree of branching and identity of the sugars in hemicelluloses tends to vary depending on the type of plant. Lignin contains no sugars and is covalently linked to hemicelluloses via ferulic acid ester linkages. It encloses the cellulose and hemicellulose molecules (Gray et al., 2006).

Lignin is hardly biodegradable and composed of three major phenolic components, with extensive cross-linking, including *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) (Lee et al., 2007; Rubin, 2008). A general detail of the lignocellulose structure, based on above description, is presented in Figure 2-2(Wyman & Yang, 2009; Rubin, 2008; Himmel, et al., 2007).

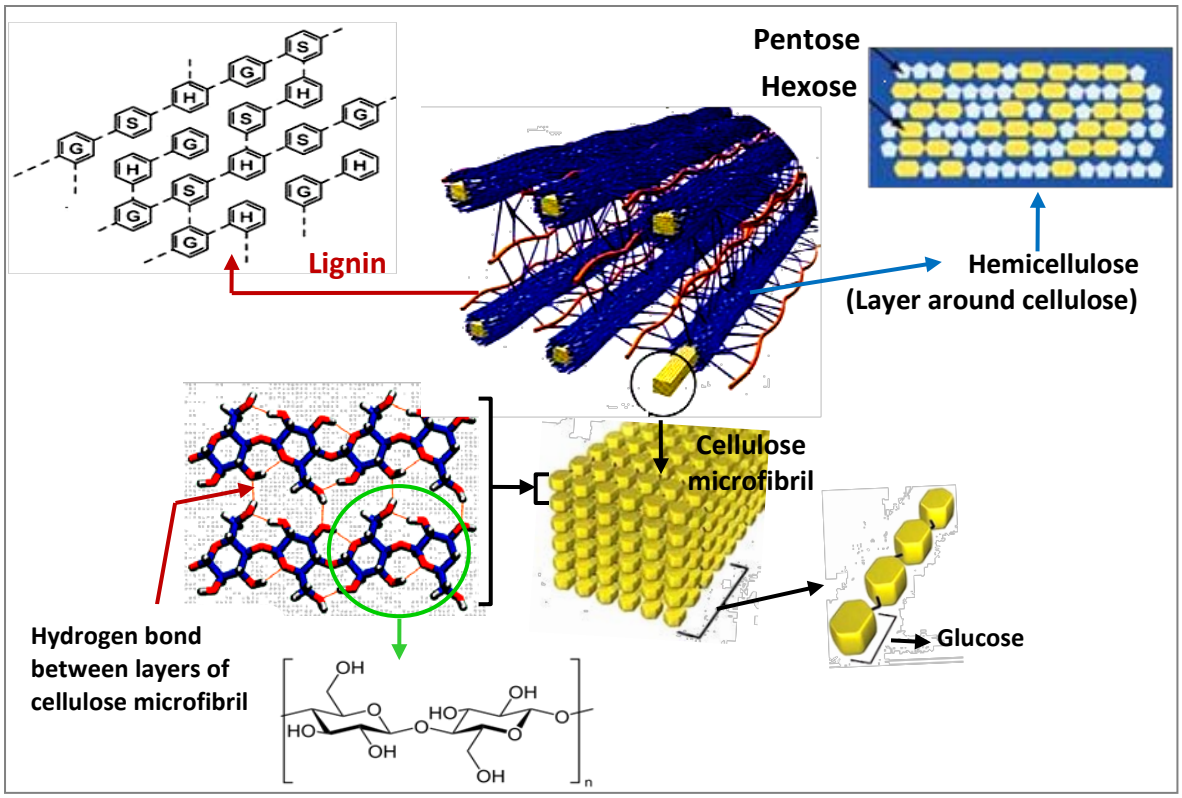

Figure 2-2 Structure of lignocellulose (Wyman & Yang, 2009; Rubin, 2008; Himmel et al., 2007)

Lignocellulosic feedstock composition differs among lignocellulosic substances. For instance, corn stover contains 41% cellulose, 21% hemicelluloses, and about 17% lignin (Gong et al, 1999). Kumar et al. (2009), and Sun and Cheng (2002), had an extensive reviews on efficient pretreatment methods based on compositional analysis of lignocellulosic material. Indeed, understanding the complexity involved with the nature of lignocellulosic substrate helps to design effective pretreatment. Composition of the major lignocelluloses is presented in Table 2-3 (Kumar et al., 2009; Sun & Cheng, 2002).

Table 2-3 Composition of agricultural residue and wastes (Kumar et al., 2009; Sun & Cheng, 2002)

Lignocellulosic Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-32
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-90	0	0-15
Wheat Straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Costal bermudagrass	25	35.7	6.4
Switchgrass	45	31.4	12
Swine waste	6	28	NA

NA; not applicable

The inherent properties of lignocellulosic biomass (i.e. lignin shealth, crystallinity of cellulose, and particle size) can limit the digestibility of the hemicelluloses and cellulose. A variety of

technologies encompass a wide range of physical, chemical, and biological pretreatments that have been developed to release polysaccharides trapped in the complex structure of lignocellulose (da Costa Sousa et al., 2009). These are categorized below (Kumar et al., 2009; Taherzadeh & Karimi, 2008; Hsu, 1996):

- Physical treatment: comminution (i.e. ball milling), irradiation (i.e. microwave heating), steaming/steam explosion, hydrothermolysis (i.e. high temperature coking in water),
- Chemical treatment: hydrolysis with dilute acid (DA) (i.e. dilute sulfuric acid, dilute nitric acid), alkalin (using sodium hydroxide alone or with other chemicals such as peroxide), organic solvent, ammonia, sulfur dioxide-catalyzed steam explosion, carbon dioxide-steaming explosion,
- Biological treatment: enzymatic hydrolysis (i.e. functional enzymes, lignin-solubilizing microorganisms to render cellulosic materials amenable to enzyme digestion).

Lignocellulosic pretreatment is described in many reports including Chang and Holtzapple (2000), Sierra et al. (2008), Hamelinck et al. (2005), Hendriks and Zeeman (2009), and Taherzadeh and Karimi (2008), with a detailed view on different lignocellulosic biomass. All of the above papers are considered the forefront of the field and highly co-cited (Thomson Reuters, 2009). Pretreatment of lignocellulosic substrate is meant to:

- Disrupt the structural bonds: break the lignin shealth, separate hemicelluloses from cellulose
- Alter crystallinity of cellulose
- Alter the degree of polymerization(DP)[1] of cellulose and hemicellulose

[1] average number of glucose units in the cellulose polymer

- Expand the structure to increase pore size and accessible surface area (of cellulose and hemicelluloses) for enzymatic pretreatment (i.e. cellulases, and hemicellulases)
- Produce fermentable sugars: hexoses and pentoses

The steps mentioned above are presented in Figure 2-3, through a cross-section view of the lignocelluloses structure (Mosier et al., 2005) in association with pretreatment function (physical, chemical and enzymatic stages) and final products (Baugh & McCarty, 1988). Pretreatment technology is considered the most costly process in a bioethanol technology system. A successful pretreatment should have the following qualities (Galbe & Zacchi, 2007):

- High recovery of all carbohydrates
- High digestibility of the cellulose in the subsequent enzymatic hydrolysis
- Low sugar degradation products, which inhibits fermentation
- low energy demand, and low cost efficiency

Assessment of the pretreatment efficiency is usually based on the following factors (Galbe & Zacchi, 2007):

- Sugar content liberated during pretreatment to the liquid, measured as a combination of monomers and oligomers as well as water-insoluble solid
- Enzymatic hydrolysis of slurry substrate originated from chemical pretreatment
- Fermentation of the pretreated substrate to assess inhibitors on the basis of two model:
  i. liquid fraction of pretreated slurry (Separate Hydrolysis and Fermentation SHF)
  ii. whole slurry via simultaneous saccharification and fermentation

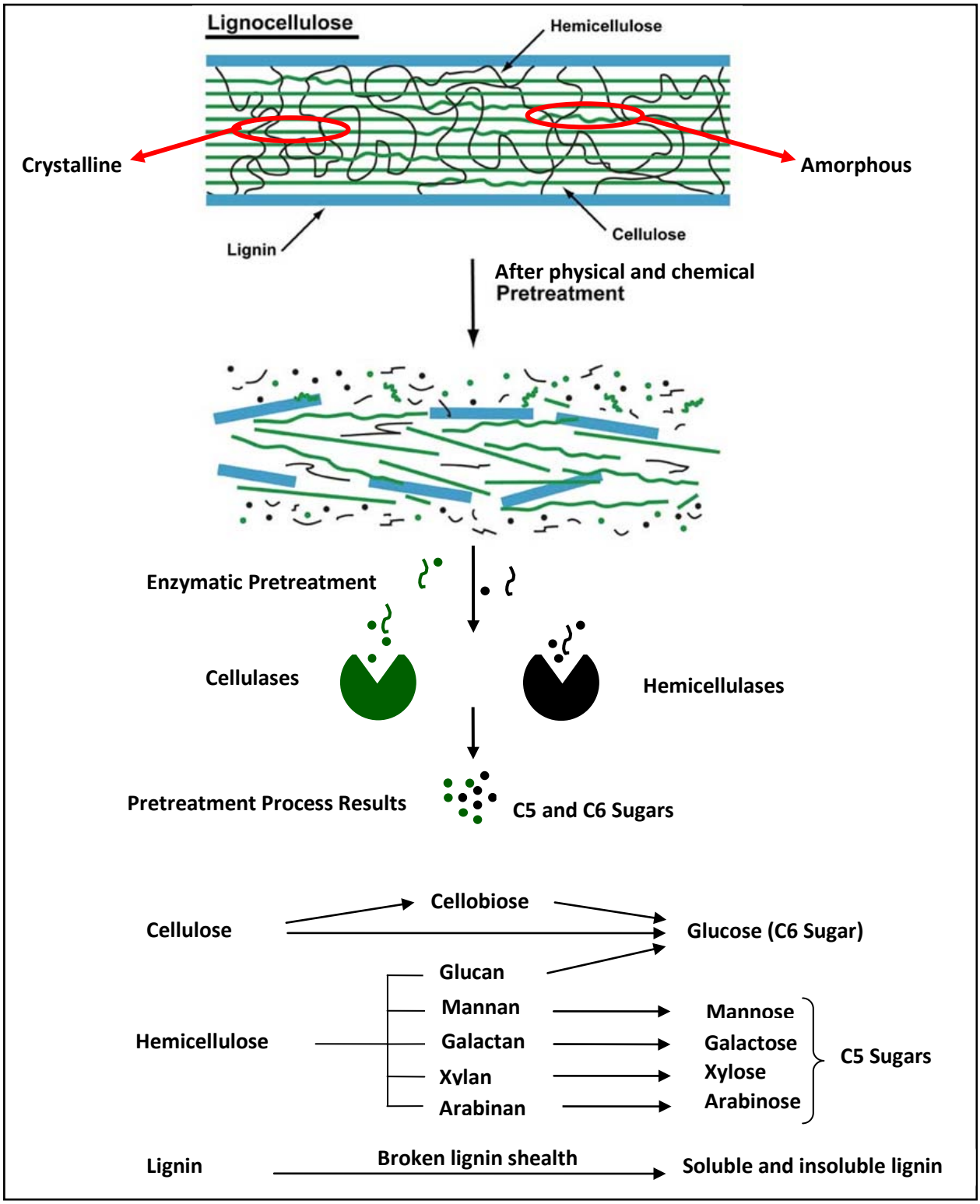

Figure 2-3 Pretreatment production of lignocellulosic biomass (Mosier et al., 2005; Baugh & McCarty, 1988)

Typical pretreatment categories with examples are summarized (Table 2-4), which is based on comprehensive studies published by Hamelinck et al. (2005); Hendriks and Zeeman (2009); Sierra et al. (2008); Taherzadeh and Karimi (2008). Deployed pretreatments in this sourcesare mainly dilute acid, ammonia fiber explosion (AFEX), and lime for evaluating different aspects including sugar recovery and design values (Wyman et al., 2005b). So far, the AFEX and the DA hydrolysis are the most common pretreatment (Hamelinck et al., 2005). Teymouri et al. (2005) emphasized the AFEX treatment based on its unique features that differentiates from other biomass treatments. Some of these distinctive features are:

- Nearly all of the ammonia can be recovered and reused while the remaining serves as nitrogen source for microbes in downstream processes.
- There is no wash stream in the process. Dry matter recovery following the AFEX treatment is nearly 100%. Treated biomass can be stable for long periods and can be fed at very high solid loadings in enzymatic hydrolysis or fermentation process.
- Cellulose and hemicellulose are well preserved in the AFEX process, with little or no degradation.
- Compared to the DA hydrolysis, the AFEX-treated biomass prior to the enzymatic hydrolysis does not require neutralization
- Enzymatic hydrolysis of AFEX treated biomass produces clean sugar streams for subsequent fermentation process.

Table 2-4 Comparison of available pre-treatment methods

Type			Examples	Temperature or Pressure	Effect on Biomass	Effect on Product
Mechanical (Physical pretreatment)			Comminution, Ball milling, Compression milling, Radiation	NA	– increase in specific surface area, – reduction of DP and the shearing	Reduces the technical digestion time by 23–59%
Chemical	Acid	H_2SO_4, HNO_3, H_3PO_4, peroxyacetic acid	Dilute/concentrated acid hydrolysis	25-75°C	loss of carbon in form of volatile compounds, strong acid pretreatment is not attractive, due to the formation of inhibiting compounds, dilute acid is one of the promising pretreatment methods due to prohibiting secondary reactions during the process	Glucose and xylose yield 75-90%
	Base	Alkalin	Alkaline hydrolysis NaOH / lime $Ca(OH)_2$	~ 25-60 °C	loss of fermentable sugars and production of inhibitory compounds make the alkaline pretreatment less attractive	xylose yield 60-70% glucose yield[1] 55%
	Thermal (mostly at high Temperature)		Liquid hot water (LHW)	190-230°C	lower risk on degradation products like furfural and the condensation and precipitation of lignin compounds which causes an increase in enzymatic hydrolysis	xylose yield 88–98% glucose yield[1] >90
			Steam retreatment/steam explosion (ST/SE) [2]	121-260°C	risk on condensation and precipitation of soluble lignin components, making the biomass less digestible, reducing the ethanol	xylose yield 45–65% glucose yield[1] 90%
			Acid hydrolysis (Dilute acid/ acid catalyzed steam explosion)	160-220°C	NA	xylose yield 75-90% for dilute acid, glucose yield[1] > 85-88%
			Alkalin [ammonia recycle percolation (ARP)]	~ 180°C	not capable of removing enough lignin of high-lignin biomass	NA
			Oxidative	NA	loss of hemicellulose due to reaction to carbon dioxide and water	NA
			Ammonia fiber explosion (AFEX)	90-100°C	More effective on the biomass with less lignin removal	glucose yield[1] 50–90%
			Ammonia and carbon dioxide	up to 200°C	NA	NA
			Alkaline + Oxidative [NaOH+ (H_2O_2 Or O_3)]	20-60 °C	lower formation of fermentable sugar has been observed	NA
	Oxidative		Wet Oxidation	185°C	a great deal loss sugars get lost due to the non-selective oxidation, formation of soluble lignin which is inhibitor	NA
	CO_2 explosion		CO_2 explosion	56.2 bar	NA	glucose yield[1] 72%
Biological			Fungi (i.e.*Trichoderma reesei*) / Commercial Enzymes	NA	cellulolytic enzymes (i.e. *cellulases*) efficiently hydrolyze the biomass	NA

[1] This is the efficiency of the downstream enzymatic hydrolysis of cellulose to glucose, usually in 24 h

[2] Difference between 'steam' pretreatment and 'steam explosion' pretreatment is the quick depressurization and cooling down of the biomass at the end of the steam explosion pretreatment, which causes the water in the biomass to 'explode'. NA: information is not available

In the DA pretreatment, neutralization is necessary prior to enzymatic hydrolysis and the cost of the DA pretreatment is usually higher than AFEX method (Sun & Cheng, 2002). However, the DA pretreatment is the most common method for industrial application (Taherzadeh & Karimi, 2008).

2.5.1 Dilute acid pretreatment

Dilute acid pretreatment is a function of five parameters including acid concentration (0.5-4% depending on the type of acid) (Galbe & Zacchi, 2002), temperature (121–240°C) (Saha & Bothast, 1999; Galbe & Zacchi, 2002), residence or reaction time (2–90 min) (Saha & Bothast, 1999), pressure (3-15 atmospheres), and solid loading (10-40%) (Wyman et al., 2005a). Specific setting of these parameters in each pretreatment depends on the type of lignocellulosic biomass.

The common problem with acid pretreatment is formation of inhibitory compounds, mostly at high temperature, which has a negative impact on microbial activity during the fermentation process. During the pretreatment, pentose monomers can further degrade to furfural, and hexoses to hydroxymethylfurfural (HMF), followed by formation of the levulinic and humic acid. This reduces available sugar for conversion to bioethanol. Moreover, during pretreatment acetic acid is released from acetyl groups in hemicellulose, as a consequence of deacetylation of acetylated pentosans (Ballesteros et al., 2007). Conditioning (overliming) process reduces the toxicity of hydrolysates generated from pretreatment by adding lime to adjust pH to the range 9-11, to precipitate gypsum (known as calcium sulfate), followed by filtration, and then neutralizing or re-acidifying to a value appropriate for fermentation (Mohagheghi et al., 2006).

Consequently, one of the main purposes of DA pretreatment at low temperature (mostly autoclave conditions) is to minimize formation of inhibitory compounds (Saha & Bothast, 1999). A broad range of lignocellulosic substrates have been examined and pretreated by dilute sulfuric acid (DSA), with different concentration from 0.3 to 2 % (w/w) over a temperature range from 120 to 150 ° C and residence time ranging from 15 to 90 minutes in an effort to optimize the pretreatment process for sugar production. Here are some examples of conducted experiments:

- Soft wood treated by 0.35% (w/w) DSA at 60° C for 6 hours, followed by steam explosion of the slurry (Schell et al., 1998)
- Alfalfa, reed canary grass, switchgrass with 0 to 2.5 % (w/v) DSA, autoclaved at 121 °C for 1 hour (Dien, et al., 2006)
- Coastal bermudagrass with 0.3 to 1.2 % (w/w) at temperatures from 120 to 180°C over residence times of 5 to 60 minutes (Redding et al., 2008)
- Wheat straw by 0.75 % (v/v) DSA, autoclaved at 121 °C for 1 hour (Saha et al., 2005)

A new method with broad substrate applicability was established by Zhang P. et al. (2007). It is based on phosphoric acid dissolution and organic solvent, operating 50°C and atmospheric pressure. This method effectively transformed the crystalline cellulose to amorphous cellulose, and had efficient organic solvent recycling. Description of this designed pretreatment is presented in the next section.

2.5.2 Cellulose-Solvent and Organic Solvent-Based Lignocellulose Fractionation

Early reports used phosphoric acid (85%) as a cellulose swelling and/or dissolution agent to reduce the DP and increase reactivity of cellulose by cellulase enzymes (Pandey et al, 2009). Later, swelling of crystalline cellulose of cotton fibre, in phosphoric acid, was investigated

(Pandey & Nair, 1974). Gradually, cellulose swelling in phosphoric acid was developed for cellulase activity assays on cellulose substrate (Sharrock, 1988). The cellulose swelling process differs from dissolution as the former retains the solid cellulose as a moiety of particles fibre, while the latter transforms the slurry into a single phase. The operation conditions determine the dominant process. Since then, phosphoric acid swollen cellulose (PASC) has become the most effective substrate in enzymatic hydrolysis process (Zhang et al., 2006).

Later, Cellulose solvent and Organic Solvent-based Lignocellulose Fractionation (COSLIF), a modified process based on PASC, was introduced by Zhang P., (2007). It is one of the newly developed bioethanol technologies, which was later elaborated in this study. The details of its unit processes are shown in Figure 2-4 and the mechanism involved in each unit operation is described below (Zhang, P., et al., 2007):

Operation 1: Concentrated H_3PO_4 is used to treat (wet) lignocellulose at 50°C for 30 to 60 min (based on optimized condition). Phosphoric acid performs the following:

- disrupts the lignin-carbohydrate complex bonds;
- dissolves cellulose fibrils and hemicellulose by breaking up orderly hydrogen bonds among sugar chains;
- weakly hydrolyzes cellulose and hemicellulose to a low DP fragments;
- removes acetyl groups from hemicellulose to form acetic acid.

Operation 2: Acetone is added to precipitate the dissolved cellulose and hemicellulose and to dissolve partial lignin in the liquid phase;

Operation 3: Acetone is used to wash out 99.5% of phosphoric acid from the precipitated solids and to remove the dissolved lignin;

Operation 4: Water is used to remove acetone from the solids and water–soluble hemicellulose from the solid cellulose;

Operation 5: Enzymatic cellulose hydrolysis is carried out at 50°C;

Operation 6: Black liquor is separated into phosphoric acid, acetone, acetone-soluble lignin, and acetic acid from hemicellulose. Highly volatile acetone and modestly volatile acetic acid can be easily separated by distillation, and the precipitated lignin can be separated by solid/liquid separator at the bottom of the column;

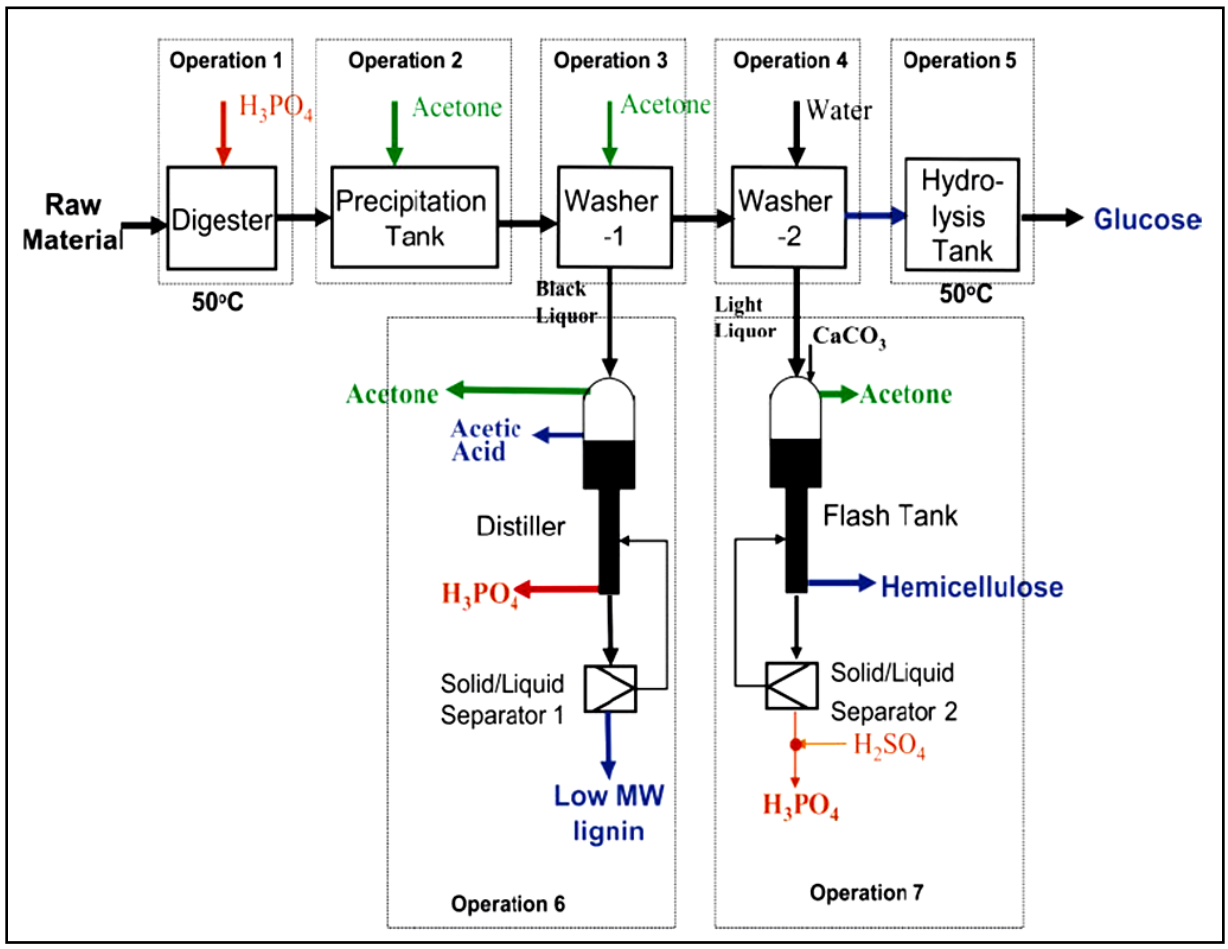

Figure 2-4 General scheme of COSLIF unit process (Zhang et al., 2007)

Operation 7: Light liquor is separated into acetone, a small amount of phosphoric acid, and water-soluble hemicellulose. Acetone can be recovered by flashing. The precipitated $Ca_3(PO_4)_2$, after the reaction with $CaCO_3$, can be used to regenerate concentrated phosphoric acid adding using the wet phosphoric acid production method; water soluble hemicellulose remains in the liquid phase.

