SACCHARIFICATION OF SPENT SUGARBEET PULP USING ENZYMES EXTRACTED FROM ANAEROBIC DIGESTER

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

“To my Mom for her endless support and patience toward my education”

ACKNOWLEDGMENTS

I would like to extend my gratitude towards many individuals who have contributed to make this research a success. Especially, I express my highest regards to Dr. Pullammanappallil, my academic advisor and committee chair, for his continual support, guidance and encouragement during my time at the University of Florida. I owe a lot to Dr. Yang at the University of Hawai‘i for introducing me to an exciting career in Bioenvironmental Engineering and recommending me to the University of Florida to pursue Doctoral research. I express my sincere appreciation to Dr. Teixeira, Dr. Svoronos, Dr. Koopman and Dr. Correll for serving on my committee and providing constructive ideas to enhance my research. I would also like thank to Dr. Chynoweth and Dr. Owens for their insightful concepts in the field of anaerobic digestion and Mr. Paul Lane for his meaningful and witty conversations as they have enriched my thoughts as an individual and as a professional engineer. In addition, I would like to thank my colleagues Douglas Renk, Cesar Moreira, Jaime Chavez, Mandu Inyang and Samriddhi Buxy for collaborating with me in conducting laboratory and extension research during my studies at the University of Florida. I would also like to extend my appreciation to Ms. Anna Maria Joykutty for her excellent contributions to my research.

On a more personal note, I express my deep gratitude towards my parents; without them this would never have been possible. I reserve my special thanks to dear friends Ioannis “Yanni” Polematidis and Amanda Diego for their moral support and inspiration to complete my Doctoral studies at the University of Florida.

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SACCHARIFICATION OF SPENT SUGARBEET PULP USING ENZYMES EXTRACTED FROM ANAEROBIC DIGESTER

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December 2009

Chair: Pratap C. Pullammanappallil
Major: Agricultural and Biological Engineering

Enzymatic saccharification of spent sugarbeet pulp was studied as a process for producing. Fermentable sugars were obtained by sequential hydrothermolysis and enzymatic hydrolysis of spent sugar beet pulp. In the hydrothermolysis stage 44% saccharification was obtained in just 1 h. Hemicellulose and pectins were solubilized to during hydrothermolysis. In the enzyme hydrolysis stage, the hydrolysate achieved almost complete saccharification (88%) in just 6 h by using a combination of pectinase (9.5 IU) and hemicellulase (0.3 IU). That is the total time to achieve complete saccharification was 7 h. It was found that there exists a strong synergism between pectinase and hemicellulase enzymes to achieve complete saccharification and pectinase was an important enzyme.

The use of anaerobic digestion (or mixed culture anaerobic fermentation) process was investigated as a means of producing enzymes for saccharification of hydrolysate. The performance of sugar beet pulp digestion at mesophilic (37 ^{o}C) and thermophilic (55 ^{o}C) temperature regimes was compared in single stage, unmixed, batch, laboratory scale anaerobic digesters. Pressed pulp as received from the factory was fed to the digesters without any pretreatment or size reduction. Three experiments were carried

out at each temperature using inoculum obtained from thermophilic and mesophilic anaerobic digesters fed sugarbeet tailings. At both mesophilic and thermophilic temperatures SBP degraded very well to yield more than 0.3 m3 of methane at STP per kg volatile solids. However, the performance of the thermophilic anaerobic digester was better than the mesophilic in most aspects that were considered like ultimate methane potential, rate of degradation, duration of digestion and maximum specific volumetric methane productivity. Hence, thermophilic anaerobic digestion was used as a source of enzymes for saccharification of sugar beet pulp.

Extracellular enzymes are indeed secreted during thermophilic anaerobic digestion of sugar beet pulp in leach bed digester, particularly, pectin and hemicellulose hydrolyzing enzymes. The maximum activity of both hemicelluloytic and pectinolytic enzymes were found in cell-free (leachate) portion of the digester, followed by biofilm-associated and cell-bound activities. The hemicelluloytic activity of the extract was 0.128 IU whereas the pectinolytic activity in extract was 0.07 IU. The activities in thermophilic digester were approximately 10 times higher than mesophilic digesters. 58% saccharification was obtained in just 24 hrs by using enzyme extracts from anaerobic digester after hydrothermolysis pretreatment.

The use of mixed culture microbial extracellular enzymes from the anaerobic digester for saccharification of sugar beet pulp is a novel concept and offers a few advantages over fungal and commercial enzymes such as 1) On-site production of highly active microbial enzymes is possible and this approach can possibly improve the economics of pretreatment, as the costs of enzymes are independent of market fluctuations 2) The anaerobic extracts possess both hemicelluloytic and pectinolytic

activities besides other activities important for degradation of pentoses in sugar beet pulp, possibly reducing the costs of using commercial enzymes 3) The method for enzyme extraction developed here can be scaled up to a continuous or a semi-continuous operation.