In all, this technology can fractionate lignocellulose into amorphous cellulose (mainly glucose after hydrolysis), lignin, hemicellulose, and acetic acid at modest reaction conditions (50°C and atmospheric pressure) with easy recycling of acetone and phosphoric acid. The COSLIF pretreatment is a promising option with high sugar yield and considerable technical benefits including (Zhang, P., et al., 2007):

- no sugar degradation,
- no inhibitor formation, and consequently no need for detoxification,
- no need for special reactor, (while dilute acid and AFEX pretreatments need special reactor),
- fast enzymatic hydrolysis rate,
- modest reaction conditions (50°C and atmospheric pressure).

2.5.3 Enzymatic Pretreatment

Enzymatic pretreatment is a bioconversion 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, and has numerous applications and biotechnological potential for various industries (i.e. chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp, paper and

agriculture). Hemicellulases and most cellulases are multi-domain proteins[2] (Howard et al., 2003).

In a natural fermentation process, potent cellulolytic organism such as *Trichoderma reesei* produces complex mixtures of enzymes composed of three major groups including endogluconases often called carboxymethylcellulase (CM-cellulases), exoglucanases or cellobiohydrolases, and ß-glucosidases or cellobiases. Digestibility of celluloses according to its structure occurs as below (Szakacs et al., 2006):

- Endoglucanases cleave the internal ß–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.
- ß–glucosidases cleave cellobiose, to release glucose.

There are 13 major hemicellulases including Exo–β-1,4–xylosidas, Endo–β-1,4 xylanase, Endo–β–1,4–mannanase, Exo–β–1,4–mannosidase, α–L-arabinofuranosidase, Endo–α–1,5-arabinanase, α–Glucuronidase, α–Galatosidase, Endo-galactanase, β–Glucosidase, Acetyl xylan esterases, Acetyl mannan esterase, Ferulic and p-cumaric acid esterases (Howard et al., 2003). Xylan is the major structure of hemicelluloses and is bonded to lignin. The depolymerisation of xylan by xylanases results in xylooligosaccharides and xyloses. The complex structure of xylan needs different enzymes for complete hydrolysis. Hemicellulose degradation is summarized as follows:

- Endo–1,4–ß –xylanases which depolymerise xylan
- α–L–arabinofuranosidase, α–D–glucuronidase, galactosidase, and acetyl xylan esterase liberate the side groups in xylan

[2] consisting of two or more domains belonging to different classes of protein

Potent producers of xylanases are cellulolytic fungi such as *Aspergillus niger* (Perma, 2006). (Perma, 2006)

This shows that efficient degradation of lignocellulosic feedstock requires a mixture of different enzymes acting sequentially or in concert. Recent discussion on multifunctional glucanases including some cellulases and hemicellulases, demonstrates the concept of "one enzyme one activity" might not hold in all cases, especially with glucanases (Ghose, 1987) which is a multifunctional protein consisting of a single type of polypeptide[3] chain, with multiple catalytic or binding activities (Howard et al., 2003).

The enzymatic process highly depends on various operating variables including type and quantity of enzymes, pH, enzyme loading, solid loading of a slurry substrate, hydrolysis time, enzyme mechanism and feedback inhibition (such as sugar concentration), and extent of digestibility of substrate (da Costa Sousa et al., 2009). Recently, a great deal of effort has been devoted to develop efficient and cost effective enzymatic hydrolysis. There is a wide range of commercial enzyme complexes available in the market. Significant advances have been made towards engineering a new generation of cellulase cocktails that would further reduce enzyme cost by enhancing the enzymatic hydrolysis efficiency and reducing the amount of enzyme required (Li, X.,et al., 2009).

In 2008, the U.S Department of Energy announced its goal to develop commercially viable biofuel focusing on new generation of enzymes with low protein content and high activity to decreasing the dosage of enzyme required to hydrolyze biomass. $33.8 million was invested among following producers (Ruggiero, 2008):

[3] chain of amino acids

- Genencor, a Danisco Division, USA, Inc.
- Novozymes Inc.
- DSM Innovation Center Inc.
- Verenium Corporation

In 2009, two enzyme manufacturers, Genencore and Novozymes, rolled out the new generation of cellulosic ethanol enzymes (Retka Schill, 2009). For example, Accellerase 1500 is Genencor's new generation of enzyme product, a significant step toward more cost-effective, commercial-scale production developed for second generation of biorefineries[4]. It contains multiple enzyme activities, mainly exoglucanases, endoglucanases, β–glucosidase, with hemicellulase side activity. The synergistic effect of the enzyme activities lead to hydrolyzing the complex lignocellulosic biomass into fermentable monosaccharides as well as aid materials handling through liquefaction and viscosity reduction. It has been shown to successfully hydrolyze a wide range of lignocellulosic feedstocks. In contrast to conventional celluclases such as Celluclast 1.5L, (product of Novozymes[5]), Accellerase 1500 has the following benefits (Genencor, 2007):

- Enhanced saccharification performance on a variety of feedstocks,
- Ability to operate in SSF, and SHF processes,
- Higher β–glucosidase activity to minimize residual cellobiose, which can lead to higher rates of saccharification and ultimately to faster ethanol fermentation.

[4] using lignocellulosic feedstocks for bioethanol production

5 Cellic CTec and HTec are Novozyme's new commercial cellulases

According to the above, performance of Accellerase 1500 and Celluclast 1.5L was compared in this study.

2.5.4 Ethanol-Producing Organisms

An ideal ethanologenic organism (ethanol biocatalyst) should be capable of fermenting all biomass sugars to ethanol. Required characteristics have been investigated and cited through many studies and prominent qualities are summarized in Table 2-5 (Dien et al., 2003; Zaldivar et al., 2001).

Table 2-5 Classified characteristics of ethanol biocatalyst (Zaldivar et al., 2001; Dien et al., 2003)

Essential trait	Desirable trait
- Broad substrate utilization range - High ethanol yields and productivity - Minimal byproduct formation - High ethanol tolerance - Increased tolerance to inhibitors - Tolerance to process hardiness: Transient adverse condition such as change in pH and temperature and/or increase in salt, sugar, or ethanol concentration	- Simultaneous sugar utilization - Hemicellulose and cellulose hydrolysis - GRAS status: Generally regarded as safe, as defined by the USA Food and Drug Administration - Recyclable - Minimal nutrient supplementation
Determined Trait	**Requirements**
Ethanol yield	>90% of theoretical
Ethanol tolerance	$>40 gl^{-1}$
Ethanol productivity	$>1 gl^{-1} h^{-1}$
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperatures

Many reports and reviews have been published on production of ethanol fermentation by microorganisms. The most common ethanol-producing organisms are summarized in Table 2-6 (Ward and Singh in 2002; Dien et al., 2003; Lin and Tanaka, 2006; Leschine & Warnick, 2007). Depending on their biological family, fermentation quality can be optimized by manipulating

operating pH and temperature. As shown, many of these species have been genetically modified for fermenting both pentose and hexose.

A wide-ranging mix of hexoses and pentoses can be released from the hydrolysis of lignocellulosic materials. Most cellulolytic bacterial strains prefer to utilize glucose as a substrate and are incapable of fermenting other sugars such as pentoses. This is a major technical roadblock to the development of bioethanol industry. Researchers are pursuing potential solutions to this problem by investigating recombinant DNA techniques to genetically modify strains in order to maximize the cellulosic ethanol yield, increase the microbe's tolerance to ethanol, eliminate unwanted byproducts, and expand their substrate selection (towards metabolism of pentose sugars) (He et al., 2009).

Up until now, great advances have been made in genetically modifying strains of *Saccharomyces cerevisiae* (a kind of yeast) and bacteria such as *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*, and thus these strains are industrially favorable (He et al., 2009). A potentially powerful strategy is to use Consolidate Bioprocessing (CBP) system, which features cellulase production, lignocellulose hydrolysis, and fermentation in one step. This bypasses the pretreatment steps and lowers the overall processing cost. For example various cellulolytic clostridia investigated for CBP system such as *Clastridiume thermocellum*, a thermophilic bacteria (Lynd et al., 2005), and *Clastridum phytofermentans* (Leschine, 2007)

Table 2-6 Ethanol-producing organisms (Ward and Singh in 2002; Dien et al., 2003; Lin and Tanaka, 2006; Leschine & Warnick, 2007)

Microorganism	Fermentation Capability	pH	Temperature (°C)
Yeasts			
Saccharomyces cerevisiae	Ferments hexose with high ethanol yield, genetically engineered to ferment pentoses	3-7	30 – 35
Candida shehatae	Ferments both hexose and pentose with moderate formation of xylitol	3.5 - 4.5	28 – 32
Pachysolen tannophilus	Efficiently ferments glucose and xylose, produces significant amount of by-product xylitol	2.5 - 5	28 – 32
Pichia stipitis	Ferments all sugars found in wood and straw hydrolysates, some strains ferment xylan, genetically engineered to rapid anaerobic growth	4 – 4.5	28 – 32
Klyuvermyces maraxianus	Ferments a wide range of sugars including xylose	3 - 7	30 - 35
Filamentous fungi			
Fusarium oxysporum	Ferments a wide range of carbon sources including xylose	5 - 6	28 – 34
Neurospora	Ferments glucose and xylose with low ethanol production	5 - 6	28 - 37
Mucor sp.	Ferments both hexose and pentose	5 - 6	28 - 32
Bacteria			
Clostridium phytofermentans	Ferments both hexose and pentose	6.0 - 9.0	30 -37
Clostridium thermocellum	Ferments cellulose, glucose, and xylose Low ethanol production	4 - 8	65 - 70
Clostridium thermohydrosulfuricum	Ferments glucose, xylose, and arabinose	4.7 - 8	65 – 70
Thermoanaerobacter ethanolicus	Ferments a wide range of sugars including xylose and arabinose	4.4 – 9.5	65 - 70
Zymomonas mobilis	Rapid glucose and sucrose fermentation, genetically engineered to ferment pentoses	4 – 6.5	30 - 37
Klebsiella oxytoca	Rapid xylose and cellobiose fermentation genetically engineered to ferment cellulose	6 - 8	30 - 37
Escherichia coli	Ferments xylose and glucose with organic acids, genetically engineered to produce high ethanol from hydrolysates	6 - 8	30 - 37

Experimental Investigation and Design for Converting SSO to Sugar

3.1 Introduction

There are limited studies on conversion of food waste to bioethanol. The most prominent peer–reviewed studies are summarized in Table 3-1. As shown, laboratory made and/or selected carbohydrate-rich food wastes with diverse characteristics have been subjected to different pretreatment. *Saccharomyces cerevisiae* is the common microorganism used for fermentation process. The yield of fermentable sugar and/or ethanol production varies as the authors focused on influential factors such as kinetic model (Davis, 2008), and statistical optimization of glucose yield through response surface methodology (Kim, J., et al., 2008). Evidently, conversion process scenario relies on function of multi- step pretreatment and multi-enzyme complex.

The preliminary studies on SSO conversion to ethanol have portrayed a promising future. Despite the holocellulose and starch content as major sources of sugars, complexity on its composition is still an obstacle in biomass hydrolysis, which is a rate-limiting step in conversion process. Laboratory investigation on SSO-ethanol is still in the empirical stages and requires far more studies in terms of characterization and pretreatment (Moon et al., 2009).

Following the objectives of this research, experimental plan was developed based on available pre-treatment methods (Table 2-5), and similar studies on organic food waste (Table 3-1). In this chapter, experimental design of each acid pretreatment is discussed. The main goal is to investigate glucose and xylose yield at moderate pretreatment condition followed by enzymatic hydrolysis and fermentation. The complexity involved in the nature of SSO substrate and experimental specifications are fundamental elements, which differentiate this analysis from others. All experimental data are presented and discussed in Chapter 4.

Table 3-1 Overview of laboratory experiments on organic food waste to sugar and ethanol production

No.	Reference	Substrate	Fermentation Condition	General Characteristic	Pretreatment	Microorganism	Product
1	Moon et al. (2009)	Food wastes collected from Cafeteria in Gyeonggi (Suwon, Korea)	Batch	Weight % based on dry mass: Starch 30.1%, Fiber 14.6 %, Salt 1.7%, protein 21%, Reducing Sugar 17.6%	- Homogenization by crushing in lab blender. - Enzymatic hydrolysis by two commercial enzyme including Amyloglucosidase (*Aspergillus niger*) and Carbohydrase (*Aspergillus aculeatus*)	*Saccharomyces cerevisiae*	Producing fermentable: glucose 58g/L (high glucose yield 0.46 g/g of dry biomass, Ethanol yield: 29.1g/L (~0.30g/g dry biomass)
2	Wang et al. (2008)	Kitchen garbage from dining room of University (Beijing)	Batch	TS 17.22%, Dissolved Solid 2.58% SS 14.64%, Total sugar 62.68%, Starch 46.12%, Protein 15.56%, Fat 18.06%, Cellulose 26%	Not available	*Saccharomyces cerevisiae*	Glucose conversion 84%) Max. ethanol 33.05g/l
3	Davis (2008)	Two types of food wastes (lab-made): a) Pre-prepared mixture b) Rice mixture	Batch	a) 26% starch, 34% sugars b) 52% starch, 12% total sugars	- Homogenization - Enzymatic hydrolysis with Stargen enzyme (mixture of α-Amylase & Glucoamylase)	*Saccharomyces cerevisiae*	Kinetic model was designed via Matlab Simulink,
4	Kim et al. (2008)	Food waste from Cafeteria	Batch	pH 5.12, TS 12.9%, VOC 89.5%TS, COD 85.1g/L	- Homogenization (mixing with water and crushing into small particles about 1-2mm) Enzymatic hydrolysis with Spirizyme (*Aspergillus niger*)	*Saccharomyces cerevisiae*	Statistical optimization of enzymatic saccharification and ethanol product
5	Li et al. (2007)	Lab made kitchen waste	Batch	Different combinations of the following material were tested: carrot peelings (CP) potato peelings (PP), grass(G) , newspaper (NP), scrap paper (SP)	- Homogenization via shredding, grinding and screening - Dilute acid (1 & 4)% (effect of H_2SO_4, HCl, HNO_3 were separately tested) - Enzymatic hydrolysis was carried out with two enzymes (from *Trichoderma reesei* &*Trichoderma viride*)	Not applicable	High glucose yield: single substrate (70-89)% Multi-substrate (41-52)%
6	Mtui & Nakamura, (2005)	Solid waste from selected dumping sites in Tanzania –Dar es salaam	Batch	Lignocellulose 93%, Metals 0.2%, Plastics 2%, organics 2%, Glass 1%, Rubber 1%, Others 0.8%	- Homogenization via shredding and then crushing by grinding miller - Dilute acid hydrolysis (by 0.5 M H_2SO_4), Enzymatic hydrolysis by *Trichoderma reesei*	*Saccharomyces cerevisiae*	(Per 1 g of biomass) Max. conversion of samples 21.45% (w/v) Glucose: 0.13 & 0.05 g/L; ethanol: 0.15g/l
7	Han & shin (2004)	Lab made food waste	Leaching-bed reactor	Mixture of Grains (61.1%TS), Vegetables (29.7%TS), Meats (9.2%TS), VS/TS= 0.95, C/N = 14.7	Not available	Clostridium sp.	Ethanol: 17%
8	Claassen et al.,(2000)	Domestic Organic Waste in Netherlands	Batch	Acid soluble lignin 1.2 %, acid insoluble lignin 10.5 %, glucose 25.1 % xylose 8.4 %, arabinose 2.3 %, mannose 1.6 %, galactose 1.6 %,	- Steam explosion - Enzymatic hydrolysis (Celluclast 1.5L & Novozymes 188)	*Solventogenic Clostridia*	Acetone, butanol and ethanol

3.2 Experimental Investigation Plan

Pre-treatment technique is a central unit process in bioethanol technology (da Costa Sousa et al, 2009) due to the cross effects amongst four steps: biomass structural features, chemical pretreatment, enzyme-catalyzed hydrolysis, and fermentation (Zhu et al., 2008).

One aspect of this study is to carry out a series of experiments based on two forms of acidic treatment: 1) Dilute Acid (DA) pretreatment with sulfuric acid; and 2) cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF). The main goal is to compare their intensity and overall efficiency on total fermentable sugar yields of subsequent enzymatic hydrolysis. Each method is evaluated in the context of the entire unit, namely acid pretreatment, followed by enzymatic hydrolysis and finally fermentation, as shown in Figure 3-1. Enzymatic hydrolysis and fermentation are pH dependant processes and range of pH, presented in Figure 3-1, is based on their optimal conditions.

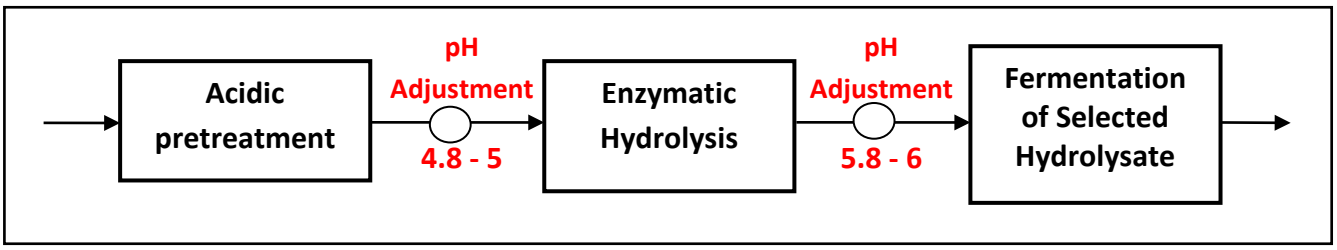

Figure 3-1 General scheme of process units

Figure 3-2 presents a general view of experimental plan. Samples for each set of experiments were duplicated. In order to prevent any iteration, description of laboratory procedures, materials and methods used in this research are not included due to being the same as applied references, except for those which were modified based on available materials and laboratory conditions.

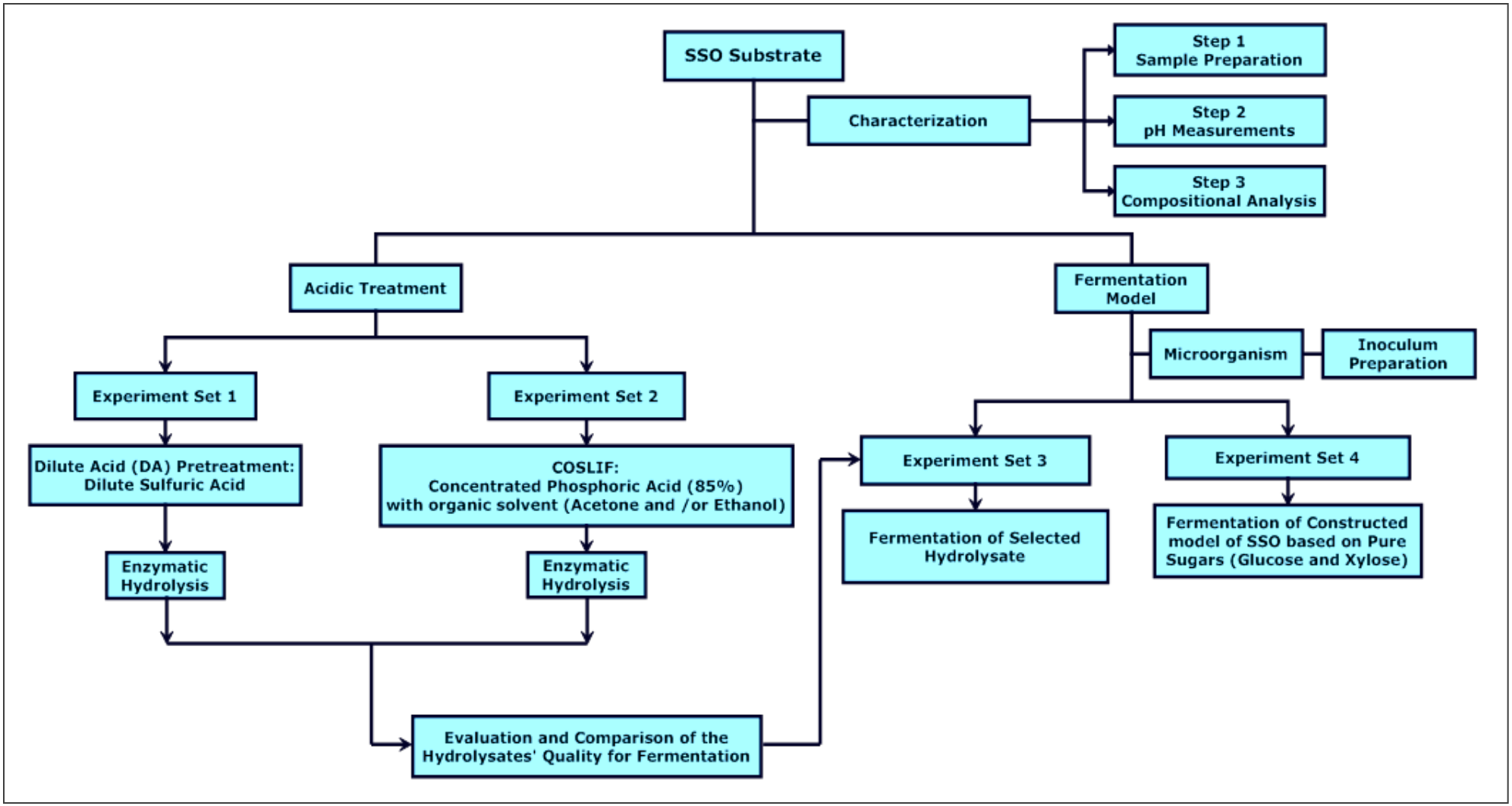

Figure 3-2 Experimental investigation plan

Quantitative analysis on samples was carried out by High Performance Liquid Chromatography (HPLC), showed in Figures 3-3 and 3-4, combined with refractive index detector. Organic compounds contained in the liquid fraction of tested samples were determined as follows:

- soluble sugars by Bio-Rad Aminex HPX-87P column equipped with de-ashing guard cartridge
- volatile fatty acids, furfural, HMF and ethanol by Bio-Rad Aminex HPX-87H column, equipped with H guard cartridge

Figure 3-3 HPLC - Perkin Elmer- LC Autosampler; ISS 200

Figure 3-4 HPLC – Perkin Elmer- LC Autosampler; Series 200

3.3 Substrate Characterization

Compositional analysis of SSO is the research gateway to identify elemental composition of this type of substrate. Generally, SSO biomass is a chemically complex mixture and a fundamental feature for microbial growth. Understanding the fundamental composition associated with energy content, and natural variability are essential for scientists and engineers to conduct and develop a research on bioethanol technology (McDermitt & Loomis, 1981). Hence, for a reasonable use of any SSO bioresource feedstock, it is justifiable to determine technical parameters and unlock the leading factors for estimation on overall efficiency, and desirable product yield in a defined process. In this respect, a well-organized quantitative and qualitative analysis is essential to support and enable a technical design.

Analytical measurements routinely referenced in biomass industry are: 1) standard procedures of the National Renewable Energy Laboratory (NREL) - a division of US Department of Energy (DOE); 2) Technical Association of the Pulp and Paper Industry (TAPPI); and 3) American Society for Testing and Materials (ASTM). The NREL procedures are the most valid and common used references (NREL, 2008). They were also applied in this study. General scheme of experimental methods and references are presented in the Figure 3-5. Among indicated parameters, it was necessary to highlight specific items, including homogenization as a core centre of chemical analysis, biodegradability because of lignin content, and extractives due to interference of inorganic material as well as non-structural compounds in downstream analysis of the SSO. Furthermore, laboratory procedures for determination of extractives and acid soluble lignin were modified in accordance with available facilities and defined experimental conditions.

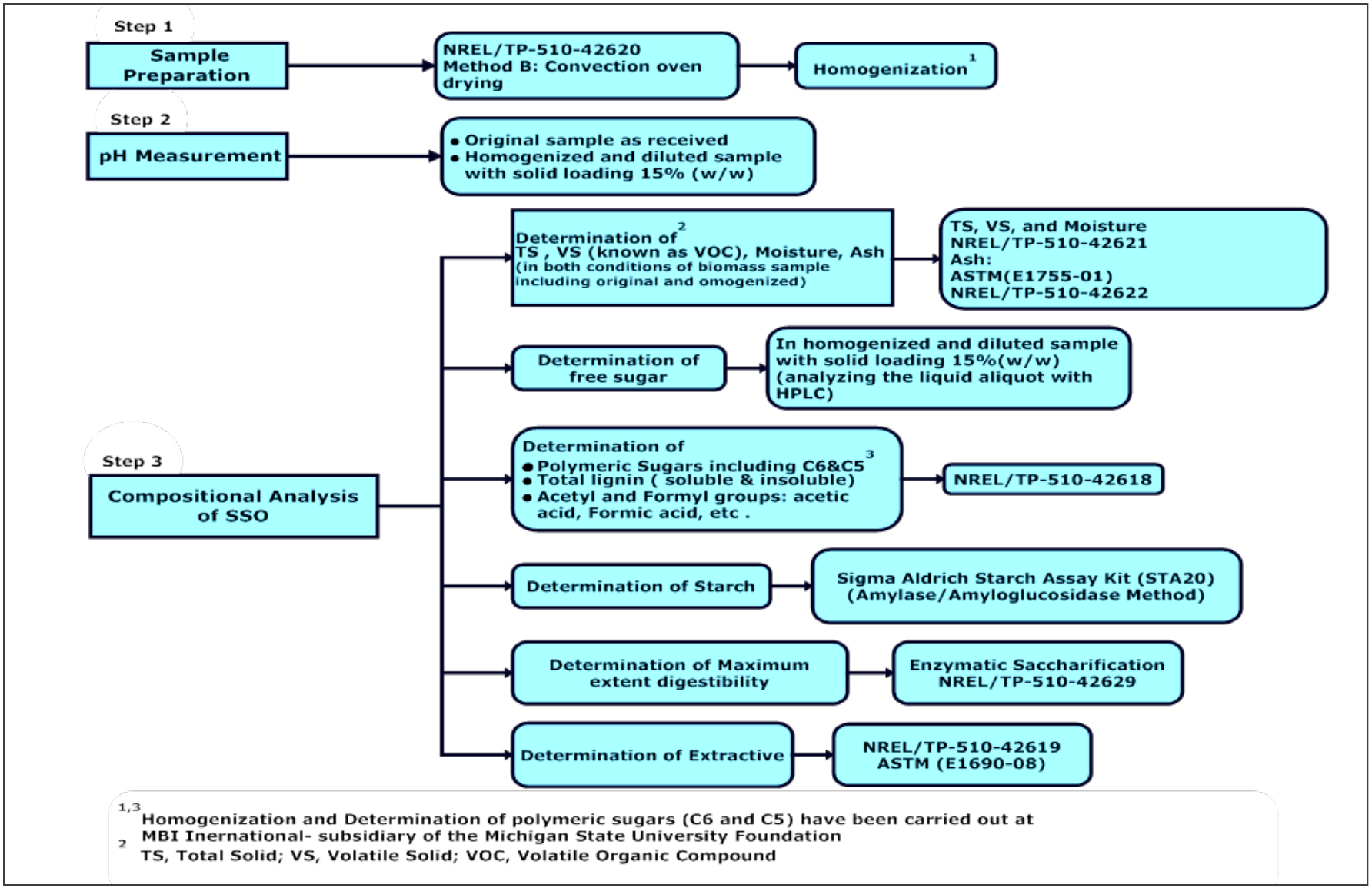

Figure 3-5 General scheme of SSO characterization procedure (NREL, 2008)

Both SSO composition and its analysis methods are highly debatable. Selected compositional analysis methods have been studied in the master thesis of Mirzajani (2009). The main parameters of the current plan are further elaborated in Table 3-2 (NREL, 2008; CCME, 2005; Celesceri et al., 1989; Zhang, R., et al., 2007). All indicated procedures were determined according to referenced procedures in Figure 3-5. Protein content, nutrient value, trace elements, and salts analyses were not measured in this study.

Table 3-2 List of physical and chemical analyses

No.	Characteristic	Importance	Measurement Reference
1	pH	Identifying the initial condition of biomass and influence of biosynthesis process	-
2	Homogenization and Particle size	For obtaining reliable data, designing fermentation system	NREL/TP-510-42620, method B
3	Solid Analysis: – Total Solid (TS) – Total Volatile Solid (VS)	Moisture content, reporting results on a dry weight basis, designing fermentation system, estimation of biomass utilization	NREL/TP-510-42621
	– Ash	Amount of inorganic material in biomass, either structural (bound in the physical structure of the biomass) or extractable (can be removed by washing or extracting)	NREL/TP-510-42622
4	Structural Carbohydrate (Cellulose and Hemicellulose)	Contribution to overall sugar measurement fermentable sugar	NREL/TP-510-42618
5	Lignin	Estimation of biodegradability	
6	O-Acyl content (Acetyl and Formyl group)	It is necessary for biomass with containing hemicellulose with xylose backbone	- NREL/TP-510-42618 - NREL/LAP017
7	Extractives	Water and ethanol soluble materials which are non chemically bound components and potentially interfere with biomass analysis (i.e., chlorophyll wax, protein, nitrate/nitrite)	NREL/TP-510-42619 ASTM (E1690-08)
8	Starch (a large chain of glucose)	Contribution to overall glucose measurement as fermentable sugar	Sigma Aldrich Starch Assay Kit (STA20)
9	Protein	Indirect nitrogen measurement, nitrogen-to-protein conversion factors	NREL/TP-510-42625
10	Nutrient value	Nitrogen and phosphorus (N and P), which are microbial nutrient [The nutrient contents of food waste indicate that food waste contained the required nutrients for anaerobic microorganisms. On average, food waste has carbon and nitrogen ratio (C/N) of 14.8 (Zhang, R., et al., 2007)	Standard methods for the examination of water and wastewater (Celesceri et al., 1989)
11	Trace elements	Possibility of negative impact inorganic compounds	CCME guidelines for compost quality
12	Salt	Changing pH, influential impact on microbial cell and growth	-

3.3.1 Homogenization

In this study, SSO sample, contained 20% woodchips, was already pretreated by the ATS thermal screw machine. The woodchips are typically wood wastes primarily Douglas fir originated from home construction furniture, flooring, cabinet, and doors. Further, samples were also oven-dried at 45° C for 48 hours in laboratory (NREL/TP-510-42620, method B), prior to homogenization. All sharp foreign matter such as metal needles, plastic and rubber wastes, and broken glasses were collected, as much as it was possible. The dried SSO biomass was sent to MBI International, a subsidiary of the Michigan State University Foundation, for grinding and determination of polymeric sugar content. Figure 3-6 presents a summary of the sample preparation.

Figure 3-6 Summary of the sample preparation

3.3.2 Biodegradability

Dry organic substrate consists of volatile solid and ash. Only the biodegradable fraction of volatile solid has the potential for bioconversion. The non-biodegradable fraction is mostly lignin, which hinders biodegradation (Kayhanian et al, 2007). The following equation was developed by Chandler et al, (1980), to provide the best estimation for substrate biodegradability (Haug, 1993; Kayhanian et al., 2007):

$$B = 0.83 - (0.028)\ X$$

where, B = biodegradable fraction of the volatile solids (VS), and X = lignin content of the VS expressed as a percent of dry weight.

The equation suggests that a substrate containing no lignin would only have a maximum degradability of 83%. This is reasonable since the decomposition of the organic waste is coupled with bacterial by-products which are not readily degradable (Haug, 1993). Kayhanian et al., (2007) had a comprehensive review on biodegradability of organic wastes and their estimation on biodegradable fraction of organic waste components in MSW is presented in Table 3-3.

Table 3-3 Biodegradability of organic fraction of MSW (Kayhanian et al, 2007)

Component	Lignin content (% VS)	Biodegradable fraction (% VS)
Food waste	0.1 - 0.7	82
Newspaper	20 - 23	22
Office paper	0.2 - 1	82
Yard wastes	4 - 10	72
Mixed blend (19% newsprint, 53% office paper, 15% yard waste, 13% food waste)	4 - 7	67.6

3.3.3 Determination of Extractives

Both laboratory references for determination of extractives, NREL/TP-510-42619 and ASTM (E1690–08) are reasonably similar in structure. Nevertheless, the procedure was modified by boiling sample in solvent for 3 hours. The soxhlet extraction apparatus associated with constant heating and reflux time (maximum24 hours) was replaced by direct boiling (using hot plate) for 3 hours (Mirzajani, 2009). The ASTM (E1690-08) procedure was also modified by three times solvent exchanges with the following considerations:

- 7 g of oven-dried and moisture free SSO was added to the extraction thimble as suggested in ASTM (E1690–08) with 1 cm of free space in the top of the thimble, and then the thimble was sealed with cotton string.
- Prepared thimble was placed in 500 Erlenmeyer flask filled with 150 ml ethanol 75% (V/V).
- Solvent was exchanged every hour (three times in total). DDW was used for second and third solvent exchange.
- All liquids were filtered, transferred to aluminum dish to evaporate to near dryness.
- Evaporated samples placed in an oven with temperature adjusted at 103-105°C for 20 minutes. Figure 3-5 shows results after drying in the oven.
- Total extractives (in water and ethanol) were determined through following calculation:

  % Extractives = $[(W_1-W_2)/W_1] \times 100$, where: W_1=Initial weight of SSO and W_2 = Weight of dry residue.

The final residue resulted from this experiment is shown in Figure 3-7.

Figure 3-7 Extractive residue after drying in oven

3.4 Dilute Sulfuric Acid (DSA) Pretreatment

The DSA pretreatment at relatively low temperature to minimize the formation of inhibitory compounds, followed by enzymatic hydrolysis, may be a workable process for generating fermentable sugars from SSO substrate. Table 3-4 presents a summary of the most common low temperature-DSA pretreatment design condition on three different substrates carried out in the last decade, thereby guiding to map out a model for the SSO pretreatment with DSA.

Table 3-4 DA pretreatment designed conditions at low temperature

<table>
<tr><th colspan="3">Corn fiber</th><th colspan="2">Switchgrass</th><th colspan="3">Saline crop[6]</th></tr>
<tr><th colspan="2">H_2SO_4 conc.</th><th rowspan="2">Optimal Condition</th><th>H_2SO_4 conc. % (w/v)</th><th rowspan="2">Optimal Condition</th><th>H_2SO_4 conc. % (w/w)</th><th>Temperature & Residence Time</th><th rowspan="2">Optimal Condition</th></tr>
<tr><th>% (v/v)</th><th>% (w/w)</th><th>% (w/v)</th><th>% (w/w)</th></tr>
<tr><td>0.5</td><td>0.92</td><td rowspan="4">0.5 % (v/v), 1 hr, average glucose yield > 30%</td><td>0.5</td><td rowspan="4">1.5% (w/v), 60 min, glucan conversion of 91.8%</td><td>0.9</td><td>140° C, 20min</td><td rowspan="4">1.4 % (w/v), 165° C, average glucose yield > 30%</td></tr>
<tr><td>0.75</td><td>1.38</td><td>1</td><td>1.2</td><td>150° C, 10 min</td></tr>
<tr><td>1</td><td>1.84</td><td rowspan="2">1.5</td><td rowspan="2">1.4</td><td rowspan="2">165° C, 8 min</td></tr>
<tr><td>1.25</td><td>2.3</td></tr>
<tr><td colspan="3">Solid loading (based on dry mass): 15% (w/v)
Temperature 121° C
Residence Time: 15, 30, 45, and 60 minutes</td><td colspan="2">Solid loading (based on dry mass): 3% (w/v)
Temperature 121° C
Residence Time: 30, 45, and 60 minutes</td><td colspan="3">Solid loading 10%</td></tr>
<tr><td colspan="3">Saha et al., (1999)</td><td colspan="2">Yang et al., (2009)</td><td colspan="3">Zheng et al., (2009)</td></tr>
</table>

[6] Salin crop including athel (tamarix aphylla L), eucalyptus (eucalyptus camaldulensis), jose tall, wheatgrass (agropyron elongatum), and creeping wild ryegrass (leymus triticoides)

In the same way, similar framework was deployed in this research and details of the experimentation plan are presented in Table 3-5. Sulfuric acid at 0.5, 1.0 and 1.5% (w/w) was used for chemical pretreatment of the oven-dried SSO samples prepared in 125ml Wheaton glass serum bottle with 15% (w/w) solid loading. The mixtures were autoclaved at 121 °C and 16.2 psi for 15, 30 and 60 minutes.

Table 3-5 DA-Experimental design in this research

Pretreatment Condition	H_2SO_4 Conc. % (w/w)	g acid per g biomass	Residence time (min)
Solid loading (based on dry SSO): 15 % (w/w)	0.5	0.4	15, 30, and 60
Autoclaving at: 121° C, 0.115 mPa (16.2 psi)	1	0.8	
	1.5	1.2	

A close image of DA samples is shown in Figure 3-8. Upon completion of the autoclave cycle, samples were removed and allowed to cool to room temperature for pH adjustment and further preparation for enzymatic hydrolysis.

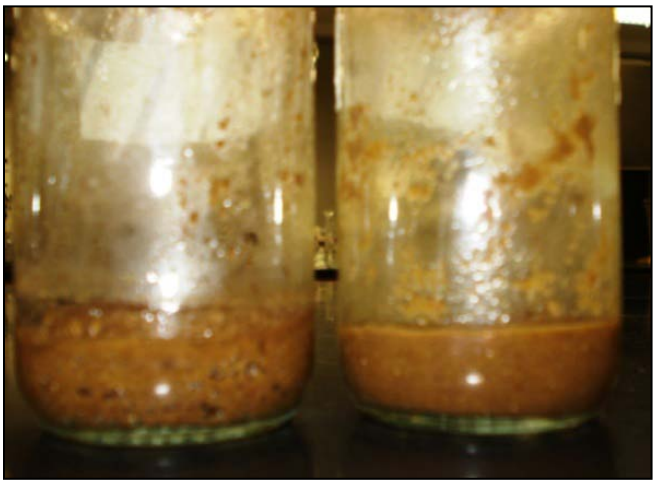

Figure 3-8 A close shot of DA pretreated sample before diluting for enzymatic hydrolysis

3.5 COSLIF Process

Concentrated phosphoric acid has proven to be ideal cellulose solvent (Zhang et al., 2006), mainly for the following reasons (Zhang, P., et al., 2007):

- Cellulose dissolution occurs at low temperature,
- Regenerated cellulose remains in an amorphous form suitable for hydrolysis with high reactivity with cellulase enzymes, and finally
- Residual phosphorous acid has no inhibitory effects on the sequential hydrolysis and fermentation.

Effective performance of COSLIF pretreatment relies on: 1) phosphoric acid concentration to act as a cellulose solvent; 2) sufficient reaction to dissolve biomass but short enough to prevent complete hydrolysis, and 3) reaction temperature below 60°C (Zhu et al., 2009).

Zhang P. et al. (2007), reported that this treatment technology is applicable to a broad range of substrates except for treating softwood Douglas fir. Nevertheless, digestibility of 75% was achieved using this treatment, compared to only 44% digestibility using SO_2 steam explosion as pretreatment. Following Zhang P. et al. (2007), results of recent attempt on COSLIF pretreatment of bermudagrass, reed, and rapeseed followed by simultaneous saccharification and fermentation, validated the performance and high yield of phosphoric acid plus acetone pretreatment in high bioethanol production (Li H. et al., 2009). Since 2007, there have been several comparative studies on optimization of COSLIF pretreatment on different substrates. Some well-known studies in this field were used as guidance for Set Two of experimentations, which presented in the experimental investigation plan (Figure 3-2). These studies are summarized in Table 3-6.

Table 3-6 COSLIF pretreatment design conditions

No.	Substrate	H_3PO_4 Conc. (%)	Incubation		Optimal Condition	Reference
			T (°C)	Time (minute)		
1	*Phragmites australis* (common reed) with different moisture content: 5, 10 and 15 %	85	40, 50, 60	30, 60, 90	85% H_3PO_4 at 50 °C 60 min	Sathitsuksanoh et al. (2009)
2	Corn stover	84	50	20, 45	84% H_3PO_4 45min	Zhu et al., (2009)
3	Flax shives	40.8, 50, 63.5, 77, 86.2	50	9.6, 30, 60, 90, 110.5	86.5% H_3PO_4 110.5 min	Kim & Mazza (2008)
4	Industrial hemp stalks	76, 81, 84, 85.9	40, 50, 60	30, 60, 120	84% H_3PO_4 60min	Moxley et al., (2008)

In view of presented techniques, experiment Set Two was conducted with the following considerations:

1. Pre-assessment on COSLIF performance on SSO Substrate using same procedure described by Zhang P.et al. (2007).
2. Assessment on proper reaction time, among 1, 2, and 3 hours - on the basis of optimized condition presented in Table 3-6, considering similarities among compositional analysis of flax shives (Kim & Mazza, 2008) and corn stover (Zhu et al, 2009) (Moxley et al, 2008).
3. Assessment on modified COSLIF pretreatment followed by fermentation: Ethanol 95% (v/v) was used instead of acetone in the last two wash and centrifugations with organic solvent for phosphoric acid removal, due to the advantages highlighted by Sathitsuksanoh et al. (2009):
    i. Non-toxic to ethanol-producing bacteria,
    ii. more stable and less corrosive than acetone,

iii. acceptable recycling efficiency compared to acetone regarding remaining fraction of organic solvent in the hydrolysate and amorphous cellulose phase,

iv. decrease in consumption of organic solvent.

Figure 3-9 exhibits the general scheme of Set Two experiments (Zhang P. et al., 2007). It is divided into three major steps including cellulose dissolution, washing and centrifugation, and finally enzymatic hydrolysis. The modification made to the cellulose dissolution step is as follows:

- A 50 ml disposable plastic centrifuge tube was substituted by a 250 ml centrifuge bottle,
- In the cellulose dissolution step, the water bath at 90 rpm was replaced by incubator (Thermo-Lab-Line-Barnstead MAX Q MINI 4450-Benchtop shaker) operating at an agitation rate of 150 rpm,
- Centrifugation was carried out by Sorvall RC-5C PLUS super speed floor model, adjusted at 7000 rpm, at 24°C for 10 minutes.

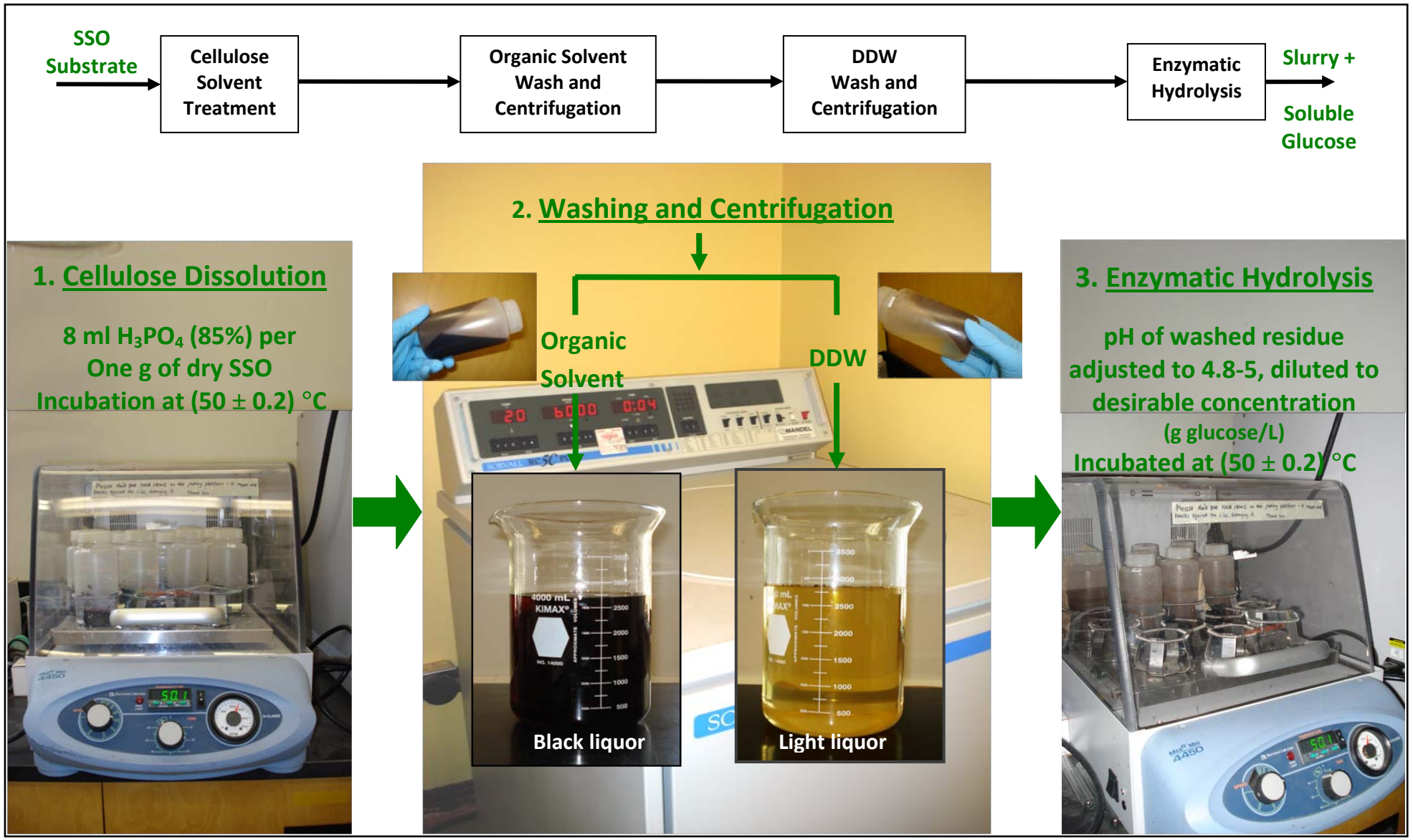

Figure 3-9 General scheme of COSLIF procedure

3.6 Enzymatic Hydrolysis

Enzymatic process is an important bridge with central role between acidic pretreatment and fermentation, as shown in Figure 3-1 on page 36. The pH adjustment is needed 1) before enzymatic hydrolysis due to the pH of enzymes reactivity, optimum range at pH 4.8-5, and 2) after enzymatic hydrolysis, optimum range at pH 5.8-6, which relies on proper pH value for microbial activity in fermentation. The heterogeneous nature of SSO and the multiplicity of enzymes make it difficult to fully understand the interactions of enzymes and substrates. In order to eliminate any complexity involved with the experimental investigation, constant condition of enzymatic digestibility was maintained in both sets of acid pretreatment.

Characteristics of the three commercial enzyme mixtures used in this research are summarized in Table 3-7 (details of measured parameters in appendix II). Celluclast 1.5 L and Novozyme 188 were purchased Sigma Aldrich, and Accellerase 1500 was kindly provided by the Genencore Inc, a Danisco Division, Rochester, NY, USA. Unlike Celluclast 1.5L, Accellerase 1500 has higher activity with lower protein content and does not require ß-glucosidase supplementation to enable more complete degradation of cellobiose.

Table 3-7 Commercial enzymes used in enzymatic hydrolysis

Enzyme	Protein Content (mg/mL)	FPA	ß-glucosidase	Endoglucanase
Accellerase 1500	95[1]	72.5[2]	674 *p*NPG U/g[10]	2638 CMC U/g[12]
Celluclast 1.5L	125[4]	65[3]	74 *p*NPG U/g[6]	797 EG U/g [13]
				1513 CMC U/g [8]
Novozyme 188	115[5]	-	258 CBU/g[11]	39 CMC U/g[9]
			330 *p*NPG U/g[7]	

[1, 2, 3] determined & measured in this research and details are presented in appendix II; [4, 5] reported by (Mohagheghi et al, 2008); [6,7,8,9] reported by (Saha & Cotta, 2006); [10, 11, 12, 13] reported by manufacturer

All prepared hydrolysates were diluted to the defined concentration of solid residue or glucose content as below with pH adjusted at (4.8-5):

- 10% (w/w) Solid loading for hydrolysate obtained from DA pretreatment
- 10 and 20 (g / L) glucose for amorphous cellulosic pellets after washing and centrifugation in COSLIF process

The dilutor includes DDW, the essential amount of ammonium hydroxide (NH_4OH) mainly for pH adjustment and as a source of nitrogen, the volume of sterile enzyme complex, and finally 1% (w/w) of sodium azide (NaN_3) solution (2%) in order to avoid microbial contamination. Despite autoclaving DA samples, the risk of cross-contamination still exists. It may occur through pH adjustment and/or dilution. The growth of organisms can be prevented by adding sodium azide. As shown in Figure 3-10, prepared samples for enzymatic hydrolysis experiments in both treatment methods were incubated in Thermo-Lab-Line-Barnstead MAX Q MINI 4450-Benchtop shaker. The incubator was adjusted at 250 rpm, sufficient to keep solids in constant suspension, and with temperature at 50 ± 0.2°C, for a period of 72hours. Figure 3-10 presents prepared sample for enzymatic hydrolysis including the DA samples with enzymes content, and the COSLIF pretreated samples.