CHAPTER 1
INTRODUCTION

1.1 Background

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increasing energy demand. Petroleum is the largest single energy source in the United States (~40%) and the world (~35%). Although production and consumption of most US energy sources (e.g., coal, natural gas) are almost balanced, almost two thirds of petroleum consumed in the US is imported, creating balance of trade deficits and energy security concerns (Wyman et al., 2005). Presently, considerable of attention has been focused on renewable fuels due to concerns about energy security, trade deficits, climate change, rising natural gas and crude oil prices, and continuous depletion of limited fossil fuel stocks (Wyman, 1999; Lynd, 2004; Herrera, 2006; Lin and Tanaka, 2006; Dien et al., 2006). In this respect, biomass will be a major contributor in the future supply of energy, chemicals and materials (Herrera, 2006; Ragaukas et al., 2006).

The NREL (National Renewable Energy Laboratory) has developed a biofuels approach for conversion of biomass to biofuels that are based on two different platforms. The "sugar platform" is based on biochemical conversion processes for the fermentation of sugars extracted from biomass feedstocks. The "syngas platform" is based on thermochemical conversion of biomass to syngas and the catalytic conversion of syngas to fuels and products. This study focuses on the “sugar platform” or biochemical conversion of biomass. There are mainly two steps involved in the conversion of biomass: hydrolysis of cellulose and hemicellulose to produce sugars and

subsequent fermentation of sugars to produce biofuels, bioproducts (e.g., biogas, bioethanol etc.). In fact, fermentable sugars from biomass are the largest known renewable carbohydrate source (Jorgensen et al., 2007). Thus, conversion of cellulosic biomass to fuels and chemicals presents a powerful opportunity to improve energy security, reduce the trade deficit, dramatically reduce greenhouse gas emissions (Wyman, 1999).

Nearly 40% of all refined sugar produced in the USA is made from sugarbeets grown in the North Central and North western regions of the United States. Approximately 400 million tons of sugar beets are produced annually, with production in the USA being between 20 and 30 million tons. Beet sugar processing generates significant quantities of both solid (tailings, beet pulp) and liquid (raffinate waste water) organic wastes and by-products. Figure 1-1 is a generalized schematic flow diagram of the process of making sugar from sugar beet highlighting daily waste and by-product generation. Sugarbeets are first washed and separated from “tailings” which mainly consist of sugarbeets, weeds, sugarbeet tops, debris and soils held by sugarbeets when harvested. After the process of extraction of sugar from sugar beets, large amount of pulp is produced. Finally, desugarized syrup called “Concentrated Separated Byproduct” raffinate, an effluent from chromatographic separators, is also produced.

There are several sugar beet processing companies in the states of Minnesota, Michigan and North Dakota that produce sugar from sugar beets. The American Crystal Sugar Company (ACSC) operates five processing plants along the Red River Valley in Minnesota and North Dakota. For example, at the East Grand Forks (EGF) plant, 8,310 tons of sugarbeets are processed daily to produce 1,320 tons of sugar. The plant also

produces every day 3500 m^3 of wastewater, 432 tons of tailings, 1273 tons of pressed pulp and 332 tons of raffinate. The amounts shown in Figure 1-1 are typical generation rates at EGF plant. The plant was operated for 219 days (duration of a campaign) in 2006/07.

Among the waste and by-products streams generated at American Crystal Sugar Company (ACSC), tailings (15% dry matter) and pressed spent pulp (22% dry matter) being rich in polysaccharides (see Table 1-1), could be used as potential feedstocks for production of biofuels. On a dry weight basis 13.5% of mass of sugar beets processed ends up as spent pulp where as only 3% is wasted as tailings. Between the two feed stocks available for biofuels production in a sugar beet plant it may cost more to prepare sugar beet tailings for biofuels production because, 1. extensive cleaning and sterilizing is required as tailings are covered with soils/mud. 2. harsher pretreatments might be necessary to cause enough structural breakdown for the enzymes to hydrolyze tailings efficiently unlike beet pulp which undergoes mechanical shear/squeezing during extraction of sugar from sliced beets. Moreover, the fraction of tailings generated is only one-third of pulp on a dry weight basis. This can increase the production cost substantially. Beet pulp is abundantly available, more easily digested by enzymes, and does not appear to contain any strong inhibitors of growth (Doran and Foster, 2000). Currently, spent pulp (the feedstock of interest for this work) is dried and sold as animal feed after mixing with concentrated raffinate. Due to the increases in cost of natural gas and other fuels the sale of dried pulp is yielding diminished returns. Therefore, more profitable avenues for utilization of spent pulp need to be developed.

Much of the research concerning production of biofuels from sugar beet pulp has focused on anaerobic digestion of spent sugar beet pulp to generate biogas. Extensive studies have been carried out on biogasification of spent sugar beet pulp (Weiland, 1993, Stoppok and Buchholz, 1985, Hutnan et al., 2000 and Hutnan et al., 2001, Koppar and Pullammanappallil, 2008, Brooks et al., 2008).