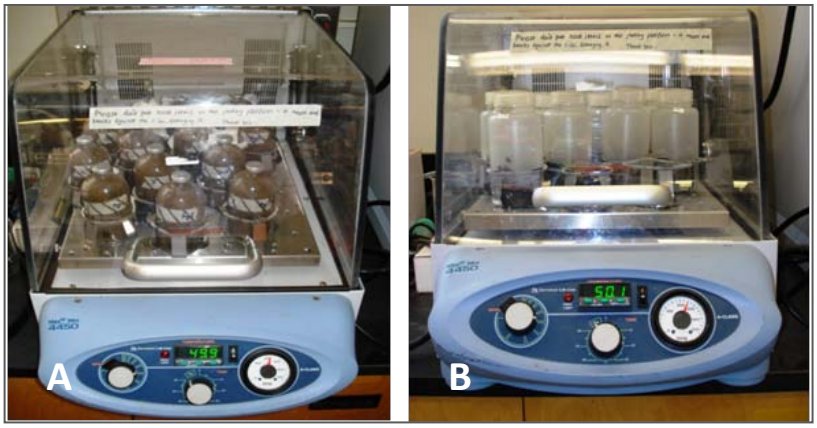

Figure 3-10 Rotary shaker, A) DA samples, B) COSLIF samples

3.7 Fermentation with Bacterial Strain of Interest

The primary focus of this study on acid pretreatment has been the evaluation of glucose and xylose yield and not necessarily the estimation of the final ethanol yield from fermentation. However, fermentation is crucial in investigating the potential inhibitory factors and effects generated during acid and/or enzymatic hydrolysis, which may have significant impact on ethanol biocatalyst. In addition, it is a key stage for evaluating the overall process of cellulosic ethanol production (Yang et al., 2009). Hence, fermentation experiments in this study do not cover all aspect of microbial growth condition associated with metabolic pathway, growth rate and kinetic model on SSO substrate, and efficiency of bioethanol production, except for feasibility of fermentation performance on acid treated hydrolysate under defined laboratory conditions.

3.7.1 Microorganism

Choice of bacterial strain was made based on performance of current ethanologenic strains, as well as content and type of monomeric sugars in SSO substrate. So far, recombinant strains of *Zymomonas mobilis*, developed and evaluated on various agricultural residues, can convert simple sugars to ethanol quicker than other species such as yeasts (Rogers et al., 2007). Therefore, *Zymomonas mobilis 8b* was applied for fermentation. It is a new genomic DNA-integrated glucose- and xylose-cofermenting strain developed and described elsewhere by Zhang et al., (1995), and Mohagheghi et al., (2004). The strain was kindly provided by Dr. Ali Mohagheghi (NREL, Golden, Co, USA) through Material Transfer Agreement (MTA).

General characteristics of *Zymomonas mobilis 8b* including cultivation condition and productivity (Zhang, 2003; Saez-Miranda et al., 2006), as a reference for designing fermentation process, are highlighted in Table 3-8.

Table 3-8 General characteristics of *Zymomonas mobilis 8b*

Characteristics: - Natural fermentative microorganism generally regarded as safe (GRAS) microorganism - Anaerobic, gram-negative bacterium that distinctively uses the Entner-Doudoroff pathway
Cultivation Condition: - **pH** at 5 - 6 controlled with KOH (2N) / NH_4OH/ NaOH - **Temperature** at (30- 37)°C - **Optimal growth condition :** pH 5.8 to 6 , Temperature 33°C
Inoculum Size: Low cell mass formation, 0.03-0.25 OD @ 600 nm (0.01 - 0.085 g cell/L)
Tolerance : - High ethanol tolerance (13% ethanol from 30% glucose) - High acetic acid tolerance (8 to 16 g /L)
Productivity: ➢ High ethanol yield from glucose (95-98% or 0.49-0.50 g/g) ➢ High specific productivity (2 to 6 g ethanol g dcw^{-1}. hr^{-1}) ➢ High sugar uptake rate (up to 10 g glucose g dcw^{-1}. hr^{-1})
By–products : xylitol, lactic acid, glycerol, acetic acid, and CO_2

3.7.2 Inoculum Preparation and Batch Fermentation

Inoculum preparation is divided into two growth stages: revive (pre-seed) and seed. The Microbial growth in pre-seed condition is important due to building cell mass from the cell stock vial. Cell were initially grown in a 15 ml sterile falcon tube (Figure 3-11a) by transferring a stock culture frozen in cryovial to inoculate 9 mL of filter-sterilized nutrient medium containing 100 (g/L) glucose, 20 (g/L) xylose, 10 (g/L) yeast extract, and 2 (g/L) KH_2PO_4.

After 8 hours of static incubation at 33°C, this culture was then transferred at 2.5% (v/v) inoculum level to a 500 mL Erlenmeyer flak containing 400 mL of the same medium (Figure 3-11 b). Incubation at 33°C was continued for 12–16 hours in a Thermo-Lab-Line-Barnstead MAX Q MINI 4450-Benchtop shaker operating at an agitation rate of 150 rpm (Mohagheghi et al, 2004; Saez-Miranda et al, 2006).

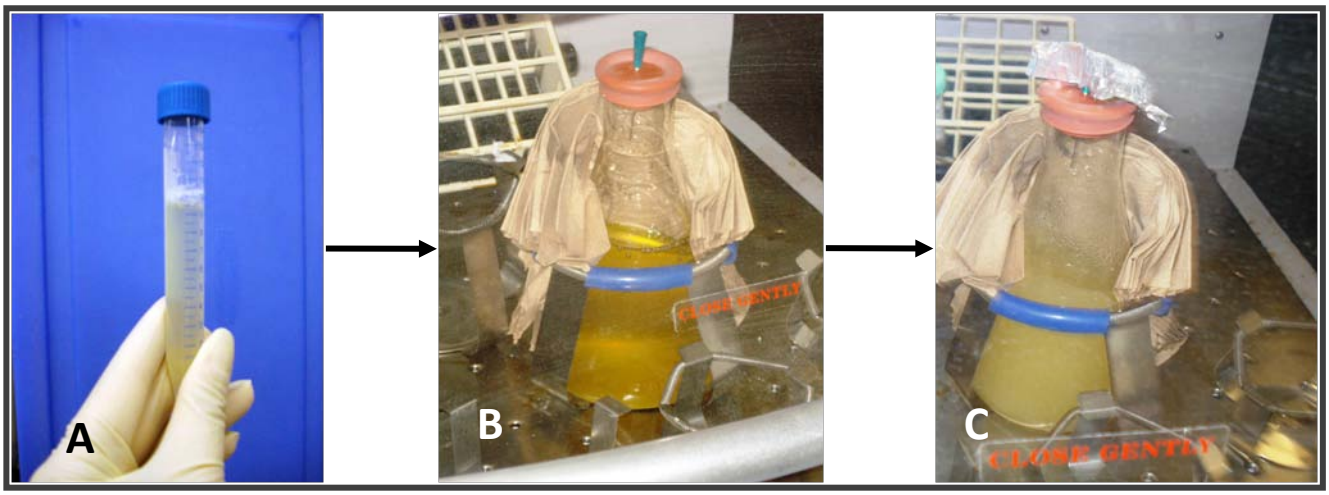

Figure 3-11 Glimpse of inoculum preparation; A) pre-seeding after 8 hr; B) 500 ml flask containing media before inoculation, C) inoculated media - seeding stage after 12 hr

After 12 hours, filter-sterilized hydrolysates obtained from acidic treatment and constructed media (freshly prepared using glucose/xylose ratio of 5:1, the same as in SSO samples) were inoculated at an optical density (OD) of 1 by transferring the specific amount of revive culture from flask of seed media (Figure 3-11c) to fermentor. Figure 3-12 presents the working area for inocula and samples prior to fermentation.

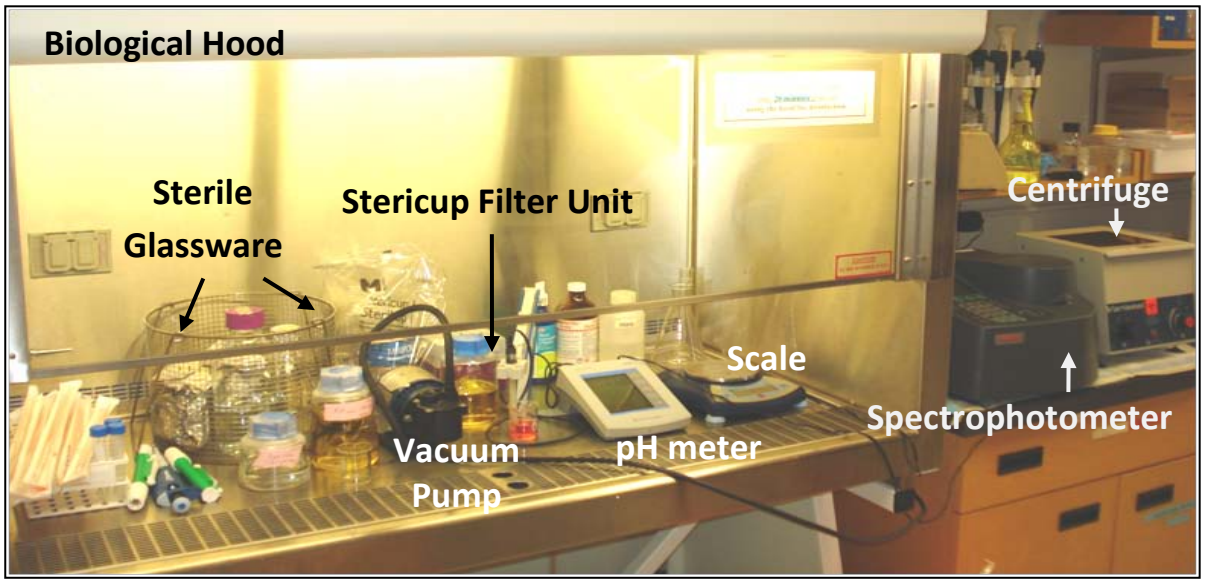

Figure 3-12 Working area for inoculum preparation and fermentation

Results and Discussion

This section presents summative analysis on practical and quantitative approach through implemented acidic treatments followed by enzymatic hydrolysis and fermentation to convert low-value lignocellulosic waste materials into high-value product that include yield of fermentable sugar to bio-ethanol.

4.1 Compositional analysis of SSO

Summarized quantitative assessment on the composition of the SSO is presented in Table 4-1. The mass balance based on total solid is given in Figure 4-1 (more details in appendix I). As shown below, approximately, two- thirds of the original sample is moisture. Key polymeric sugars content data in oven-dried SSO was kindly provided by MBI International. The results in triplicate showed that the SSO sample includes on average 27% glucan, 5.4% xylan, 1.2% arabinan, 5.7% mannan and 1.2% galactan.

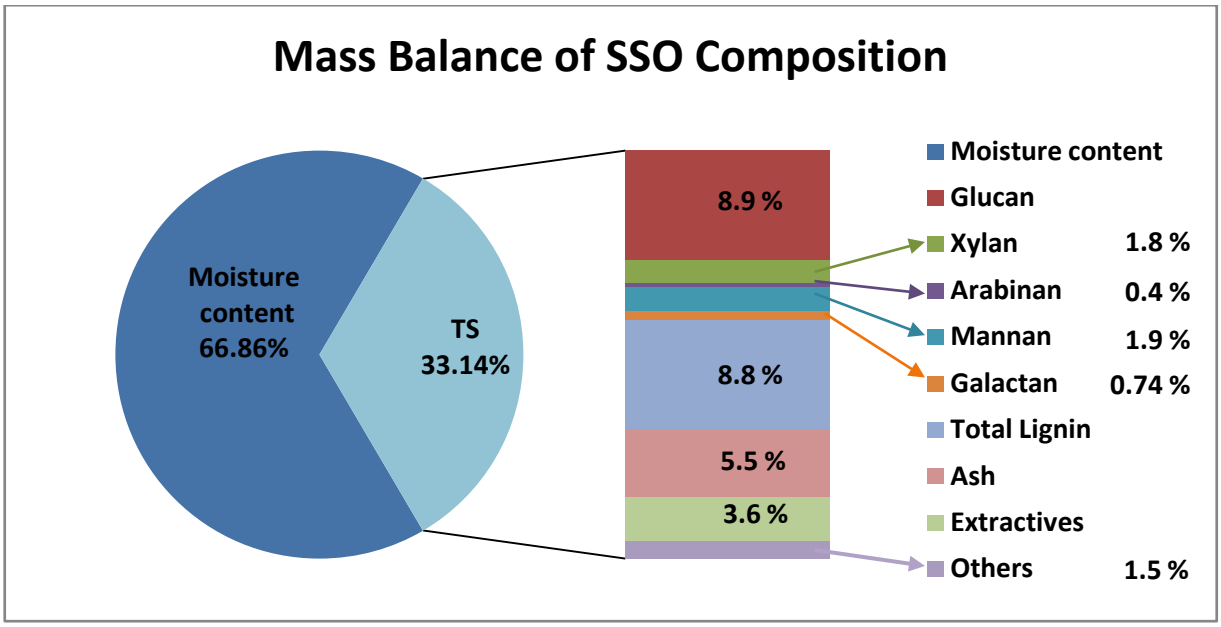

Figure 4-1 Mass balance of the SSO composition

This sample with acidic nature (pH at 5 -5.5) has the lowest content of the food waste. Unlike the quality of kitchen waste that must to be rich in starch, free sugar and volatile fatty acids, these compound were not significant and detectable in the tested SSO sample.

Table 4-1 Compositional analysis of SSO

Parameters	Value
A. Physical Properties	
Biomass as received	
pH	5 @ 25°C
TS	33.14%
Moisture content	66.86%
VOC per dry mass	28.00%
Ash per dry mass	5.14%
Oven-dried and homogenized biomass	
pH	5.5 @ 25°C
Moisture content	6.60%
TS	93.40%
VOC	83.40%(TS)
Ash	16.60% (TS)
B. Structural Carbohydrate and Lignin (per oven-dried and homogenized biomass)	
Starch	NS
Free Sugar	NS
Glucan	26.80%
Xylan	5.40%
Arabinan	1.20%
Mannan	5.70%
Galactan	2.20%
Total sugars	41.26%
Acid Insoluble Lignin (AIL)	25.40%
Acid Soluble Lignin (ASL)	1.20%
Total Lignin	26.60%
VFA (Acetic acid, Lactic acid, and Formic acid)	NS
C. Others	
TKN*	5450 µg/g
Extractives	11%
Digestibility	12.70%
Biodegradability	82%

NS= not significant ; *TKN was reported by AMEC Earth & Environmental Ltd.

Figure 4-2 presents a quick test as a comparison between non-sterilized (vial A) and sterilized (vial B) substrate. Both presented vials have the same concentration of SSO. Vial A shows microbial activity while liquid phase in vial B is transparent.

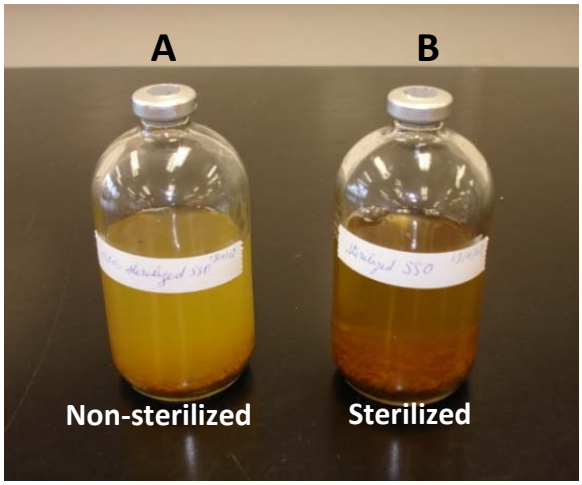

Figure 4-2 Substrate sterility check for microbial activity

In view of the above facts and characterization results, the following calculation hypothesizes that during pretreatment of SSO with the ATC thermal screw machine, a high degree of organic matter degradation occurs:

Total Glucose = Free Glucose + Polymeric Glucose (originated from cellulose and starch)

Then, in one kilogram (kg) of SSO:

Woodchips = 20% = 200 grams (g), Moisture content= 66% = 660 g,

Total glucose = 100.98 g glucose (29.7% on dry matter basis, calculated from glucan)

Dry Solid=340 g including 200 g woodchips and 140 g other waste material (mostly supposed to be food waste)

Theoretically, the minimum cellulose content (glucose) in woodchips is 35% and maximum 40-45% (Kumar et al, 2009). With regards to minimum content of cellulose, starch can be calculated as follows:

100.98 g glucose = [(200 * 35)/100] + starch, Starch = 28.98 g which is equal to 8.5% of dry SSO, (or 3 % of one kg SSO)

The above calculation suggests that total glucose (29.7%) content in dry SSO is comprised of 21.2% glucose from woodchips and 8.5% from food waste. In support of above statement about glucose, low xylose and Total Kjeldahl Nitrogen (TKN), and the absence of starch in the SSO substrate, indicate that each component of food waste including starch, cellulose and protein can be degraded by some members of the microbial consortia under a suitable environmental conditions such as natural anaerobic processes, (Han & Shin, 2004; Gea et al., 2004; Bayer et al., 2007). Apparently, about 67% moisture, surrounding and impregnating the solids, is sufficient to facilitate biodegradation of organic matter in liquid phase (de Guardia et al., 2002). Finally, in spite of 82% biodegradability, 12.7% digestibility of non-pretreated SSO substrate validates the highly recalcitrant nature of lignocellulosic fraction which relies on type and percent of the woodchips in composting process.

4.2 Effect of Dilute Acid Pre-treatment on SSO Substrate

SSO samples after pretreatment with dilute sulfuric acid at 0.5, 1.0 and 1.5% (w/w) for 15, 30 and 60 minutes were analyzed for sugar content and inhibitory compounds including furfural and HMF. Results are summarized in Table 4-3 (page 61), and indicate that pretreatment condition was not sufficient enough to solubilize cellulose and hemicelluloses. Consequently, sugar degradation by-products were not formed. In fact, dilute sulfuric acid pretreatment did not greatly remove lignin.

However, based on the DA results, condition of pretreated samples with 1% (w/w) H_2SO_4, at 121°C for 1 hour was chosen for enzymatic treatment. Accellerase 1500 at different dosage rate was used for saccharification of cellulose and hemicelluloses portion in the DA hydrolysate, as given in the Table 4-2.

Table 4-2 Different dosage rate of enzyme used in saccharification

Samples no.	(ml) Enzyme / (g) Glucan		FPU per g Glucan		Protein content mg
	Accellerase 1500	Novozyme 188	Accellerase 1500	Novozyme 188	
1	0.21	-	15	-	20
2	0.21	0.14	15	42	36
3	0.34	-	25	-	33.3
4	0.5	-	35	-	49

Efficiency of released glucose in enzymatic conversion of pretreated cellulose was between 8% and 10%. In fact, summarized results of enzymatic treatment efficiency in Figure 4-3 confirm ineffectiveness of DA pretreatment. Furthermore, adding ß–glucosidase (Novozyme 188) did not significantly change the performance of Accellerase 1500, and highlights the unique product formulation of this enzymes complex. This is similar to the results of conducted research on Accellerase family of enzymes conducted by Ko et al., (2009). They confirmed that use of Accellerase does not require b-glucosidase supplementation to enable more complete degradation of cellobiose unlike Celluclast 1.5L. Finally, the percent glucan conversion was not considerable and comparable to similar DA condition reported by Saha et al., (1999) and Yang et al., (2009). Typically, DA pretreatment is a chemical process governed by five parameters: temperature, residence or hydrolysis time, acid concentration, pressure, and solid loading. In order to increase the efficiency of DA pretreatment, laboratory available variables such as dilute acid concentration and residence time should be subject to further investigation.

Table 4-3 Dilute sulfuric acid pretreatment with 1% (w/w) H_2SO_4

Acid conc. % (W/W)	Acid (g) per biomass (g)	Released Sugar																	
		15 min						30 min						60 min					
		pH (aver.)	Glucose		Xylose		Total: 5 type of sugars (g/L)	pH (aver.)	Glucose		Xylose		Total: 5 type of sugars (g/L)	pH (aver.)	Glucose		Xylose		Total: 5 type of sugars (g/L)
			Conc. (g/L)	Yield (%)	Conc. (g/L)	Yield (%)			Conc. (g/L)	Yield (%)	Conc. (g/L)	Yield (%)			Conc. (g/L)	Yield (%)	Conc. (g/L)	Yield (%)	
0	0	5.78	0.716	1.36	0.054	0.50	3.552	5.95	0.824	1.57	0.057	0.53	2.193	5.98	0.863	1.64	0.053	0.49	2.128
0.5	0.033	5.15	0.886	1.69	0.164	1.52	2.473	5.35	0.838	1.60	0.052	0.48	2.359	5.51	0.968	1.84	0.050	0.46	2.390
1	0.067	4.3		1.56	0.088	0.81	2.593	4.54	0.902	1.72	0.055	0.51	2.865	4.71	0.996	1.9	0.055	0.51	2.896
1.5	0.1	2.7	1.002	1.91	0.129	1.19	3.691	2.94	0.613	1.17	0.12	1.11	3.450	3.15	1.291	2.46	0.197	1.82	4.729

Conc., concentration; aver. average

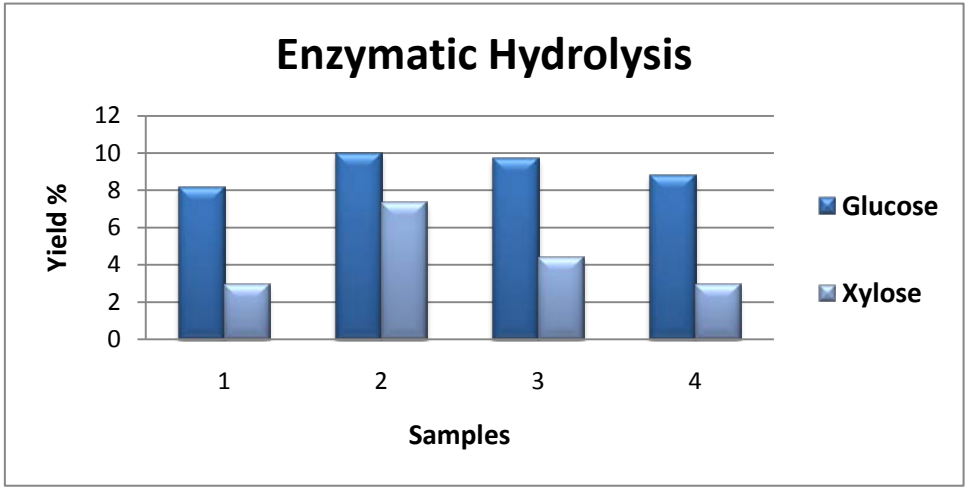

Figure 4-3 Enzymatic hydrolysis of DA pretreated SSO by 1% (w/w) H_2SO_4

In summary of the above facts, new sets of SSO samples with 4% (w/w) H_2SO_4 at 1 and 4 hours, and 1% (w/w) for 2 hours, were prepared and autoclaved at 121°C as shown in Table 4-4.

Table 4-4 DA pretreatment with 4% and 1% (w/w) H_2SO_4 with different residence time

Samples	Autoclave time (hr)	pH	g acid per g biomass	Glucose		Xylose	
				Ave. Conc. (g/L)	Yield (%)	Ave. Conc. (g/L)	Yield (%)
4% acid	4	0.885	0.267	0.030	0.06	0	0
4% acid	1	0.94		3.108	5.92	4.108	38.00
1% acid	2	3.24	0.06	1.347	2.57	0	0
1% acid	1	4.71		0.996	1.90	0.055	0.51

As seen from the Table 4-4, increasing the residence time enhanced the loss of sugar. On the other hand, increasing the DA concentration made a drastic decrease in pH value and it could release more sugar. In this case, low pH means more volume of base (salt) was needed to adjust the pH for enzymatic treatment, which led into salt inhibition in biological process. As an ultimate attempt, the DA pretreatment with 2% (w/w) H_2SO_4 followed by enzymatic pretreatment with cellulase enzymes loading at 60 FPU per g glucan, was conducted. Two separated and duplicated sets of SSO samples pretreated by 2% (w/w) H_2SO_4 were used for enzymatic hydrolysate, (one with Accellerase 1500, and the second one with Celluclast 1.5L). As shown in Figure 4-4, efficiency of sugar production is similar to previous results presented in Figure 4-3.

These DA pretreatment did not present acceptable results for further investigation through fermentation. Detailed results of DA pretreatment on the SSO are presented in appendix III.

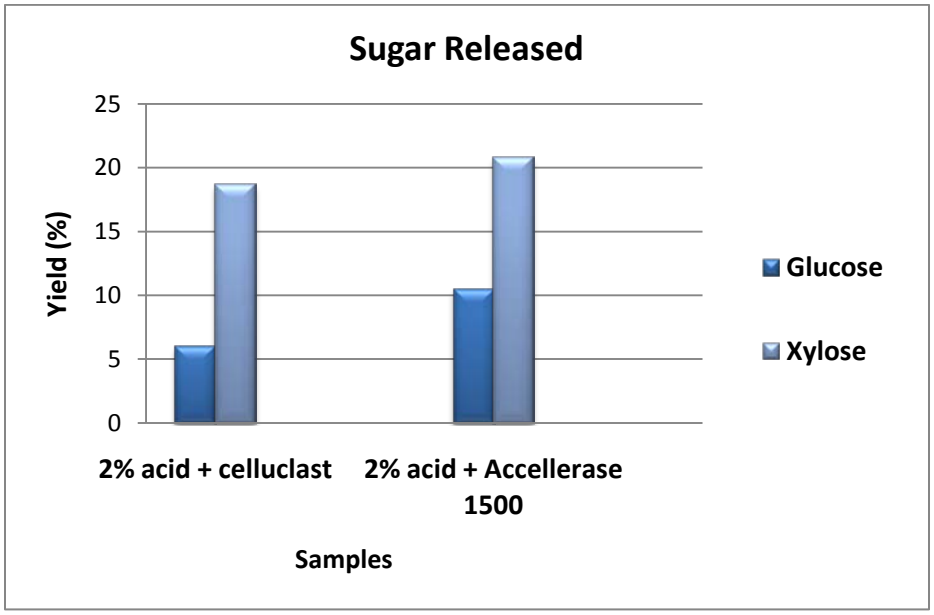

Figure 4-4 Enzymatic hydrolysis of DA pretreated SSO by 2% (w/w) H_2SO_4

4.3 Effect of COSLIF on SSO Substrate

The new lignocellulose pre-treatment featuring modest reaction conditions, 50°C and atmospheric pressure, was demonstrated to fractionate lignocellulose portion of SSO to amorphous cellulose, hemicellulose, lignin, and acetic acid by using: 1) concentrated phosphoric acid (85%), as a non-volatile cellulose solvent; 2) acetone and ethanol, highly volatile organic solvents; and 3) Distilled Deionized Water (DDW) (Zhu et al., 2009).

4.3.1 Pre-assessment on COSLIF Performance on SSO Substrate

Two gram of oven-dried SSO substrate was placed in a 250 ml centrifuge bottle and mixed with 16 ml concentrated phosphoric acid (85%) with a glass rod. Then slurry was incubated at 50°C for 3 hours. After the reaction, slurry was washed with 40 ml cold acetone and centrifuged. Then

centrifugation with 160 ml acetone was repeated three times and black liquor (supernatants) was discarded. The pellet was washed with DDW and centrifuged. Finally, the residual pellets, containing amorphous cellulose, were neutralized to pH 4.8-5 by DDW and NH_4OH, and then diluted to 10g glucan /L for enzymatic hydrolysis. Accellerase 1500 and Celluclast 1.5 L loading at 60 FPU per g glucan were tested on the obtained slurry from COSLIF pretreatment. As expected, Accellerase 1500 with yield value of 65.77% presented higher glucose than Celluclast 1.5L with value of 64% (Figure 4-5).

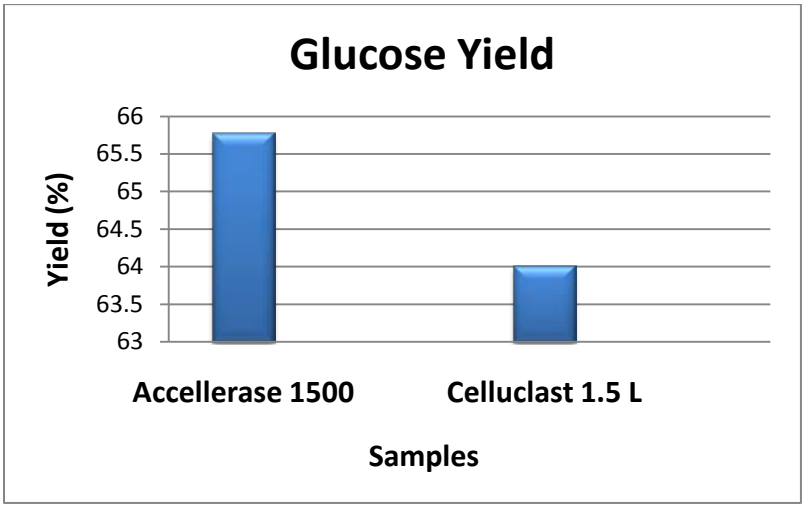

Figure 4-5 COSLIF pretreatment at 50°C for 3 hr

4.3.2 Assessment on Proper Reaction Time

The same procedure, as described in subsection 4.3.1, was implemented with different incubation times of 1, 2, and 3 hours. Treated slurry after each incubation time was tested for glucose content. Based on the results (Figure 4-6), the lack of glucose released from the slurry validated that cellulose is in the amorphous state. When treated with different enzymes, these slurries released both cellobiose and glucose. Since cellobiose is an intermediate compound for cellulose conversion to glucose, and glucose concentrations are varied for each treatment time, optimal parameters (low cellobiose remaining, high glucose released) were selected for each enzyme.

This evidence was further clarified as samples for each hydrolysis were taken initially and after 72 hours, and these measured concentrations were significantly different. The exhibited results were consistent with pre-assessment mentioned in subsection 4.3.1.

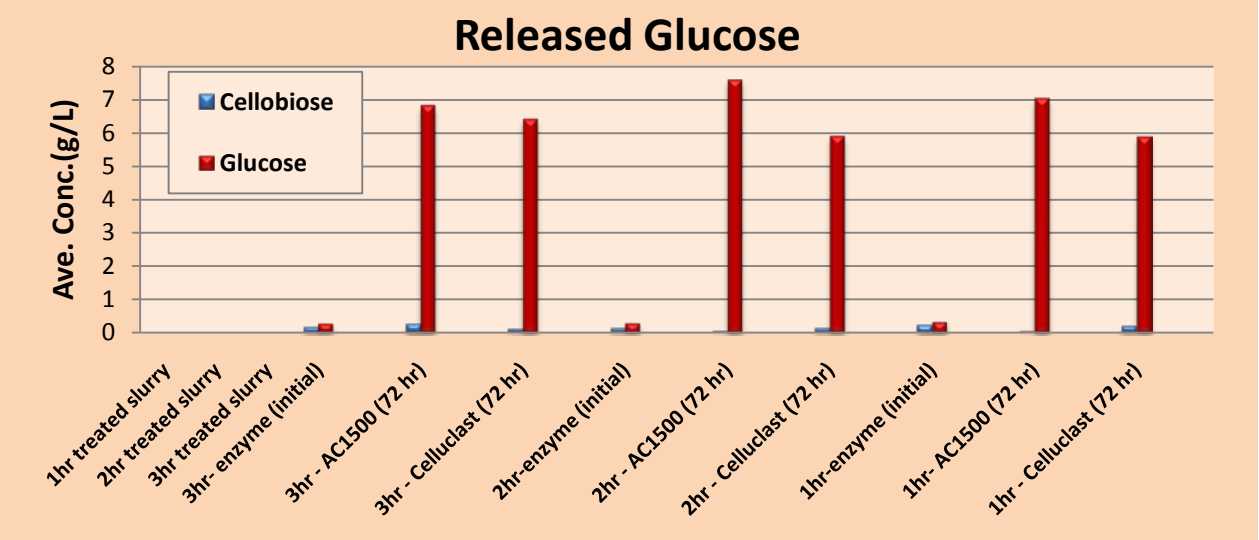

Figure 4-6 COSLIF pretreatment results followed by enzymatic hydrolysis

The highest sugar yields after enzymatic hydrolysis was attributed to little, or no, sugar degradation during early steps and the high enzymatic cellulose digestibility. Figure 4-7 presents the comparison of pretreated samples.

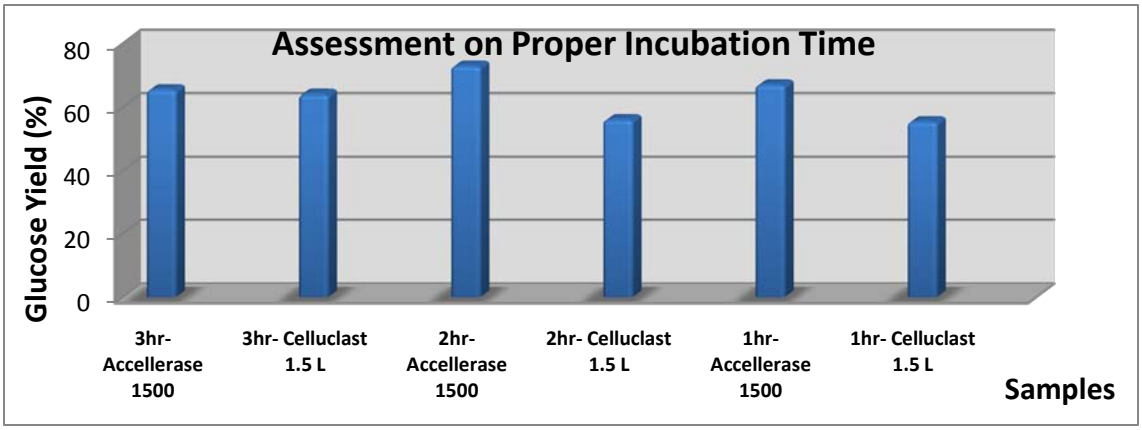

Figure 4-7 Comparison of glucose yield in different incubation time

It was observed that the appearance of COSLIF pretreated residues with different incubation time were slightly different. Inevitably, for samples incubated for 1, 2 and 3 hours, partial loss of particles occurred during the wash and centrifugation process, and probably influenced the final glucose yield of the process. This phenomenon highlights the physics of particle-particle adhesion and interaction mechanism for the crystalline-to- amorphous transformation.

To summarize briefly, COSLIF pretreated biomass under 2 hours incubation and enzymatic digestion with Accellerase 1500 loading at 60 FPU per g glucan, presented higher glucose yield (70%) than other options (Figure 4-7). This hydrolysate was selected for further investigation in the fermentation process

4.3.3 Modified COSLIF Pretreatment and Fermentation

Six samples of 5 g SSO substrate in each of its content were prepared based on the selected results from the above section. During phosphoric acid removal, after first wash and centrifugation with cold acetone, acetone was replaced by ethanol 95% for subsequent wash and centrifugation. At the end, residual pellets were diluted to 20 g glucan/L. The average glucose yield among six samples was 89.5%, nearly 20% increase from the highest yield of the previous experiment (Figure 4-8). However, as observed from the figure, there is substantial variation between the samples, from glucose yield of 84% in sample 1 to a yield of 94% in sample 2. This may be attributed to the observed loss of particles during wash and centrifugation.

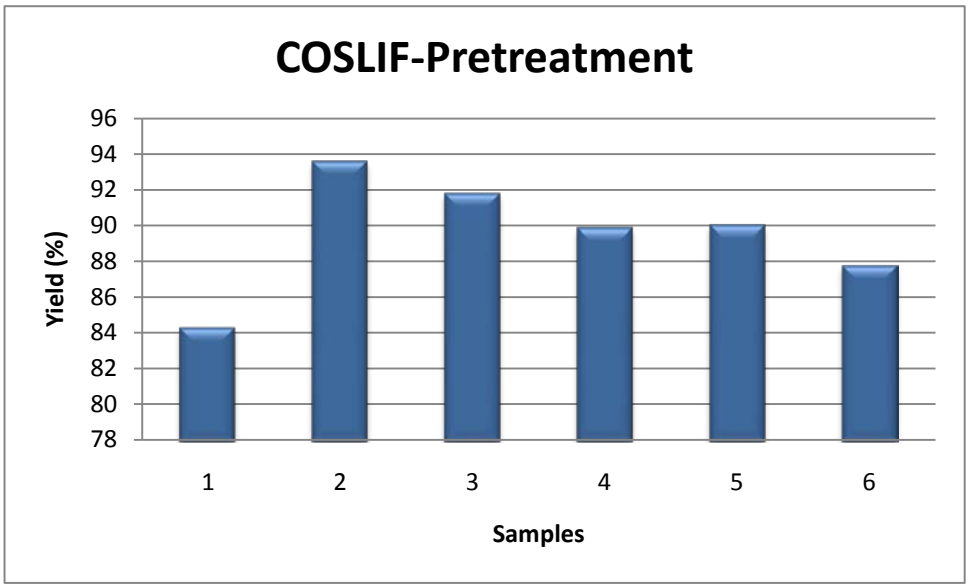

Figure 4-8 Glucose yield after modified COSLIF pretreatment

Three samples were randomly chosen for Separate Hydrolysis and Fermentation process (SHF). Prior to the fermentation, enzymatic hydrolysates were sterilized by filter sterile. At the same time, a culture media based on glucose to xylose ratio in SSO composition (5:1) was constructed. Three 125 ml Erlenmeyer flasks were filled with 50 ml of constructed media. Fermentation in those six batch culture samples were carried out with *Zymomonas mobilis 8b.*

After 24 hours of inoculation, sugar content as a measure of substrate utilization in each batch culture (Erlenmeyer flask) was quantified and results are presented in Table 4-5 and 4-6. In the constructed model, glucose (100%), and xylose (40%) were utilized while microbial growth in COSLIF pretreated samples, with approximate 17.6% glucose consumption, was not significant, as expected.

Table 4-5 Sugar content after 24 hrs fermentation in constructed model media

Constructed Samples	Before Fermentation		Remained Glucose After 24 hrs Fermentation			
	Glucose (g/L)	Xylose (g /L)	Glucose (g/L)	Xylose		
				Conc. g/L	Average	
					g / L	%
CX1	100	20	0	9.43	7.95	39.75
CX2			0	4.91		
CX3			0	9.52		

Table 4-6 Glucose content after 24 hrs fermentation in COSLIF pretreated hydrolysate

Randomly Selected Samples	Glucose before Fermentation (g/L)	Remained Glucose After 24 Fermentation (g/L)	Utilized sugar (%)
2	19.83	15.29	22.89
4	19.09	16.23	14.98
6	18.7	15.92	14.87

Low bacterial activity in fermentation of SSO hydrolysate may attribute to many factors including: longer lag phase for *Zymomonas mobilis 8b* as the adaptation time to growth condition, low growth rate on SSO hydrolysate, unavoidable contamination during sample preparation despite sterilizing hydrolysates before fermentation, and finally, lack of micronutrient (nitrogen and phosphorus). Detailed results of COSLIF pretreatment on the SSO are presented in appendix IV.

4.3.4 Scanning Electron Microscopy (SEM)

SEM images from three conditions of oven–dried SSO substrate, as a qualitative comparison, with three magnitudes, are presented in Figure 4-9.

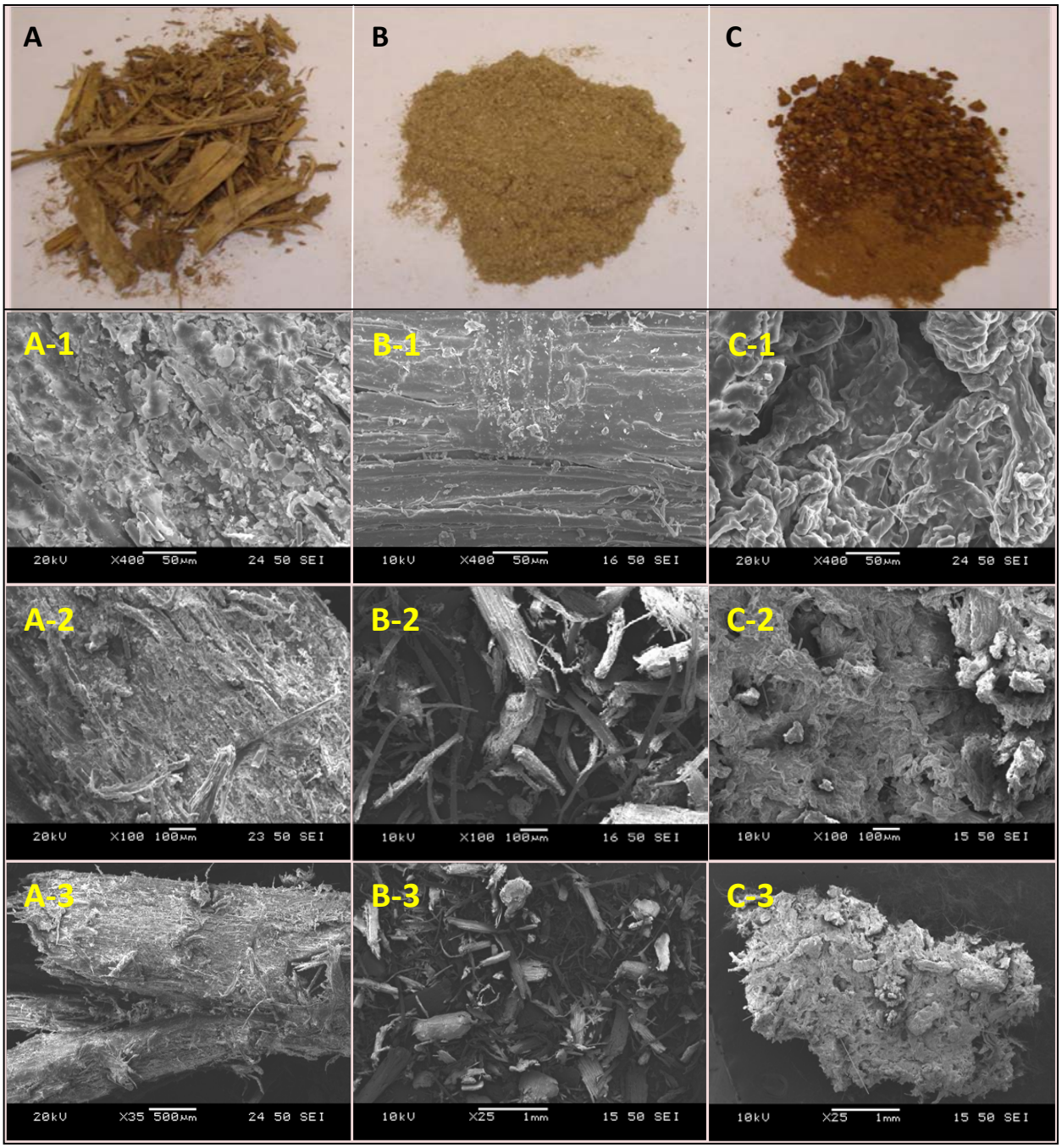

Figure 4-9 Scanning electron microscopic of pretreated SSO

These images show the appearance of SSO before grinding (A), after grinding (B), and after COSLIF pretreatment (C), and each one with three magnifications as describe below:

- Series 1 × 400, including A-1, B-1, and C-1
- Series 2 × 100, including A-2, B-2, and C-2
- Series 3: A-3 × 35, B-3 and C3 × 25

Each pretreatment process (physical and chemical) has altered the supramolecular structure of the SSO biomass. The SEM images from A and B present changes in particle size. While C shows dramatic changes in which all fibrous structures (cellulose, hemicelluloses and lignin) in a COSLIF pretreated lignocellulosic sample were completely disrupted (Figure 4-10). These images are reasonably comparable with similar studies on avicel and corn stover shown in Figure 4-10 which draws attention to the resemblance of COSLIF pretreated SSO to them.

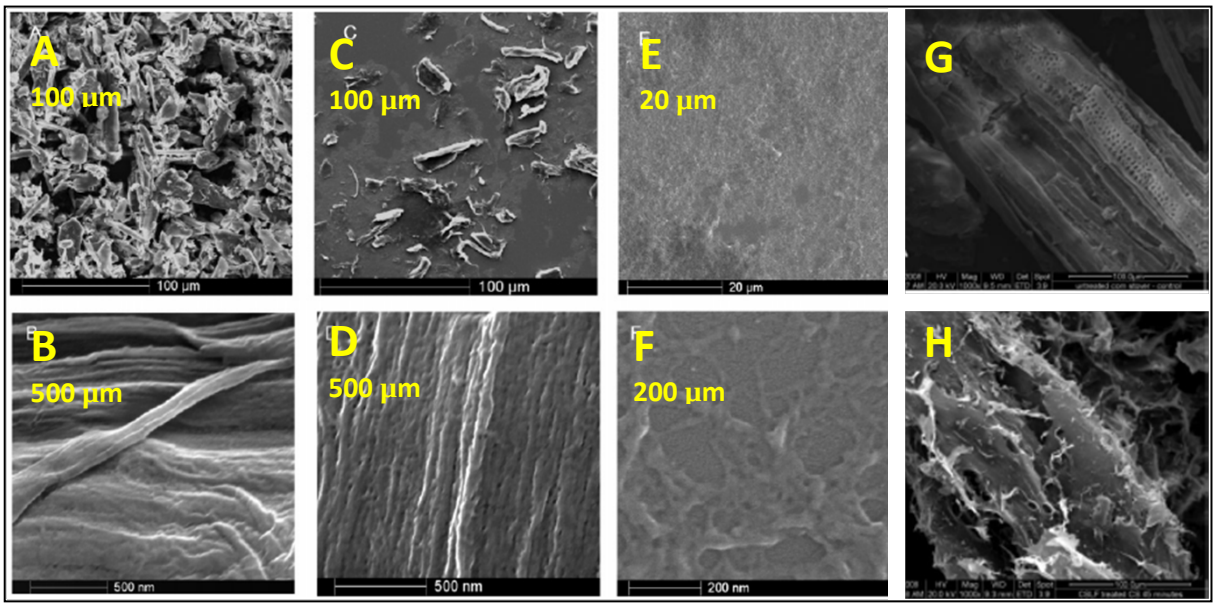

Figure 4-10 Referenced SEM images compared with pretreated SSO

Figure 4-10 exhibits the SEM images of two conducted researches on COSLIF pretreatment. Zhang et al. (2006) presented the structural difference on avicel as a pure cellulose (A and B), avicel treated by 77% phosphoric acid (C and D), treated by 83% phosphoric acid (E and F). Similarly, Zhu et al. (2009) demonstrated corn stover substrate (G) before pretreatment and (H) after COSLIF pretreatment with 84% phosphoric acid.

Conclusions and Scope for Future Work

5.1 Conclusions

This study investigated:

1. Composition of thermally-processed source-separated organic waste by the ATS thermal screw machine
2. Feasibility of two acidic pretreatments, followed by enzymatic hydrolysis
   - Dilute sulfuric acid at 121°C and 16.2 psi
   - Concentrated phosphoric acid (85%) with organic solvent, acetone and ethanol, known as cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF)
3. Comparing two different cellulases, commercial enzyme complexes
4. Evaluation of selected pretreatment through fermentation by selected strain of bacteria

Results of compositional analysis (i.e. low xylose and undetectable amounts of starch) confirmed the continuous microbial degradation of food waste components including starch, cellulose and protein, under a suitable environment such as natural anaerobic conditions facilitated by sufficient moisture content, surrounding SSO feedstock. Furthermore, quantity and type of added woodchips can significantly alter the pretreatment results and production of fermentable sugars due to the strength of recalcitrant lignocellulosic biomass involved in the structure of chemical bonds among cellulose, hemicelluloses and lignin, which differs in any single lignocellulosic matter. For instance, Douglas fir, a softwood, has been using for furniture, flooring, cabinet, and doors. This type of wood is much harder in the mechanical sense, and likely exhibits more resistance to fractionation and enzymatic hydrolysis than the hardwoods.

Dilute sulfuric acid, at 121°C and 16.2, hydrolyzes SSO substrate with 15% solid loading followed by enzymatic hydrolysis with 10% solid loading and different dosage of commercial biocatalysts, Accellerase 1500 and Celluclast 1.5L, was not effective or severe enough to alter the lignin shealth and produce fermentable sugar. The maximum efficiency was 10% glucose yield while concentrated phosphoric acid with acetone (COSLIF) presented a significantly higher glucose yield (70%). The efficiency of COSLIF pretreatment was improved by 20% with modified process using ethanol instead of acetone. On average glucose yield from the modified process was 89.5%. SEM images of COSLIF pretreated SSO substrate were compared with similar studies and validated the obtained results of altered crystalline cellulose to amorphous.