Very little information is available on utilization of sugar beet pulp as a potential feedstock for conversion to ethanol. From ~ 30 million tons of sugar beets processed annually and in the US about 4.5 million tons of sugarbeet pulp is generated from sugar beet processing. Micard et al., (1996) conducted a detailed analysis on carbohydrates in sugar beet pulp (see Table 1-1). Based on this information the theoretical yield of ethanol from spent sugar beet pulp would be 124.5 gallons per dry ton of beet pulp. Hence sugar beet pulp alone can produce 831 million gallons of ethanol/year from the US sugar industry. At American Crystal Sugar Company, the daily yield of ethanol would be 34,867 gallons or 7.6 million gallons per year from EGF plant. The overall ethanol yield for five plants at ACSC will be about 38 million gallons per year by only utilizing spent sugar beet pulp without affecting sugar production. At a gate price of $1.15 per gallon, ACSC can earn ~44 million dollars/campaign from sale of fuel ethanol.

The biochemical platform is attractive because nearly theoretical yields of sugars are possible and is more net energy positive when processing wet feedstocks key to economic success. Fermentable sugars are derived from pectin (e.g., sugar beet pulp, citrus pulp), hemicellulose and cellulose, but these are not readily available to enzymatic hydrolysis. Pretreatment is an essential step, which disrupts cell wall physical barrier (e.g., pectin layer on beet pulp), as well as cellulose crystallinity and association

with lignin so that hydrolytic enzymes can access the biomass macrostructure. Hydrolysis of cellulose is a sequential breakdown of the linear glucose chains, whereas hemicellulases must be capable of hydrolysing branched chains containing different sugars and functional groups (Jorgensen et al., 2007). Cellulose comprises ~30 – 40 % of sugars in most biomass feedstocks and glucose is the preferred carbon source for many microorganisms.

Traditionally most of the research has been focused on improving cellulases and decreasing the costs associated with the enzymatic hydrolysis of cellulose (Himmel et al., 2007; Schubert, 2006; Zhang et al., 2006). The presence of hemicelluloses has long been neglected when considering the performance of enzyme mixtures for hydrolysis of cellulose. However, sugarbeet pulp is a different type of feedstock that is equally rich in cellulose, hemicellulose and pectins. The structure of sugar beet pulp is highly complex and solubilization of sugar beet pulp requires synergistic action of variety of different enzymes. Cellulase alone cannot saccharify beet pulp unless the pectin layer is broken down by pectinase to make hemicellulose and cellulose accessible. The development of microorganisms capable of fermenting hemicellulose sugars efficiently is rapidly progressing (Hahn – Hägerdal et al., 2006; Jefferies, 2006). Many pretreatment methods also partially remove and degrade the hemicelluloses (Jorgensen et al., 2007). Some pretreatment methods leave the hemicellulose in the material and efficient hydrolysis of these materials therefore also requires use of hemicellulases. Due to complex structure of hemicelluloses a number of enzymes are required. It has been shown that addition of xylanases and pectinases significantly improves the performance of cellulases and increases cellulose conversion of pretreated corn stover, hardwoods

and softwoods (Berlin et al., 2005, 2006, 2007). As hemicelluloses vary between different plant species, the optimal enzyme mixture is most likely to be adjusted for each type of plant material.

Significant progress has been achieved in developing more efficient and cheaper enzymes for cellulose hydrolysis, these have only been optimized for one specific substrate and cannot be necessarily be applied to other substrates successfully (Zhang et al., 2006). Besides, other auxiliary enzyme activities can improve the performance of the enzyme preparation even when not directly involved in hydrolysis of cellulose (Berlin et al., 2005, 2006). Production of enzymes on the target lignocellulosic material for hydrolysis has shown that these enzyme preparations perform better than standard commercial enzyme preparations produced on substrates such as purified cellulose (Jorgensen et al., 2006; Baker et al., 1997; McMillan et al., 2001). This could beneficially be employed in biorefineries to produce enzymes on-site (Jorgensen et al., 2007). Moreover, on-site enzyme production could reduce enzyme costs due to less need for purification and stabilization of enzyme preparations (Himmel et al., 1999).

Recent study on thermophilic, high solids, leach bed anaerobic digestion of sugar beet pulp (Koppar and Pullammanappallil, 2008) has shown that 95% methane potential can be achieved in just 7 days, with a dry matter reduction of 92%. The digester reached a peak activity in just 2 days, implying rapid hydrolysis of beet pulp. These results indicate significant presence of polysaccharide hydrolyzing extracellular enzymes in the leach bed digester.

Thermophilic/Mesophilic bacteria (aerobic or anaerobic) are known to produce cellulases. Bacteria belonging to *Clostridium, Cellulomonas, Bacillus,*

Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibria, Microbispora, and Streptomyces can produce cellulases (Bisaria, 1991). Cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteriodes cellulosolvens* produce cellulases with high specific activity (Duff and Murray, 1996). Lai et al., (2001) have extensively studied the celluloytic activity in the anaerobic digester treating MSW. Buchholz et al., (1990) reported presence of significant amounts of extracellular polysaccharide-hydrolysing enzymes in two – stage mesophilic anaerobic digester system digesting sugar beet pulp. These studies indicate that there exist enzymes in the digester that can hydrolyze polysaccharides in beet pulp.