Accellerase 1500, as a new generation of commercial enzyme complex utilized in this study, is preferred over Celluclast 1.5L, the first generation of cellulases, as it is a more complex mixture of enzymes, containing multiple enzyme activities, mainly exoglucanase, endoglucanase, β–glucosidase, and hemicellulase. It exhibits higher activity and efficiency with lower protein content.

Efficiency of COSLIF pretreatment was evaluated by conducting two sets of batch culture fermentation with selected strain, *Zymomonas mobilis 8b*, on enzymatic hydrolysate obtained from COSLIF pretreatment and constructed model of SSO substrate. In the constructed model, after 24 hours, 100% of glucose and 40% of xylose were consumed. In the enzymatic hydrolysate obtained from COSLIF, on the other hand, the fermentation slowly processed while 75% of glucose remained untouched after the same period of time. It can be concluded that this strain of bacteria favours glucose over xylose. As described before, xylose was separated from the SSO hydrolysate in the COSLIF process. Therefore, due to the lack of xylose in the system,

the above conclusion for the preference of glucose over xylose cannot be extended to the fermentation of enzymatic hydrolysate of SSO. Low efficiency in fermentation of SSO hydrolysate may attribute to longer lag phase for the adaptation of the strain to growth condition, unavoidable contamination during sample preparation (despite sterilization of hydrolysate before fermentation), and lack of micronutrient (N and P).

It was observed that the nature of COSLIF pretreated residues with different incubation time were slightly different. Inevitably, for samples incubated for one, two, and three hours, partial loss of particles occurred during the wash and centrifugation process, and probably influenced the final glucose yield of the process. This phenomenon highlights the physics of particle-particle adhesion and interaction mechanism for the crystalline–to–amorphous transformation.

5.2 Scope for Future Work

It is foreseen that the results of this study will reinforce the understanding of the Simultaneous Saccharification and Co-Fermentation (SSCF) process. However, a comprehensive study of the nature of lignocellulosic degradation is recommended in order to decipher the complex pathway of this degradation. To summarize, the recommendations for the future works and directions are listed as below:

1. Investigation of DA pretreatment at higher temperature and pressure facilitated by proper reactor.
2. Optimization of DA pretreatment followed by enzymatic hydrolysis based on temperature, pressure, acid concentration, solid loading, and various enzymes doses. It

should be mention that DA pretreatment on SSO waste at high temperature is not feasible in industrial application.

3. Optimization of COSLIF pretreatment condition under different temperature, enzyme loading, and incubation time

Optimization on pretreatment method should be given to the feasibility of the proposed technology as applied to SSO waste.

4. Detailed investigation on COSLIF pretreatment which is needed to quantify soluble and insoluble compounds in solvent streams of wash and centrifugation including extracting hemicellulose from light liquor for fermenting xylose. Furthermore, evaluation on macronutrient reduction during wash and centrifugation is needed.
5. Detailed investigation on the nature of *Zymomonas mobilis 8b* including kinetic model of microbial growth, inhibitory factors.
6. Performance of *Zymomonas mobilis 8b* on SSO substrate, pretreated with either DA or COSLIF method, should be investigated on the efficiency of ethanol production using a proper bioreactor.

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Appendix I

Compositional Analysis

A. Total Solid, Volatile Solid (VS) known as volatile organic compounds (VOC), and ash

Table I-1 TS, VS, and ash in SSO Biomass as received

No	Dish (g)	Dish and sample (g)	Net weight of Sample (g)	After 105° C					After 550°C				
				Dish and sample (g)	TS (g)	TS (%)	Moisture (g)	Moisture (%)	Dish and Sample (g)	Ash (g)	Ash (%)	VOC (g)	VOC (%)
1	31.353	34.354	3.001	32.369	1.016	33.86	1.985	66.145	31.616	0.263	8.764	0.753	74.114
2	30.489	33.503	3.014	31.493	1.004	33.31	2.010	66.689	30.658	0.169	5.607	0.835	83.167
3	35.658	38.662	3.004	36.638	0.980	32.62	2.024	67.377	35.829	0.171	5.692	0.809	82.551
4	32.223	35.223	3.000	33.228	1.005	33.5	1.995	66.500	32.390	0.167	5.567	0.838	83.3831
5	35.964	38.980	3.016	36.941	0.977	32.39	2.039	67.606	36.171	0.207	6.863	0.77	78.813
Average			3.007	-	0.996	33.14	2.011	66.863	-	0.195	6.499	0.801	80.406

Table I-2 TS, VS, and ash in oven-dried and homogenized SSO

No	Dish (g)	Dish and sample (g)	Net weight of Sample (g)	After 105° C					After (575 ± 25)°C				
				Dish and sample (g)	TS (g)	TS (%)	Moisture (g)	Moisture (%)	Dish and sample (g)	Ash (g)	Ash (%)	VOC (g)	VOC (%)
1	35.964	36.966	1.002	36.904	0.940	93.81	0.062	6.188	36.117	0.153	16.277	0.787	83.723
2	32.223	33.231	1.008	33.163	0.940	93.25	0.068	6.746	32.382	0.159	16.915	0.781	83.085
3	30.839	31.843	1.004	31.775	0.936	93.23	0.068	6.773	30.988	0.149	15.919	0.787	84.081
4	35.658	36.661	1.003	36.595	0.937	93.42	0.066	6.580	35.814	0.156	16.649	0.781	83.351
5	31.353	32.359	1.006	32.291	0.938	93.24	0.068	6.759	31.514	0.161	17.164	0.777	82.835
Average				-	0.940	93.39	0.066	6.609	-	0.156	16.585	0.783	83.415

B. Compositional analysis based on dry weight of , reported by MBI International

Table I-3 Sugar content of SSO

Samples	Concentration (%)					Average (%)					TOTAL
	Glucan	Xylan	Galactan	Arabinan	Mannan	Glucan	Xylan	Galactan	Arabinan	Mannan	Sugars (%)
Wood standard sample # 3	41.26	12.76	0.98	0.53	2.16	41.10	12.76	0.95	0.55	2.15	57.52
Wood standard sample # 4	40.95	12.77	0.92	0.56	2.13						
Ryerson University #5	26.25	4.60	2.00	1.06	5.69	26.80	5.39	2.23	1.16	5.68	41.26
Ryerson University #6	26.26	4.87	2.08	1.04	5.71						
Ryerson University #7	27.90	6.70	2.62	1.38	5.65						

In samples' preparation for DA and COSLIF experiments, glucan and xylan conversion to glucose and xylose were based on the following equations (Schwietzke et al, 2009):

- $W_{Glucose} = W_{Glucan} \times (180/162)$,
- $W_{Xylose} = W_{Xylan} \times (150/132)$,

C. Free Sugars

Table I-4 Sample's description

no	Parameter	Value
1	Working Volume	50 g (42.5 g liquid including DDW and moisture + 7.5 g Dry SSO)
2	Solid loading	15%
3	Moisture content	6.6%

Table I-5 Free sugar

Sample no	pH (25°C)	Glucose			Galactose			Mannose			Total
		Area	Conc. (g/L)		Area	Conc. (g/L)		Area	Conc. (g/L)		Sugars
		(µV*sec)	Sample	Ave.	(µV*sec)	Sample	Ave.	(µV*sec)	Sample	Ave.	(g/L)
FS1	5.5	717848.2	0.906	0.969	61099	0.176	0.199	1073277	1.309	1.312	2.479
FS2	5.5	839964.1	1.051		120720	0.271		1135490	1.380		
FS3	5.5	806168.4	1.011		63847.6	0.181		1087056	1.325		
FS4	5.5	719047.4	0.907		53652.77	0.165		1005771	1.232		
Average in 100 g SSO		**0.6 g**			**0.11 g**			**0.74 g**			**1.4 g**

D. Determination of lignin

Table I-6 Sample's description

Date of test	26-Jul
	11-Sep
Weight of Sample	0.3 g
Moisture content	6.61%
Weight of Overn-dried sample	0.28g

Table I-7 Acid insoluble ligning (AIL)

No	Weight		% AIL
	after 105°C	after (575± 25)°C	
1	34.494	34.427	23.929
2	34.499	34.431	24.286
3	34.496	34.423	26.071
4	28.865	28.793	25.714
5	28.455	28.380	26.786
Average			25.357

Calculation formula

% acid insoluble lignin=[(W1-ODW)/W2×(T105)/100]×100

Oven Dry Weight (ODW) = Weight after 105°C, W_2 = Weight after (575± 25) °C, W_3 = Weight of sample,

T_{105}= % total solids determined at 105°C

Table I-8 Acid soluble lignin

No	abs @ 280nm			abs @ 205nm	
	abs @ 280	**%ASL -1**	**%ASL-2**	**abs @ 205**	**%ASL-3**
1	0.934	1.230	1.246	0.196	0.055
2	0.917	1.207	1.223	0.196	0.055
3	0.915	1.205	1.220	0.178	0.050
4	0.902	1.188	1.203	0.174	0.049
5	0.871	1.147	1.162	0.171	0.048
Average		1.195	1.211	-	0.052

ASL -1: acid soluble lignin with absorptivity constant 23.6 for hard wood

ASL -2, acid soluble lignin with absorptivity 23.3 for soft wood;

ASL -3, acid soluble lignin with absorptivity 110 for hard wood

Calculation:

$$\%ASL = [\frac{UVabs \times Volume\ \ hydrolysis\ Liquor(L) \times df}{\varepsilon\ \times ODW}] \times 100$$

UV_{abs} = UV absorbance; Volume $_{\text{hydrolysis liquor}}$ = 0.087 L ; df = dilution factor (=1); ODW = Oven- dried weight of sample after 105°C; ε = Absorptivity constant at recommended wave length for selected biomass (Maekawa et al, 1989).

E. Volatile Fatty Acids

Table I-9 Volatile fatty acids

Sample	pH (25°C)	Lactic Acid			Formic Acid			Acetic Acid		
		Area	Conc. (g/L)		Area	Conc. (g/L)		Area	Conc. (g/L)	
		(µV.sec)	Sample	Ave.	(µV.sec)	Sample	Ave.	(µV.sec)	Sample	Ave.
1	5.5	179957.39	0.673	0.871	822871.16	3.134	2.913	362859.41	1.157	1.065
2	5.5	186120.43	0.686		811730.57	3.095		389442.99	1.223	
3	5.5	591362.74	1.589		803373.33	3.066		272404.58	0.933	
4	5.5	117386	0.533		600123.54	2.358		278095.97	0.948	

F. Extractives

Table I-10 Extractives

no	Weight (g)		Extractives	Extractives (%)
	Empty dish before boiling	Dish and Sample after boiling		
1	1.943	2.681	0.738	10.54
2	1.923	2.717	0.794	11.34
3	1.929	2.701	0.772	11.03
Average				10.97

G. Biodegradability

$B = 0.83 – (0.028)X$,

where, B = biodegradable fraction of the volatile solids (VS), and X = lignin content of the VS expressed as a percent of dry weight.

B = 82%

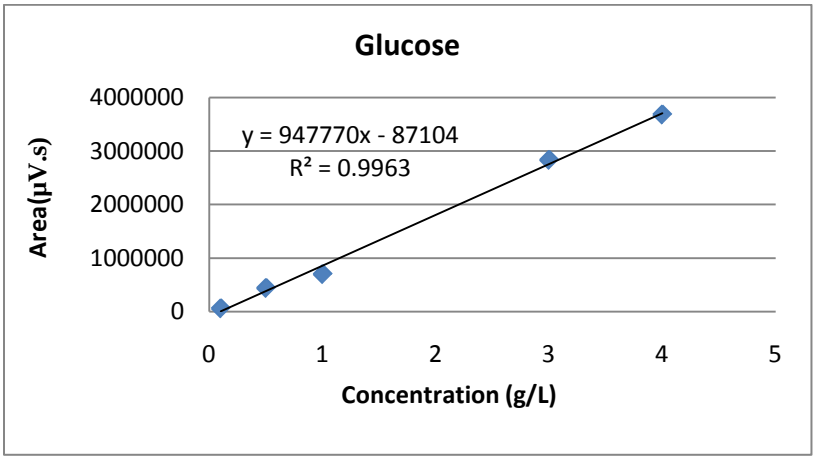
H. Digestibility

Table I-11 Sample's description

no	Parameter	Value
1	Working Volume	10 L
2	Moisture content of SSO	6.6%
3	Solid loading	0.35 g (3.5%)
4	Cellulose content	0.11

Table I-12 Standards for HPLC calibration

Standards	Conc. g/L	Glucose Area(µV.S)	Xylose Area(µV.S)
S-ST1-2	0.1	62048.2	61197.95
S-ST2-2	0.5	441673.89	414745.23
S-ST3-2	1	700542.09	648029.23
S-ST4-2	3	2832383.8	2597107
S-ST5-2	4	3678651.3	3377073



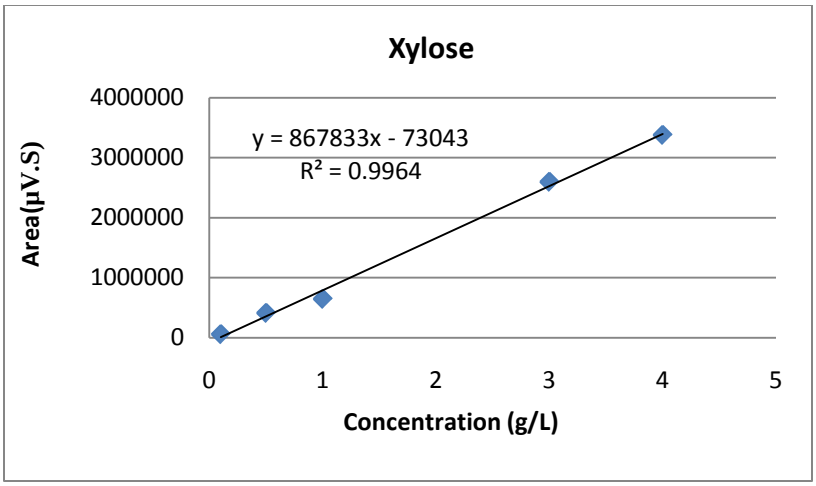
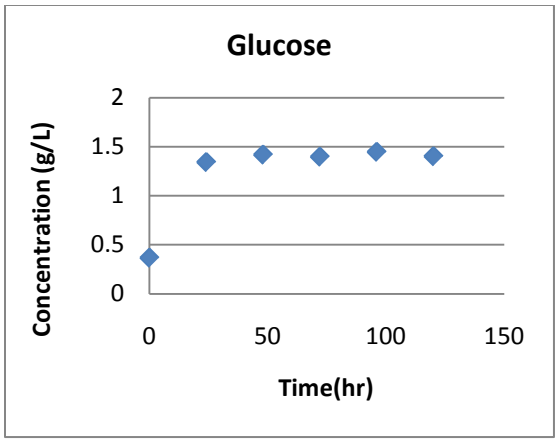
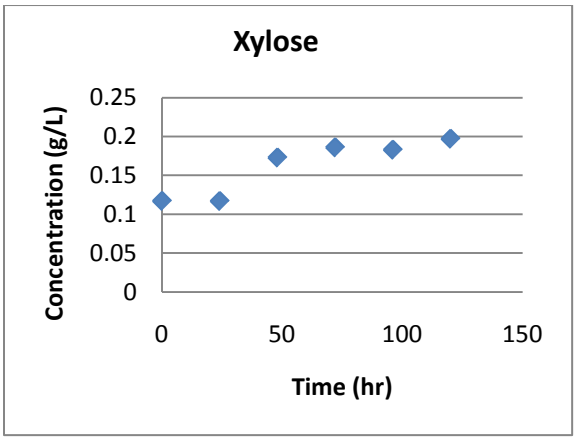


Table I-13 Results of enzymatic saccharification

Sample	Time (hr)	Glucose			Xylose		
		Area (µV.S)	Conc.		Area (µV.S)	Conc.	
			(g/L)	Average		(g/L)	Average
ES01	0	256831.4	0.363	0.368	34613.2	0.124	0.117
ES02		293456.38	0.402		42789.22	0.133	
ES03		234582.2	0.339		7956.85	0.093	
ES1	24	1059935	1.21	1.344	16949.8	0.104	0.117
ES2		1194529.6	1.352		2918.9	0.088	
ES3		1306095.27	1.470		65189.6	0.159	
ES4	48	1378143.49	1.546	1.420	77136.4	0.173	0.173
ES5		1231725.53	1.392		71372	0.166	
ES6		1166329.11	1.323		82228.4	0.179	
ES7	72	1335915.98	1.501	1.399	91055.2	0.189	0.186
ES8		1295432.43	1.459		76669.6	0.173	
ES9		1085070.31	1.237		97127.6	0.196	
ES10	96	1278150.08	1.440	1.448	84275.6	0.181	0.183
ES11		1301297.77	1.465		85784.8	0.183	
ES12		1277525.91	1.440		86815.6	0.184	
ES13	120	1248603.26	1.409	1.404	94286.4	0.193	0.197
ES14		1193823.49	1.352		105013.2	0.205	
ES15		1287410.08	1.450		94396.4	0.193	

% Digestion = (gram cellulose digested / gram cellulose content) ×100 = 12.73%


Glucose
Concentration (g/L)
2
1.5
1
0.5
0
0
50
100
150
Time(hr)


Xylose
Concentration (g/L)
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
Time (hr)

Appendix II

Enzymes

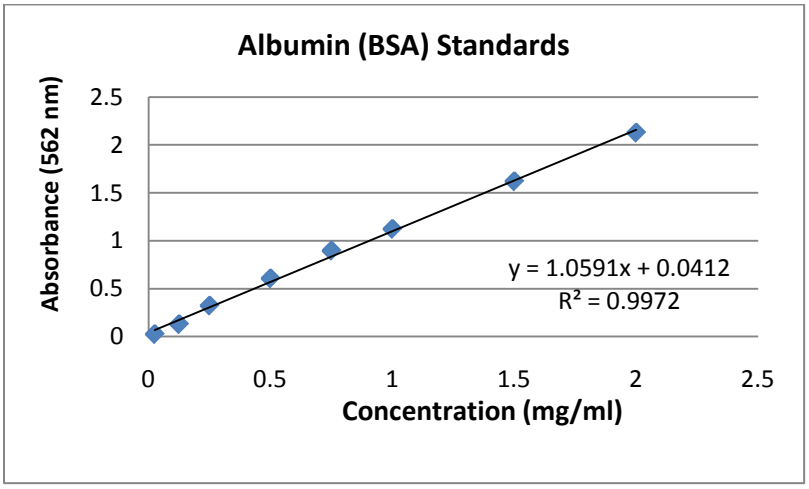
A. Protein Measurement

- Before protein measurement, enzymes were de-salted with a Zeba desalt spin columns.

- Protein measured by using Pierce's BCA protein assay kit. Bovine Serum Albumin (BSA) was used as the standard protein. Measurement procedure was provided by Thermo Fischer Scientific Inc.

Table II-1 Protein standards

No.	Abs @ 562nm	Conc.(µg/ml)	Conc.(mg/ml)
1	2.127	2000	2
2	1.619	1500	1.5
3	1.122	1000	1
4	0.895	750	0.750
5	0.606	500	0.50
6	0.322	250	0.250
7	0.128	125	0.125
8	0.024	25	0.025



Sample: Accellerase 1500

Table II-2 Results of spectrophotometer

Accellerase 1500	Abs @ 562 nm	Conc. (µg/ml)	Conc. (mg/ml)	df (10^2)
AC1	1.072	937.091	0.973	97.328
AC2	1.021	890.727	0.925	92.513
AC3	1.046	913.455	0.949	94.873
Average			0.9490	94.904

Protein content in Accellerase 1500 = 95mg/ml

B. Measurement of cellulose activity

The measurement of cellulase activity was based on procedure presented by Ghost, 1987 and NREL/TP-510-42628. Modified DNS[7] reagent presented by Miller, 1959, was used for estimation of reducing sugar.

B.1 Celluclast 1.5 L

July 22, 2009

Table II-3 Glucose standards

Standards no.	Glucose stock (ml)	Citrate Buffer (ml)	Dilution	Glucose (mg/0.5 ml)	Abs @ 540nm
S1	1	0.5	1/15	3.350	0.970
S2	1	1	1/2	2.500	0.630
S3	1	2	1/3	1.650	0.534
S4	1	4	1/4	1	0.275

Enzyme stock solution: Diluted 1:20 in citrate buffer

[7] 3,5Dinitrosalcylic acid

Table II-4 Diluted enzyme

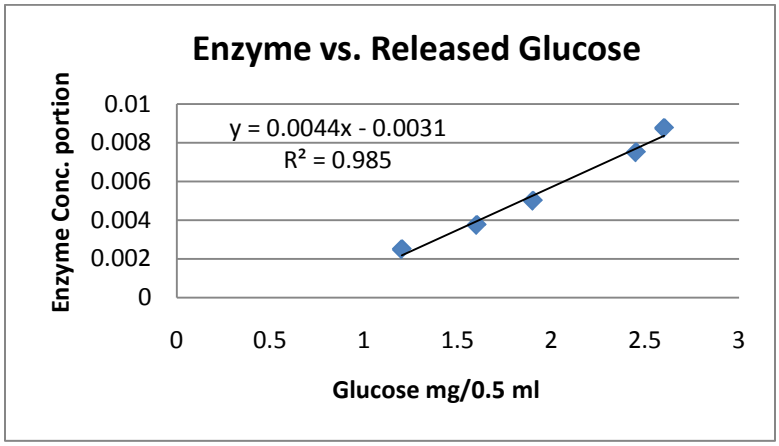
No.	Enzyme from Diluted Stock Solution (ml)	Citrate buffer (ml)	Concentration portion
1	1.75	8.25	0.00875
2	1.5	8.5	0.0075
3	1	9	0.005
4	0.75	9.25	0.00375
5	0.5	9.5	0.0025

Table II-5 Results of FPA- Celluclast 1.5L

No.	Samples no.	Abs @ 540nm	Glucose (mg/0.5 ml)	Ave. Glucose (mg/0.5 ml)
1	C1	0.797	2.833	2.629
	C2	0.641	2.266	
	C3	0.784	2.786	
2	C4	0.791	2.812	2.452
	C5	0.483	1.692	
	C6	0.802	2.852	
3	C7	0.465	1.626	1.883
	C8	0.57	2.008	
	C9	0.572	2.015	
4	C10	0.505	1.772	1.573
	C11	0.431	1.503	
	C12	0.415	1.444	
5	C13	0.283	0.964	1.160
	C14	0.358	1.237	
	C15	0.369	1.277	

Table II-6 Glucose content and dilution portion

Concentration portion	Ave. Glucose (mg/0.5 ml)
0.00875	2.6
0.0075	2.45
0.005	1.9
0.00375	1.6
0.0025	1.2



Y= (0.0044×2) -0.0031= 0.0057, FPU /mL =0.37/0.0057= 64.9 = 65

B.2 Accellerase 1500

2nd August,2009

Table II-7 Standards

Standards no.	Glucose stock(ml)	citrate buffer (ml)	Dilution	Glucose (mg/0.5 ml)	Abs @ 540nm
S1	1	0.5	1/15	3.35	0.88
S2	1	1	1/2	2.5	0.61
S3	1	2	1/3	1.65	0.36
S4	1	4	1/4	1	0.182

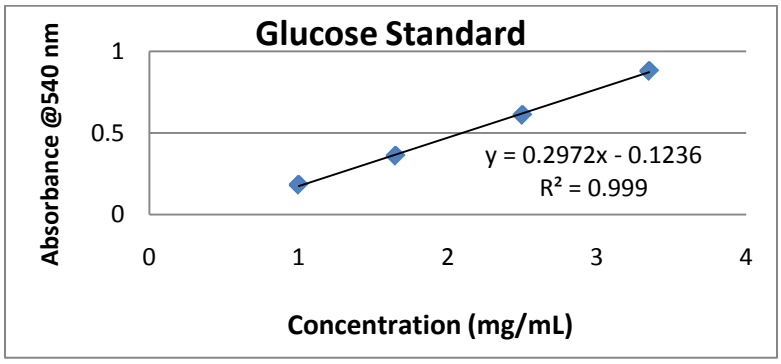


Table II-8 Diluted enzyme

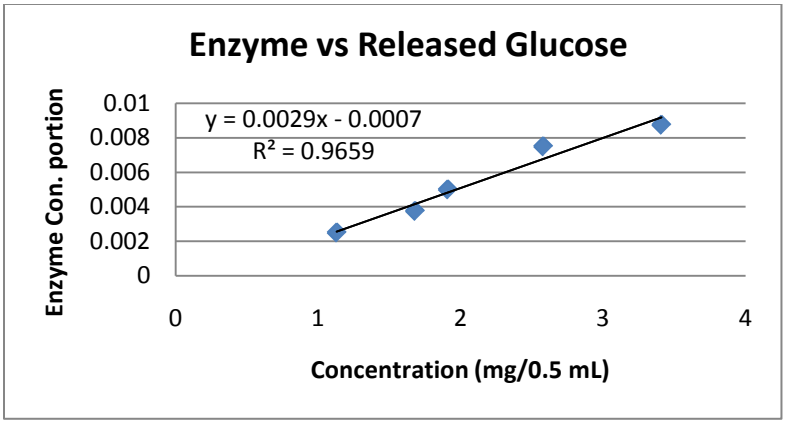
no.	Enzyme from Diluted stock Solution (ml)	Citrate buffer (ml)	Concentration portion
1	1.75	8.25	0.00875
2	1.5	8.5	0.0075
3	1	9	0.005
4	0.75	9.25	0.00375
5	0.5	9.5	0.0025