This research particularly, addresses the development of (1) methods for saccharification using commercial enzymes, (2) techniques for extraction of extracellular polysaccharide hydrolysing enzymes from thermophilic anaerobic digester and, (3) use of the extracted enzymes for solubilization of spent pulp. The monosaccharides that will be produced can serve as precursors for production of ethanol or other biobased products.

1.2 Objectives

The goal of this research was to investigate the feasibility of using extracellular enzymes extracted from a thermophilic anaerobic digester to hydrolyze beet pulp. Specific objectives were to:

- Validate a simple DNS (di-nitro salicyclic acid) assay to quantify sugar concentration in enzyme catalyzed reactions – (Chapter 2)
- Develop pre – treatment techniques for saccharification of sugar beet pulp using commercial enzymes – (Chapter 3)
- Compare effect of thermophilic and mesophilic temperature on biogasification of sugar beet pulp without using any pretreatment using single-stage leach bed digester – (Chapter 4)

- Assess the performance of single-stage leach bed digester in terms of the process in terms of its kinetics, methane yield and extent of degradation and comparison to other processes – (Chapter 4)

- Develop techniques for extraction of extracellular enzymes from the anaerobic digester – (Chapter 5)

- Determine the location of maximum extracellular enzyme activity in an anaerobic digester – (Chapter 5)

- Evaluate solubilization of sugar beet pulp using extracted anaerobic enzymes from anaerobic digester – (Chapter 5)

Table 1-1 Carbohydrates in sugar beet pulp as % dry weight of solids (Micard et al., 1996)

Component	Percentage	Component	Percentage
Rhamnose	2.4	Galacturonic acid	21.1
Xylose	1.7	Acetic acid	3.9
Arabinose	20.9	Methanol	1.8
Mannose	1.1	Protein	11.3
Galactose	5.1	Other	8.6
Glucose	21.1	Ash	3.6

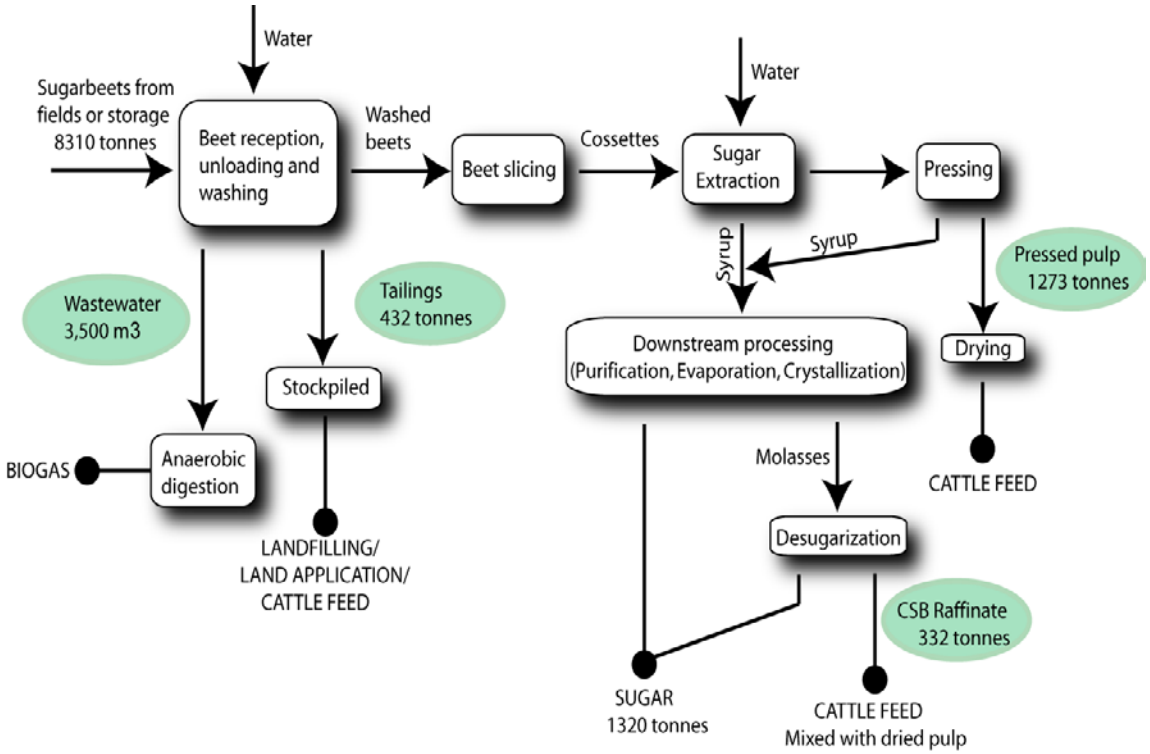


Figure 1-1 Schematic diagram of sugar beet processing highlighting daily waste and by-product flows

CHAPTER 2
MATERIALS AND METHODS

2.1 Introduction

This Chapter describes the construction and operation of thermophilic anaerobic digester employed for digestion of sugar beet pulp, methods of extraction of enzymes from the digester, preparation of assays for measuring activities of extracted enzymes, assays for testing activities of commercial enzymes on pure substrates. The Chapter also describes the equipment that was used for performing hydrothermolysis and procedures for validation of DNS (Di-nitro Salicylic Acid) for analysis of reducing sugars.