Table II-9 Results of FPA –Accellerase 1500

Dilution	Samples no.	Abs @ 540nm	Glucose (mg/0.5 ml)	Ave. Glucose (mg/0.5 ml)
1	AC 1	0.774	3.020	3.409
	AC 2	0.983	3.723	
	AC 3	0.912	3.485	
2	AC 4	0.747	2.929	2.583
	AC 5	0.593	2.411	
	AC 6	0.592	2.408	
3	AC 7	0.388	1.721	1.910
	AC 8	0.452	1.937	
	AC 9	0.492	2.071	
4	AC 10	0.362	1.634	1.679
	AC 11	0.328	1.520	
	AC 12	0.436	1.883	
5	AC 13	0.268	1.318	1.135
	AC 14	0.177	1.011	
	AC 15	0.196	1.075	

Table II-10 Glucose content and dilution portion

Concentration portion	Ave. Glucose (mg/0.5 ml)
0.00875	3.41
0.0075	2.58
0.005	1.91
0.00375	1.68
0.0025	1.13



Y= (0.0029×2) -0.0007= 0.0051, FPU /mL =0.37/0.0051= 72.5

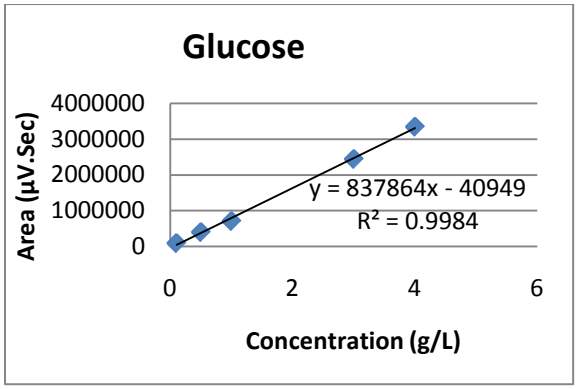
Appendix III

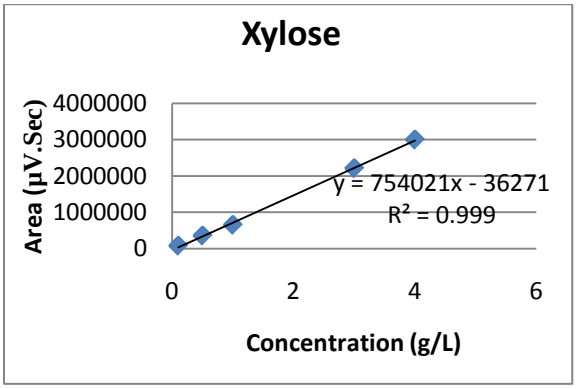
Dilute Acid Pretreatment

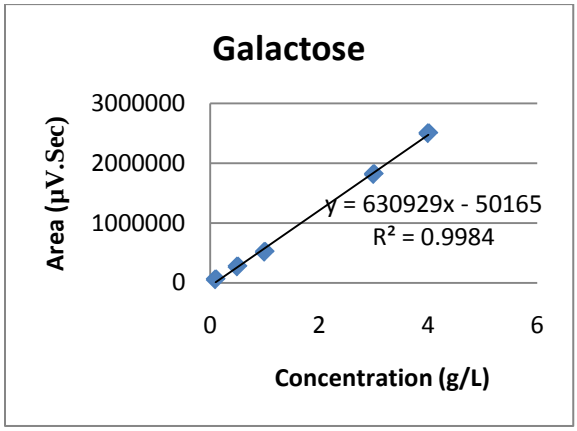
Dilute Acid pretreatment

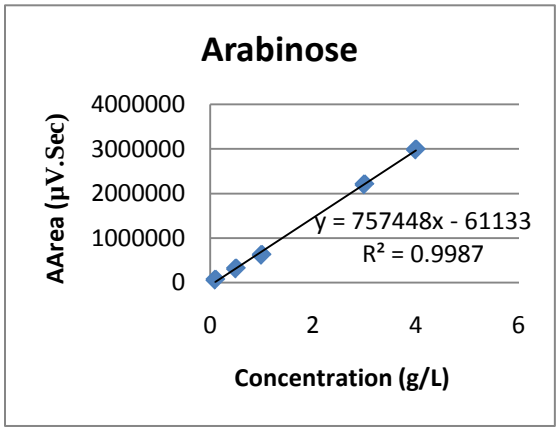
Table III-1 Standards for HPLC calibration

Standards	concentration (g/L)	Peak Area (µV*Sec)				
		Glucose	Xylose	Galactose	Arabinose	Mannose
S-ST1	0.1	94835.9	77720.97	58376.04	68813.02	68507.87
S-ST2	0.5	401882.32	357371.64	273874.29	319411.59	382219.95
S-ST3	1	710451.75	652442.75	519408.15	625177.6	712411.35
S-ST4	3	2445597.08	2212997.84	1819433.37	2206861.79	2542929.92
S-ST5	4	3348121.36	3002690.57	2504075	2988121.95	3460596.1









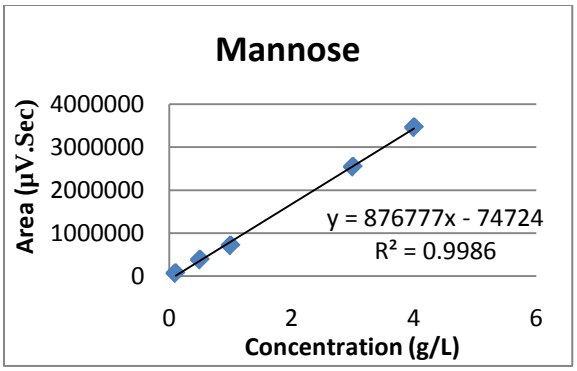


Table III-2 Sample's Description

No.	Parameter	Value
1	Working Volume	50 g 42.5 g liquid (including DDW and acid) + 7.5 g Dry SSO
2	Solid loading	15%
3	Moisture content of SSO	6.6%
4	Glucose content	52.47 g/L
5	Xylose content	10.8 g/L

Table III-3 Dilute acid pretreatment results

Time (Min)	Sample no.	Acid conc. (%)	pH	Peak Area (µV*sec)	Glucose Conc. (g/L) Sample	Glucose Conc. (g/L) Ave.	Peak Area (µV*sec)	Xylose Conc. (g/L) Sample	Xylose Conc. (g/L) Ave.	Peak Area (µV*sec)	Galactose Conc. (g/L) Sample	Galactose Conc. (g/L) Ave.	Peak Area (µV*sec)	Arabinose Conc. (g/L) Sample	Arabinose Conc. (g/L) Ave.	Peak Area (µV*sec)	Mannose Conc. (g/L) Sample	Mannose Conc. (g/L) Ave.	Total Sugars (g/L)
15	SA1	0.5	5.05	619198.6	0.788	0.886	89129.6	0.166	0.164	192083.34	0.384	0.362	22450	0.110	0.097	701949.2	0.886	0.964	2.473
	SA2	0.5	5.25	784153.9	0.985		85596.4	0.162		164276.33	0.34		2341.34	0.084		839379	1.043		
	S3	1	4.36	896093	1.118	0.816	59856	0.127	0.088	202766.28	0.401	0.302	131187.2	0.254	0.213	1250179	1.511	1.179	2.598
	S4	1	4.31	389509.6	0.514		995.12	0.049		77710	0.203		69084.61	0.172		667171.4	0.846		
	S5	1.5	2.62	785561.4	0.986	1.002	61878	0.130	0.129	202862.58	0.401	0.406	540967.2	0.795	0.754	1148032	1.395	1.399	3.691
	S6	1.5	2.85	812031.6	1.018		59812.4	0.127		209572.72	0.412		479564.1	0.714		1155960	1.404		
	SA7	0	5.8	558265.4	0.715	0.716	6702.04	0.057	0.054	1009904.6	1.68	1.682	27	0.081	0.081	805652.2	1.004	1.019	3.552
	SA8	0	5.77	559480.8	0.717		2336.25	0.051		1011811.4	1.683		34.24	0.081		832308.8	1.035		
30	SA9	0.5	5.34	686440.6	0.868	0.838	4799.27	0.054	0.052	97365.7	0.234	0.225	4700.96	0.087	0.084	999939.2	1.226	1.159	2.359
	SA10	0.5	5.36	636290.5	0.808		433.41	0.049		86733.43	0.217		763.51	0.082		883718	1.093		
	S11	1	4.5	687237.1	0.869	0.902	5698.49	0.056	0.055	113120.4	0.259	0.261	114575.6	0.232	0.226	1137722	1.383	1.421	2.865
	S12	1	4.58	742187.2	0.935		4662.16	0.054		116413.6	0.264		105889.7	0.221		1203835	1.458		
	S13	1.5	2.93	98354.55	0.166	0.613	76078.4	0.149	0.120	258161.02	0.489	0.428	673048.2	0.969	0.900	1169944	1.420	1.389	3.45
	S14	1.5	2.95	846881.7	1.060		32016.4	0.091		181314.4	0.367		568747.7	0.832		1117115	1.359		
	SA15	0	5.96	620914	0.790	0.824	13404.81	0.066	0.057	84103.07	0.213	0.221	578.72	0.081	0.081	785018.6	0.981	1.010	2.193
	SA16	0	5.95	677374	0.857		406.85	0.049		94508.8	0.229		10.81	0.081		837030.6	1.040		
60	SA17	0.5	5.48	767775	0.965	0.968	1603.86	0.050	0.050	55110.46	0.167	0.167	9223.34	0.093	0.0942	916534	1.131	1.111	2.39
	SA18	0.5	5.54	771858.8	0.970		1849.08	0.051		54804.66	0.166		11158.04	0.095		882418.8	1.092		
	SA19	1	4.72	812297.6	1.018	0.996	5718.19	0.056	0.055	78726.8	0.204	0.205	137412.8	0.262	0.258	1164678	1.414	1.382	2.896
	SA20	1	4.71	774000.4	0.973		5354.84	0.055		79380	0.205		131821.8	0.255		1108442	1.349		
	S21	1.5	3.19	1025264	1.273	1.291	110022	0.194	0.197	263323.39	0.497	0.499	836518.2	1.185	1.176	1269695	1.533	1.566	4.729
	S22	1.5	3.2	1055813	1.309		113886.4	0.199		266145.76	0.501		822564.8	1.167		1327746	1.600		
	SA23	0	5.94	648490.2	0.823	0.863	6254.65	0.056	0.053	53115.32	0.164	0.164	4320.57	0.086	0.085	764983	0.958	0.962	2.128
	SA24	0	6.02	715794.2	0.903		1359	0.050		54032.4	0.165		2133.33	0.084		773220.8	0.967		

Enzymatic hydrolysis on SSO pretreated by 1% H_2SO_4:

Accellerase 1500 = 72.5 FP/ml
ß-glucosidase 258 CBU/g
density =1.18g/ml
total solid loading = 10%
Na - Azide added to each = 0.75ml
Temperature = 50 C
pH = 4.8
Working volume=50 g= DD W 67.5 (g or ml)+7.5 g solid
Solid content in each sample = 7.5 g
glucan content in each sample = 2.01 g
Glucose content = 2.23 g
Xylan content in each sample = 0.404
Xylose Content = 0.459 g
Glucose : 33 g/L
Xylose : 6.8 g/L

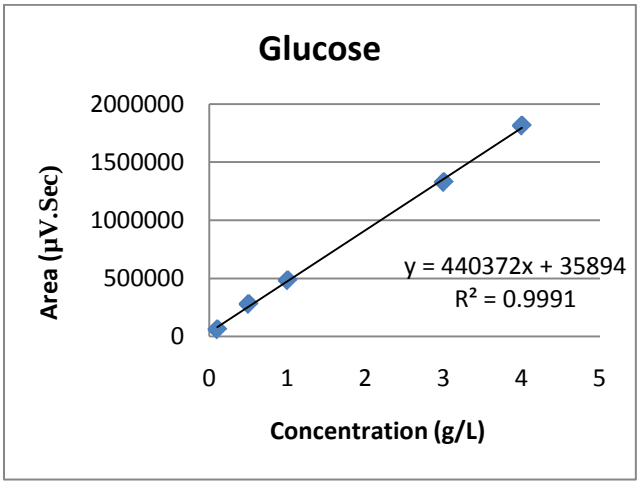
Table III-4 Enzymatic hydrolysis on SSO pretreated by 1% H_2SO_4

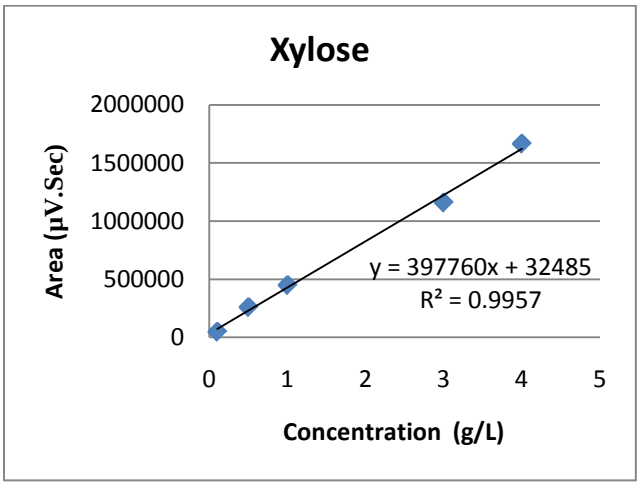
No.	Enzyme activity		Protein (mg/ml)	produced sugar			
				Glucose		Xylose	
	Accellerase 1500	Novozyme 188		(g/L)	Yield (%)	Conc. (g/L)	Yield (%)
1	15 FPA	-	20	4.3	8.182	0.4	2.941
2	15 FPA	42 CBU	36	4.5	10	0.6	7.353
3	25 FPA	-	33.3	4.5	9.697	0.4	4.412
4	35 FPA	-	49	4.9	8.788	0.4	2.941

Changing acid concentration and residence time comparing 4% (W/W) H_2SO_4, (residence time 1 and 4 hrs) with 1% (W/W) H_2SO_4 (residence time 1 and 2 hrs)

Table III-5 Standards for HPLC calibration

Standards No.	Conc. (g/L)	Glucose	Xylose
		Area (µV*Sec)	Area (µV*Sec)
S-ST1	0.1	61075.84	48580.4
S-ST2	0.5	279051.56	260104.63
S-ST3	1	483552.07	449586.34
S-ST4	3	1328289.33	1159508.03
S-ST5	4	1814703.41	1665380.14





SSO pretreated by 1% and 4% H_2SO_4

August 28 & 29

Table III-6 Results

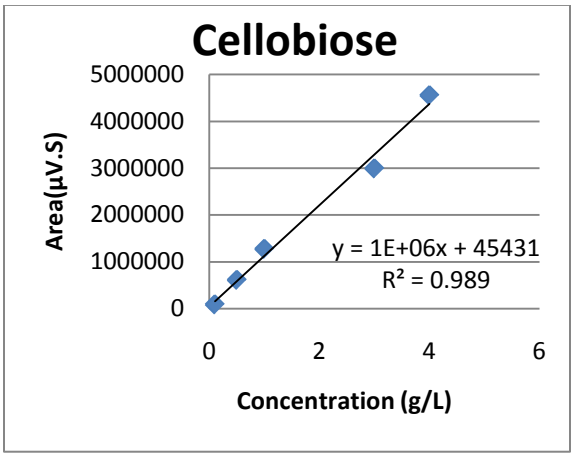
Samples	autoclave time (hr)	pH	(g) Acid per (g) biomass	Glucose			Xylose		
				Are(μV.sec)	Conc. (g/L)	Yield(%)	Are(μV.sec)	Conc. (g/L)	Yield(%)
4% acid 1	4	0.88	0.267	48128.4	0.028	0.053	15145.92	0.000	0
4% acid 2	4	0.89	0.267	50004.88	0.032	0.061	9988.11	0.000	0
4% acid 1	1	0.94	0.267	1540955.52	3.418	6.514	1847340.04	4.563	42.247
4% acid 2	1	0.94	0.267	1268087.74	2.798	5.333	1485996.99	3.654	33.836
1% acid 1	2	3.25	0.06	581747	1.240	2.362	13326.89	0.000	0
1% acid 2	2	3.23	0.06	676798.8	1.455	2.774	8320.5	0.000	0

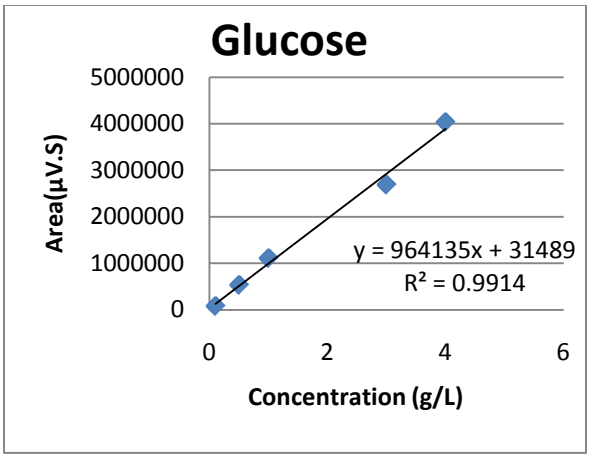
Table III-7 Summary of results

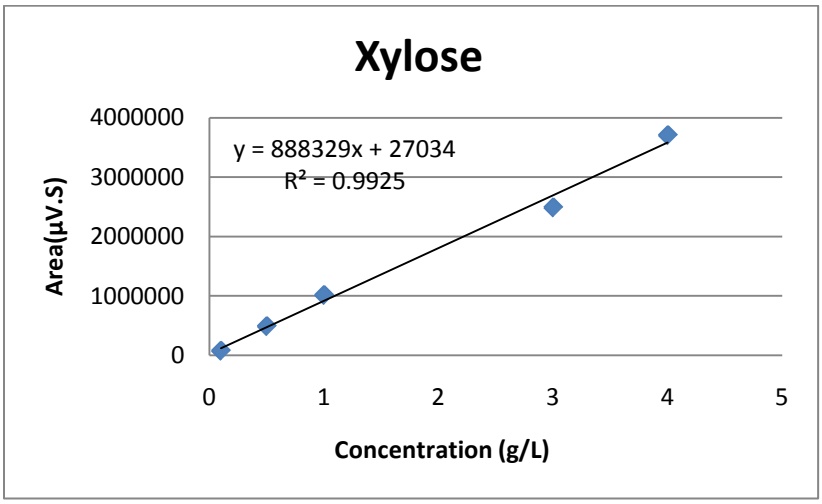
Samples	Autoclave time (hr)	pH	g acid per g biomass	Glucose		Xylose	
				Ave. Conc. (g/L)	Yield (%)	Ave. Conc. (g/L)	Yield (%)
4% acid	4	0.885	0.267	0.030	0.057	0.000	0
4% acid	1	0.94		3.108	5.923	4.108	38
1% acid	2	3.24	0.06	1.347	2.568	0	0
1% acid[1]	1	4.71		0.996	1.9	0.055	0.51

Table III-8 Standards for HPLC calibration used for SSO sample

Standards	Conc. (g/L)	CB Area(μV.S)	glucose Area(μV.S)	xylose Area(μV.S)
CB-S-ST1	0.1	90463.49	81079.86	75601.79
CB-S-ST2	0.5	613541.7	534446.4	489402.4
CB-S-ST3	1	1275535	1110304	1013983
CB-S-ST4	3	2991914	2689345	2489482
CB-S-ST5	4	4553849	4033830	3706329







Final Attempt for DA pretreatment:

Changing acid concentration and residence time: 2% (W/W) H_2SO_4, with residence time 1 hr

Samples prepared on Sept. 8 &9 and Tested on Sept. 12 &13

Glucose content in 2% EAC and 2% ECEC: 33.83 g/L
Xylose content in 2% EAC and 2% ECEC: 6.98 g/L
Enzyme Loading : 60 FPU
pH after dilute acid pre treatment= 1.51 @ 25°C

2% EAC:

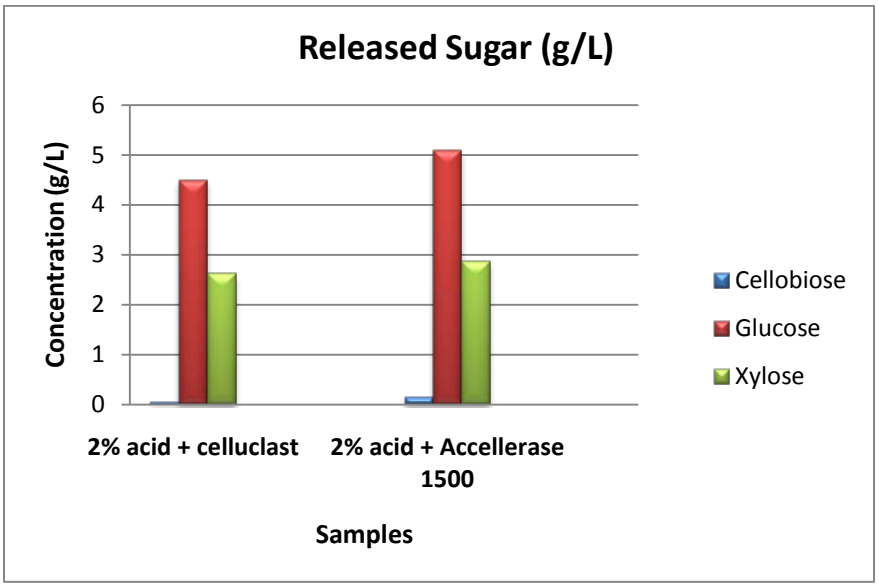
2% H2SO4 + Accellerase 1500

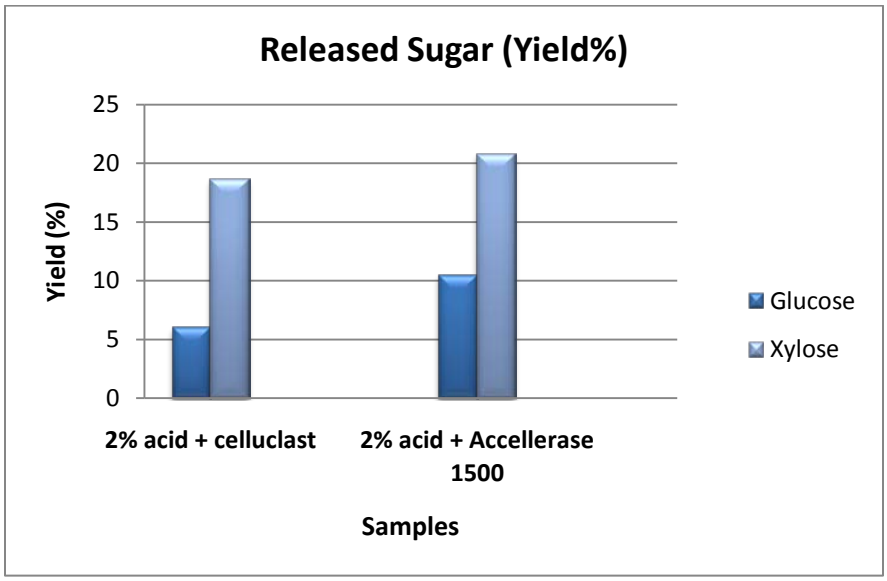
2% E Cellu:

2% H2SO4 + Celluclast 1.5L

Table III-9 Enzymatic hydrolysis of SSO pretreated by 2% H2SO4

Sample		no.	Cellobiose			Glucose				Xylose			
			Area	Conc.	Ave. Conc.	Area	Conc.	Ave. Conc.	Yield	Area	Conc.	Ave. Conc.	Yield
			(µV.S)	(g/L)	(g/L)	(µV.S)	(g/L)	(g/L)	(%)	(µV.S)	(g/L)	(g/L)	(%)
2% E Cellu-0		1	344770.4	0.299	0	2402622	2.459	2.459		1195548	1.315	1.315	
2% E Cellu-1	2% acid + Celluclast 1.5L	2	80663.55	0.035	0.0469	4153845	4.276	4.495	6.0169	2572754	2.866	2.619	18.676
2% E Cellu-2		3	103966.7	0.059		4575780	4.713			2133705	2.371		
2% E AC-0		4	48532.8	0.003	0	1510010	1.534	1.534		1282589	1.413	1.413	0
2% E AC-1	2% acid + Accellerase 1500	5	184308.4	0.139	0.145	4982097	5.135	5.081	10.496	2776457	3.095	2.865	20.804
2% E AC-2		6	195946.1	0.151		4885936	5.035			2367950	2.635		