2.2 Materials

2.2.1 Anaerobic Digester

The digester, 5 L (0.005 m3), (working volume of 4 L, (0.004 m3), was constructed by modifying Pyrex glass jar (Fig 2-3). The height and inner diameter of the digester was 0.406 m (16 in.) and 0.0610 m (2.4 in.), respectively. The digester was sealed with a top lid, outer diameter of 0.0965 m (3.8 in.), using an O-ring fitted for gas and liquid tightness and clamped with a stainless steel clamp. Three ports were provided at the top of the lid, one for gas outlet, and others for sample withdrawal. At the bottom of the digester an outlet port was provided for draining. No additional external/internal mixing device was employed. The digester set-up is shown in Figure 2-1. Gas production from the digester was measured using a positive displacement gas meter. The device consisted of a clear PVC U-tube filled with anti-freeze solution, solid state time delay relay (Dayton OFF Delay Model 6X153E), a float switch (Grainger), a counter (Redington Inc.) and a solenoid valve (Fabco Air). The U-tube gas meter was calibrated in-line to determine volume of biogas per count. A count was considered as that amount

of gas read on syringe (in milliliters) for which the gas meter completes one whole number count (e.g. one count = 0.045 L, then two counts = 0.09 L and continued on). The pH in the digester was measured daily using pH meter (Accumet pH meter, Model 805 MP).

2.2.2 Feedstock

Sugarbeet pulp was provided by American Crystal Sugar Company, Minnesota. Aliquots of 0.45 kg (wet weight as received) of spent pulp were taken in Ziploc airtight plastic bags and stored in deep freezer. In digestion experiments content of one bag (i.e., 0.45 kg) was loaded into a digester for each run. To prevent compaction of the solids, 2 kg of a bulking material (lava rocks from landscaping supplier, 0.025 m (in average size) was also mixed with the spent pulp. The liquid volume held in the digester was 2 liters (0.002 m^3) after the addition of bulking material.

2.2.3 Hydrothermolysis Equipment

High pressure (4 bars) and high temperature (160 °C) was employed for pretreating beet pulp.To achieve this a beaker dyer (Labomat BFA – 24, Werner Mathis) was employed. The beaker dyer had a temperature range between (20 °C – 160 °C), temperature gradient between (0.3 °C – 4 °C) and a rotating speed between (5 – 65 rpm, stepless). Stainless steel beakers were available in standard size (volume – 200 ml). The beaker dyer was run in an automatic mode by creating a program to control temperature during operation. Cooling was provided by chilled water circulation around the equipment.

2.3 Methods

2.3.1Anaerobic Digestion Protocol

The bags were removed from the freezer 6 hours prior to the loading the digester to allow sufficient time for thawing. Experiments were carried out in the digester serially. All digestion experiments were carried out at thermophilic and mesophilic temperature by placing the digesters in an incubating chamber set at 55 °C and 38 °C respectively. The first run in the digester was inoculated with 2L (0.002 m^3) of inoculum taken from thermophilic digester that had been digesting desugarized molasses for over a year. In addition, 10 g L^{-1} of sodium bicarbonate was also added to buffer against pH changes. Once the gas production from first experimental run tapered down, the digesters were opened and next batch (0.45 kg) of pulp charged from top. The second run in digester was initiated by the digester liquor remaining from first run, no further inoculum or sodium bicarbonate was added. The digester was then closed, placed in the incubator and digestion allowed to proceed. Similarly mesophilic digestion runs were carried out by starting the first run using inoculum from mesophilic digestion.

2.3.2 Hydrothermolysis Protocol

The bags were removed from the freezer 6 h prior to experiment to allow sufficient time for thawing. The experiment was carried out at 160 °C for 2 hours. At the start of experiment, 3 grams of sugar beet pulp (as received) was added to four beakers (labeled 1 - 4). The beakers (labeled 1 - 4) were then filled with 150 ml of deionized water using a measuring cylinder. The beakers were always filled to equal volumes to avoid risk of overheating due to unequal volumes. The beakers were than closed carefully with a specially designed stainless steel, magnetic lid and tightened using an allen screw. An embedded rubber gasket on the lid provided an air-tight fit. The beakers

were placed in Mathis equipment and the temperature program was activated to reach160 °C quickly. Beakers 1 - 4 were removed from the dye at 15 minutes, 30 minutes, 1 hour and 2 hour, respectively and allowed to cool down at room temperature for 30 minutes. The lid was unscrewed and contents (hydrolysate and residual solids) of the beakers were transferred to 50 ml sterile centrifuge tubes (Corning, 4558). The tubes were then stored in a deep freezer. Another set of experiments using 1% ortho-phosphoric acid solution instead of deionized water was also carried out. Procedures similar to that employed previously were used for these experiments.

2.3.3 Extraction of Enzymes from the Digester

Extracellular enzyme extraction methods were adapted from (Zhang et al., 2007). Activities of extracellular enzyme were defined as cell-bound, cell-free and biofilm-associated. The cell-free and cell-associated enzyme activities were to examine activities of suspended cells in the liquid phase (mixed liquor), while biofilm-associated enzyme activities were determined to evaluate activities adhered to solid phase (digesting beet pulp).

Cell-free and cell-associated enzyme activities in the liquid phase were determined as follows:

- Centrifuge 10 ml of leachate sampled from the digester for 10 minutes
- The supernatant was collected and used to measure cell-free activity.
- The pellet was washed twice by suspending 10 ml in potassium dihydrogen phosphate buffer (pH 7.0, 0.1 mol)
- Pellet was re-suspended in 2 ml sodium acetate buffer (pH 6.0, 0.05 mol) and centrifuged for 10 minutes
- The supernatant was collected for determining cell-bound activities.

Biofilm-associated enzyme activity in the solid phase was determined as follows:

- 20 g of digesting beet pulp was taken out from the digester
- Sample was washed twice by suspending in 10 ml potassium dihydrogen phosphate buffer (pH 7.0, 0.1 mol)
- Re-suspend in 2 ml sodium acetate buffer (pH 6.0, 0.05 mol) and centrifuged for 10 minutes
- The supernatant was collected for determining biofilm-associated activities.

2.3.4 Assays for Measuring Enzymatic Activities of Commercial Enzymes and Extracted Anaerobic Enzymes

Commercial enzyme preparations used were cellulase, hemicellulase and pectinase. Each of them was assayed for enzymatic activity using specific substrates. The methods were adapted from Spagnuolo et al., (1997).

Total cellulase activity was determined by the Filter-Paper assay procedure. The one ml of enzyme was incubated at 50 °C for 60 min with Whatman filter no.1 strips (1 cm x 6 cm) in 1.5 ml of 0.05 M citrate buffer pH 4.8. The soluble sugars were measured by the DNS method.

Pectinase activity was obtained by determining the amount of galacturonic acid released by hydrolysis from pectin at 50 °C for 60 min. No standard conditions for assay were available to measure activity. Based on literature review it was found that polygalacturonases have an optimal pH range of 3.5 – 5.5 and optimal temperature range of 30°C - 50°C (Jayani et al., 2005). One ml of enzyme was incubated at 50 °C for 60 min with 50 mg analytical grade pectin and one ml of 0.05 M citrate buffer pH 4.8. Soluble sugars were measured by the DNS method.

Hemicellulase activity was measured using arabinogalactan as substrate. One of enzyme was incubated at 40 °C and 50 °C for 30 min with 50 mg analytical grade arabinogalactan 1.5 ml of 0.05 M citrate buffer pH 4.8. Soluble sugars were measured by the DNS method.

Similar procedures were employed for measuring activities of extracted enzymes by replacing commercial enzymes with extracted enzymes.

2.3.5 Saccharification of Sugar Beet Pulp and Hydrolysate after Hydrothermolysis

Tubes containing hydrolysate were removed from the freezer 8 hours prior to the start of the experiment. Hydrolysate was separated from residual solids by decanting. 1 ml of hydrolysate,1 ml of Citrate buffer (0.05 M, pH 4.8) and 1 ml diluted (1000^{th}) enzyme (pectinase and / or hemicellulase) were added to four sterile test tubes. The tubes were then closed and placed in a warm water bath (45°C) to equilibrate for 5 minutes. Finally, the tubes were placed on a shaker in an incubator at 50°C. The tubes were withdrawn at different time intervals for reducing sugar analysis. Similar procedure was repeated for raw sugar beet pulp saccharification.

2.4 Analysis

For Anaerobic digestion: Methane production rate from the digesters was measured using a gas metering device described in Koppar and Pullammanappallil (2008). Analytical procedures for determination of total solids (TS), volatile solids (VS) content and methane content in biogas followed those described in Polematidis et al. (2008). Volatile organic acid concentrations were analyzed on Shimadzu GC- 9AM with a Flame Ionization Detector (FID) gas chromatograph. The GC-FID was equipped a 1.7 m long by 3 mm inner diameter glass column packed with 100/120 chromosorb WAW coated with 1% phosphoric acid. High purity nitrogen (99.9%) was used as the carrier