Released Sugar (g/L)
Concentration (g/L)
6
5
4
3
2
1
0
2% acid + celluclast
2% acid + Accellerase 1500
Samples
Cellobiose
Glucose
Xylose


Released Sugar (Yield%)
Yield (%)
25
20
15
10
5
0
2% acid + celluclast
2% acid + Accellerase 1500
Samples
Glucose
Xylose

Appendix IV

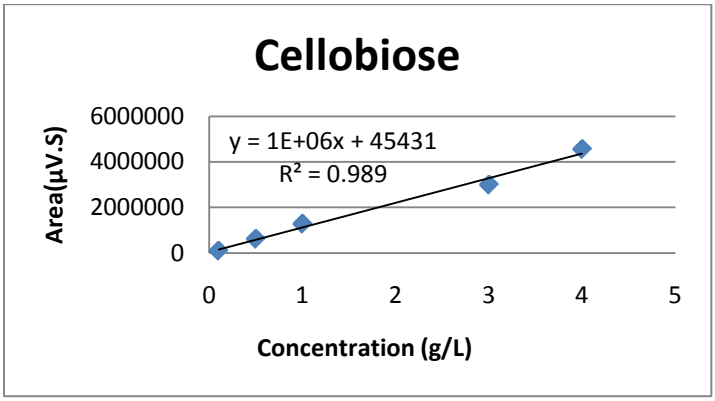
COSLIF Pretreatment

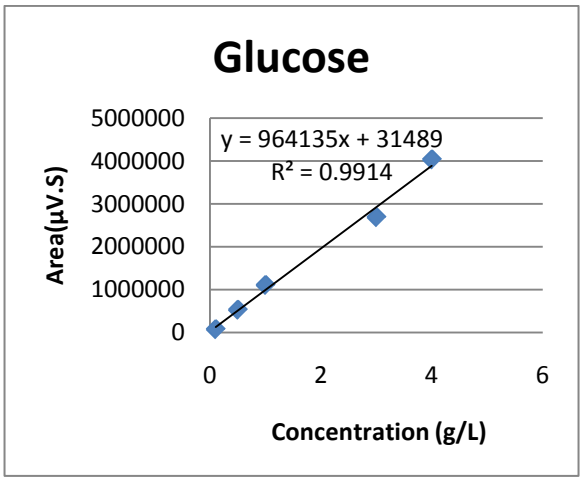
Phosphoric acid

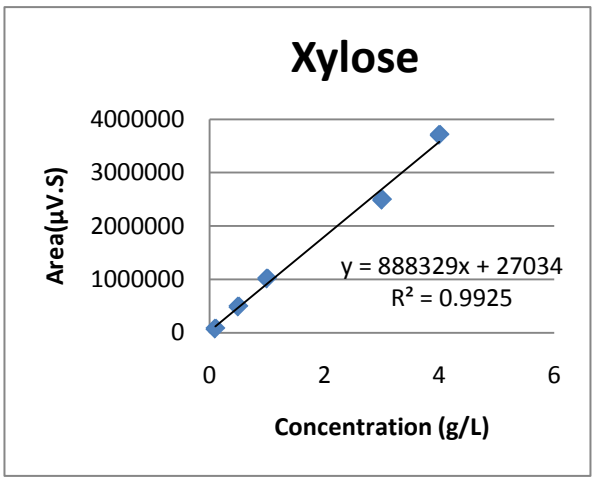
A. Pre-assessment of COSLIF pretreatment on SSO

Table IV-1 Standards for HPLC calibration

standards	Conc.g/L	CB	glucose	xylose
		Area(μV.S)	Area(μV.S)	Area(μV.S)
CB-S-ST1	0.1	90463.49	81079.86	75601.79
CB-S-ST2	0.5	613541.73	534446.36	489402.4
CB-S-ST3	1	1275534.72	1110303.81	1013982.5
CB-S-ST4	3	2991913.64	2689344.5	2489481.91
CB-S-ST5	4	4553849.4	4033829.65	3706329.28



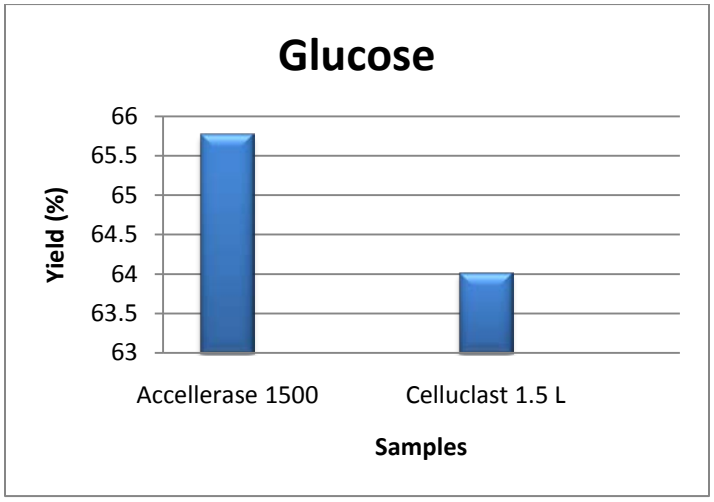




Samples prepared on Sept. 8 &9. Tested by HPLC on Sept. 12 &13
Sugar content in PhAc-CEC and Ph Ac-AC: 10 g/L
Enzyme Loading : 60 FPU

Table IV-2 Results of pre-assessment

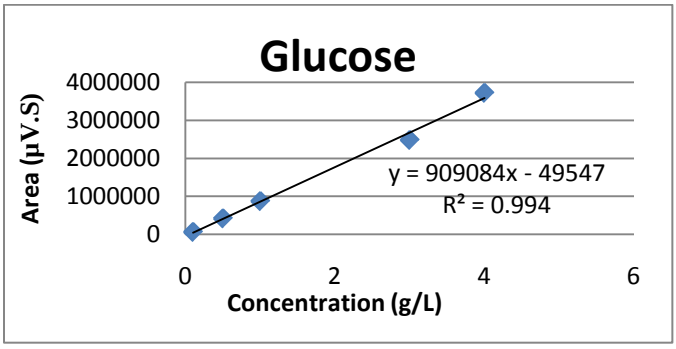
Sample		no.	CB			Glucose				Xylose		
			Area	Conc.	Ave.	Area	Conc.	Ave.	Yield	Area	Conc.	Ave.
			(μV.S)	(g/L)	(g/L)	(μV.S)	(g/L)	(g/L)	(%)	(μV.S)	(g/L)	(g/L)
Ph Ac-AC1	Accellerase 1500	7	192884.65		0.2	6589042.26	6.801	6.577	65.77	0	0	0
Ph Ac-AC2		8	245453.39	0.2		6156003.19	6.352			0	0	
Ph Ac-Cellu-1	Celluclast 1.5 L	9	140537.49	0.095	0.095	6166195.2	6.363	6.401	64.01	0	0	0
Ph Ac-Cellu-2		10	140811.8	0.095		6240244.7	6.44			0	0	

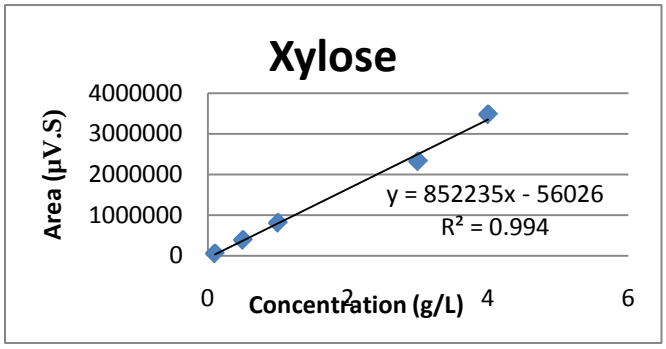


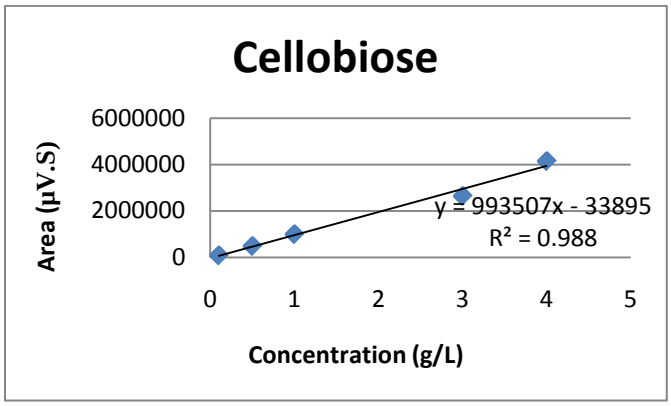
B. Assessment on proper reaction time

Table IV-3 Standards for HPLC calibration

Standards	Conc. (g/L)	CB Area(μV.S)	glucose Area(μV.S)	xylose Area(μV.S)
CB-S-ST1	0.1	86556.4	63656.21	51986.42
CB-S-ST2	0.5	489935.6	421390.26	385209.71
CB-S-ST3	1	1000263.2	875702.87	807780.69
CB-S-ST4	3	2647614.98	2484874.57	2324175.22
CB-S-ST5	4	4150319.2	3724766.15	3479942.25







Samples prepared on Sept. 17 and Tested on Sept. 20 & 21
Sugar content in each sample: 10 g/L Enzyme Loading : 60 FPU

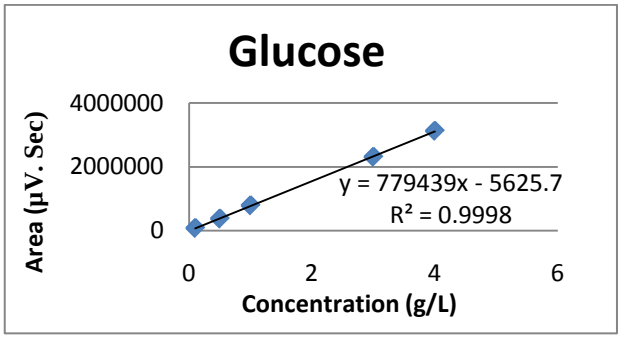
Table IV-4 Assessment on proper reaction time

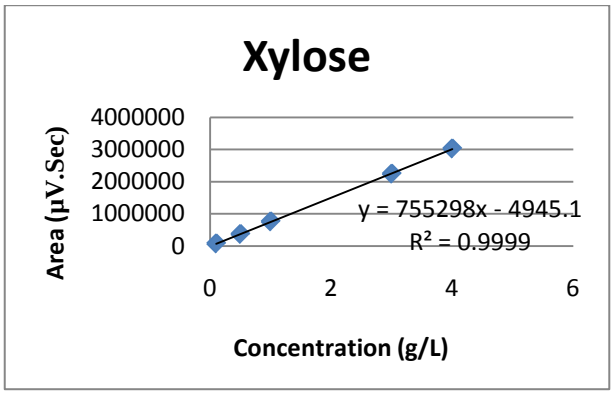
Sample		no.	CB			Glucose				Xylose			
			Area	Conc.	Ave.	Area	Conc.	Ave.	Yield	Area	Conc.	Ave.	Yield
			(μV.S)	(g/L)	(g/L)	(μV.S)	(g/L)	(g/L)	(%)	(μV.S)	(g/L)	(g/L)	(%)
1hr-BE	1hr treated slurry	1	0	0	0	0	0	0	0	0	0	0	0
2hr-BE	2hr treated slurry	2	0	0	0	0	0	0	0	0	0	0	0
3hr-BE	3hr treated slurry	3	0	0	0	0	0	0	0	0	0	0	0
3hr-01	3hr- enzyme (initial)	4	180321	0.147	0.145	298067.91	0.2733751	0.275	NA	0	0		NA
3hr-02		5	175438.2	0.142		301502.54	0.2771532			0	0		
3hr-1(AC1500)	3hr - AC1500 (72 hr)	6	239880.6	0.207	0.252	6073540.78	6.6264435	6.817	68.17	0	0	0	0
3hr-2(AC1500)		7	327820.06	0.296		6419811.49	7.0073442			0	0		
3hr-3(CEL)	3hr - Celluclast (72 hr)	9	140537.49	0.095	0.095	6166195.2	6.362912	6.401	64.01	0	0	0	0
3hr-4(CEL)		10	140811.8	0.095		6240244.7	6.4397161			0	0		
2hr-01	2hr-enzyme (initial)	8	164521.88	0.131	0.11	334972.24	0.3139702	0.285	NA	0	0	0	0
2hr-02		9	122739.15	0.089		281475.45	0.2551232			0	0		
2hr-1(AC1500)	2hr - AC1500 (72 hr)	10	79461.45	0.046	0.044	7056471.4	7.7076754	7.576	72.92	0	0	0	0
2hr-2(AC1500)		11	75254.44	0.042		6817344.37	7.4446337			0	0		
2hr-3(CEL)	2hr - Celluclast (72 hr)	12	143138.46	0.11	0.113	6278127.67	6.8514908	5.896	56.11	0	0	0	0
2hr-4(CEL)		13	148616.6	0.115		4540469.98	4.9400528			0	0		
1hr-01	1hr-enzyme (initial)	14	233379.99	0.201	0.211	447357.15	0.4375945	0.329	NA	179129.8	0.144	0.039	NS
1hr-02		15	253473.54	0.221		250736.6	0.2213102			0	0		
1hr-1(AC1500)	1hr- AC1500 (72 hr)	16	59821.9	0.026	0.029	6353676.35	6.934595	7.026	66.96	0	0	0	0
1hr-2(AC1500)		17	65932.97	0.032		6519684.8	7.1172057			0	0		
1hr-3(CEL)	1hr - Celluclast (72 hr)	18	226387.6	0.194	0.178	5684510.49	6.1985069	5.877	55.47	0	0	0	0
1hr-4(CEL)		19	195344.8	0.163		5099327.11	5.5548003			0	0		

C. Modified COSLIF pretreatment

Table IV-5 Standards for HPLC calibration

Standards	Concentration	Cellobiose	Glucose	Xylose
		Area	Area	Area
	(g/L)	(μV*Sec)	(μV*Sec)	(μV*Sec)
S-ST1	0.1	76355.55	77658.36	73544.17
S-ST2	0.5	386735.4	372202.64	367536.35
S-ST3	1	807853.89	788100.22	758190.13
S-ST4	3	2357205.8	2310624.5	2243980.1
S-ST5	4	3175334.3	3126462.98	3027584.72





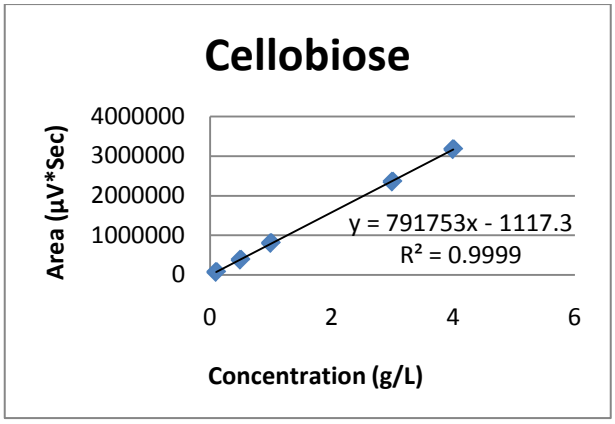


Table IV-6 Results of modified COSLIF pretreatment

	Sample	Cellobiose			Glucose			Xylose		
Samples	**no**	**Are(µV.sec)**	**Conc. (g/L)**	**Average**	**Are(µV.sec)**	**Conc. (g/L)**	**Average**	**Are(µV.sec)**	**Conc. (g/L)**	**Average**
after acid pretre.1	1	0	0.001	0	0	0.007	0	0	0.007	0
after acid pretre.2	2	0	0.001		0	0.007		0	0.007	
Enz. Hydr. Zero T1	3	80080.29	0.103	NA	901567.6	1.164	NA	346596.8	0.4654347	NA
Enz. Hydr. Zero T2	4	92042.72	0.118		842326.1	1.088		268048.8	0.3614387	
1	5	359003.3	0.455	0.3621	14002080	17.972	19.026	53175.02	0.077	0.0544
2	6	328963.92	0.4172		15452736	19.833		163876.73	0.224	
3	7	174791.09	0.222		15171428	19.472		0	0.007	
4	8	358249.02	0.454		14875488	19.092		0	0.007	
5	9	75698.24	0.097		14895948	19.118		0	0.007	
6	10	416968.45	0.528		14545363	18.669		0	0.007	

Table IV-7 Summary of modified COSLIF pretreatment

Samples	**Glucose**	
	Conc. (g/L)	**Yield (%)**
Enz. Hydr. Zero T1	1.16	NA
Enz. Hydr. Zero T2	1.09	
1	17.97	84.2
2	19.83	93.5
3	19.47	91.7
4	19.09	89.8
5	19.1	89.96
6	18.7	87.71
Average		89.5

Appendix V

Fermentation

Fermentation:

The following samples and the samples described in appendix V were tested together. Therefore, the standards for HPLC analysis of fermentation is the same as appendix V

Table V-1 Constructed model, (Glucose/xylose) = 5:1

Samples	Sample no	Cellobiose		Glucose		Xylose	
		Are(μV.sec)	Conc. (g/L)	Are(μV.sec)	Conc. (g/L)	Are(μV.sec)	Conc. (g/L)
10fold batch 12hr	11	27442.4	0.036	2097242	2.698	1398347	1.858
main batch 12hr	12	250328.33	0.318	16026489	20.569	12452278	16.493
main batch 24hr	13	56118.4	0.072	443797.1	0.577	276262.3	0.372
main batch 24hr	14	56118.4	0.072	443797.1	0.577	276262.3	0.372
Zero T1	15	324434.97	0.411	77900042	99.951	15050000	19.932
Zero T2	16	251880.63	0.320	77938140	100	15100001	19.999
10:2CX1	17	437191.65	0.554	0	0.007	7118568	9.431
10:2CX2	18	522380.07	0.661	0	0.007	3701629	4.907
10:2CX3	19	565738.4	0.716	0	0.007	7189697	9.526
						Average	39.854

Table V-2 Selected hydrolysate from enzymatic pretreatment after COSLIF process.

Samples	(g/L)	pH		Cellobiose		Glucose		Xylose	
			Sample no	Are(μV.sec)	Conc. (g/L)	Are(μV.sec)	Conc. (g/L)	Are(μV.sec)	Conc. (g/L)
10fold batch 12hr	_	6	20	0	0.001	2575816.3	3.3119	1502809.7	1.9962
main batch 12hr	_	6	21	297814	0.378	25015110	32.101	13843347	18.335
2	19.83	5.8	23	138054.16	0.176	11908703	15.286	0	0.007
4	19.09	6	25	229902.23	0.292	12645998	16.232	0	0.007
6	18.7	5.98	27	296891.64	0.376	12404266	15.922	0	0.007

Appendix VI

Project History

Since 2006, a multi-stage and collaborative research project based on cellulosic ethanol from Source-Separated Organics (SSO) waste[8] pretreated by Automation Tooling System Inc. (ATS) thermal screw machine has been conducted by Dr. G. Luk, a professor and program director of Graduate Studies at the Department of Civil Engineering at Ryerson University. Dr. Luk's research team is working on different aspects of the subject that is divided into 4 phases. It is shown in the table below. This project is unique and involved with different influential factors and parameters such as SSO characteristics, bacteria and optimal growth condition, fermentation process, and bench-scale bioreactor design. The first three phases have been successfully completed and the project would be accomplished in 2011. This thesis contributes to the last stage of phase three involving the pretreatment and saccharification investigation.

Table VI-1 Overview of the research project history

Cellulosic Ethanol Production through Fractionation and Bacterial Fermentation		
Phase No.	**Subtopics**	**Timeline**
Phase 1	Proof of Concept	2006-2007
Phase 2	Growth Kinetics and Modeling of Bacterium of Interest	2007
Phase 3	Investigation on Feasibility of Ethanol Production: ➢ Characterization analysis of SSO and monitoring seasonal fluctuation ➢ Investigation on cellulolytic bacterium in batch culture based on Consolidated Bioprocessing(CBP) system ➢ Pretreatment and Saccharification of SSO	2007-2009
Phase 4	Bench-scale Anaerobic Bioreactor Design	2009-2011

More details about the project in association with four partners are presented in the following figure.

[8] Organic Fraction of Municipal Solid Waste (OFMSW)/green bin

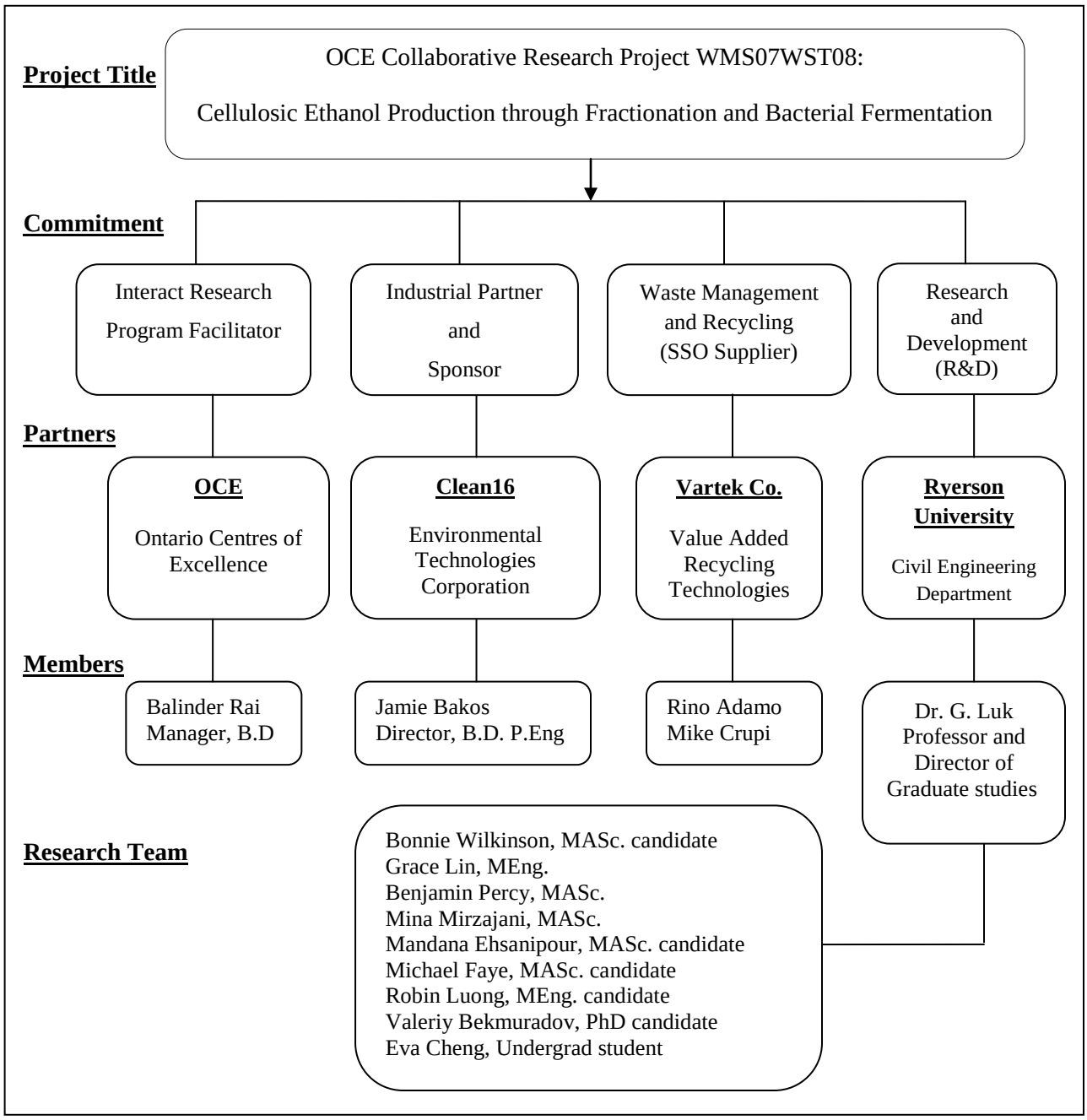


Figure VI -1 Scheme of collaborative research project