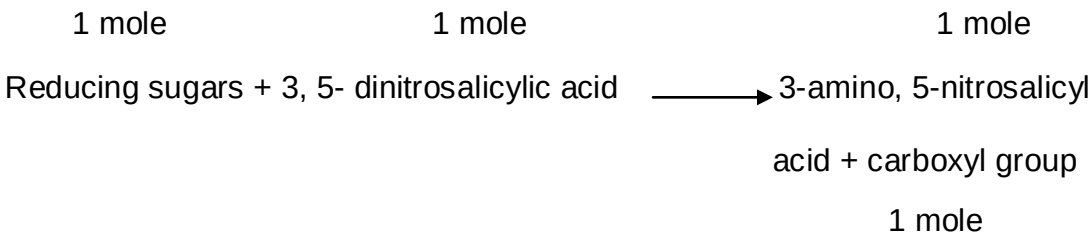
gas at a flow rate of 20 ml/min. Hydrogen and air were used as the combustion gases, flowing at 0.6 and 1.0 ml/min, respectively. Temperatures of injector, column and detector were 180 °C, 145°C and 200°C respectively. The performance of the digesters was evaluated by fitting the cumulative methane production data to the modified Gompertz equation (Polematidis et al., 2008).

Reducing sugars analysis – Definition: - Reducing sugar is any sugar that in an alkaline solution forms some aldehyde or ketone. This allows sugar to act as a reducing agent. All monosaccharides which contains ketone group are known as ketoses and those contain aldehyde group are known as aldoses. Common examples of reducing sugars are; glucose, mannose, galactose etc., (hexoses) and arabinose, xylose, etc., (pentoses).

Chemistry (Miller, 1959)**: -** A reducing sugar occurs when its anomeric carbon (the carbon which is linked to oxygen atoms) is in the free form. Since sugars occur in a chain as well as ring structure it is possible to have an equilibrium between these two forms. The aldehyde can be oxidized to a carboxyl group via redox reactions. The chemical that causes this oxidation becomes reduced. Thus the reducing sugar is the one that reduces certain chemicals.

Detection (Miller, 1959)**:**.This method tests for the presence of free carbonyl group (C=O), in the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions. 3, 5-Dinitrosalicyclic acid (DNS) is

an aromatic compound that reacts with reducing sugar and other molecules to form 3-amino-5-nitrosalicyclic acid which absorbs light strongly at 546 nm.



The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described (Lindsay, 1973). The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3, 5-dinitrosalicylic acid (Miller, 1959).

2.5 Validation of Method

Figure 2-1 shows the individual calibration for glucose, arabinose and galacturonic acid and mean calibration (as glucose equivalent). The individual calibrations of arabinose and galacturonic acid fall within 1 std. dev and 20% error bound. Hence the mean of concentrations of all three sugars were plotted as glucose equivalent. But the stoichiometry of reducing sugars reacting with DNS agent suggests that different color intensities are obtained by different reducing sugars. This fact has been elucidated in Figure 2-1. Therefore, various mixtures of sugar concentrations (1.5 mg/ml. 0.75 mg/ml,

0.45 mg/ml and 0.15 mg/ml) were measured for absorbance to get a comprehensive calibration for measuring reducing sugars in sugar beet pulp. Tables 2-1, 2-2, 2-3, 2-4 summarize the results for calibrations. A plot of mean concentration and absorbance was plotted for final calibration (Figure 2-2). Once again the errors in measurements were found to be within 1 std. dev from mean and within 20% error bound around mean. An internal standard was prepared for an unknown sample (leachate from digester) and color was fully developed for the unknown sample by DNS reaction and measured for absorbance. Then a known amount of sugar was added to this sample. The increase in the absorbance upon the second color development is equivalent to the incremental amount of sugar added. Table 2-5 shows the % recovery of sugars after spiking with known amount of sugars to the unknown sample. The % recovery was above 90% for both arabinose and glucose and ~80% for galacturonic acid.

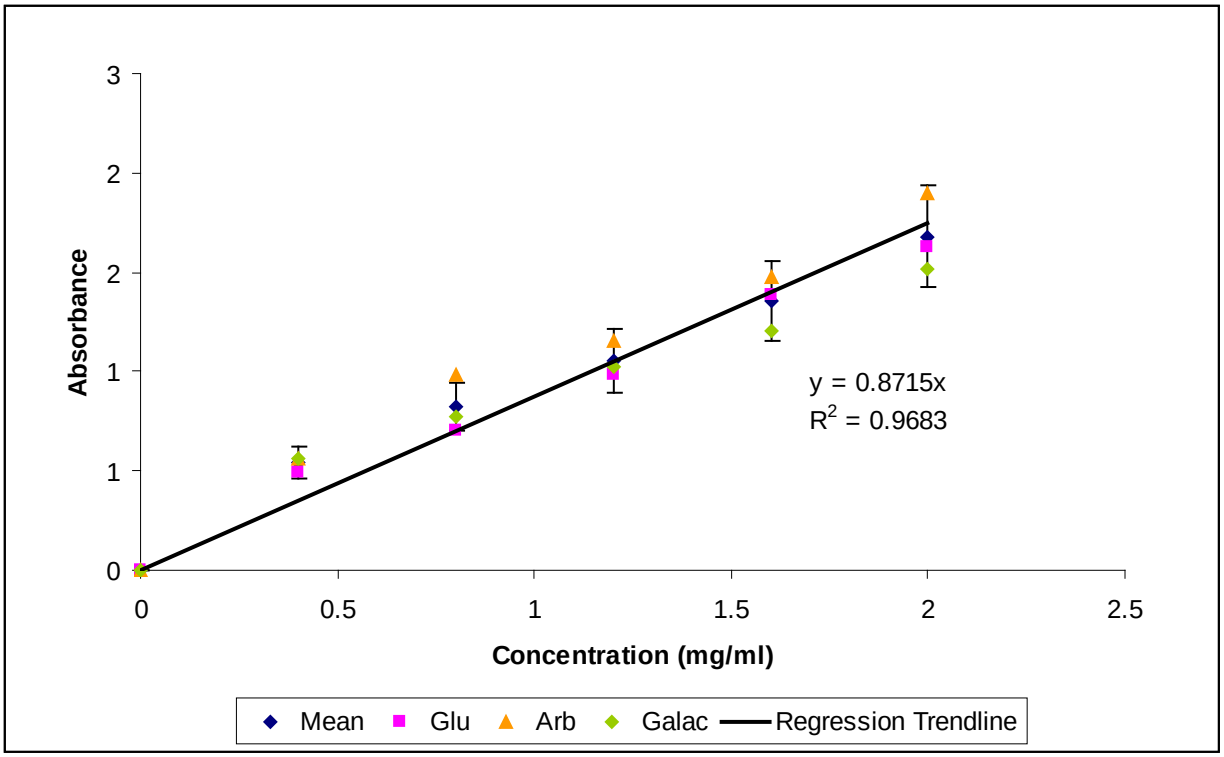


Figure 2-1 Individual sugars calibration of DNS method - (1 std. dev - 20% error bound)

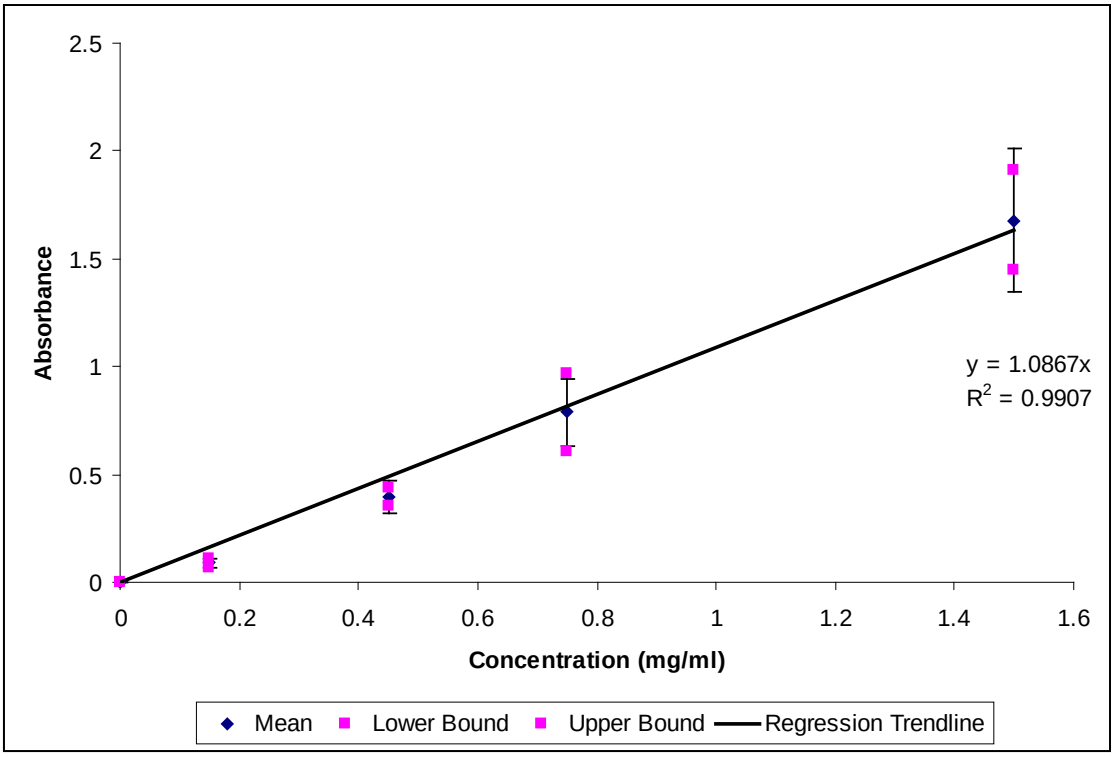


Figure 2-2 Final calibration of DNS method - (1 std. dev - 20% error bound)

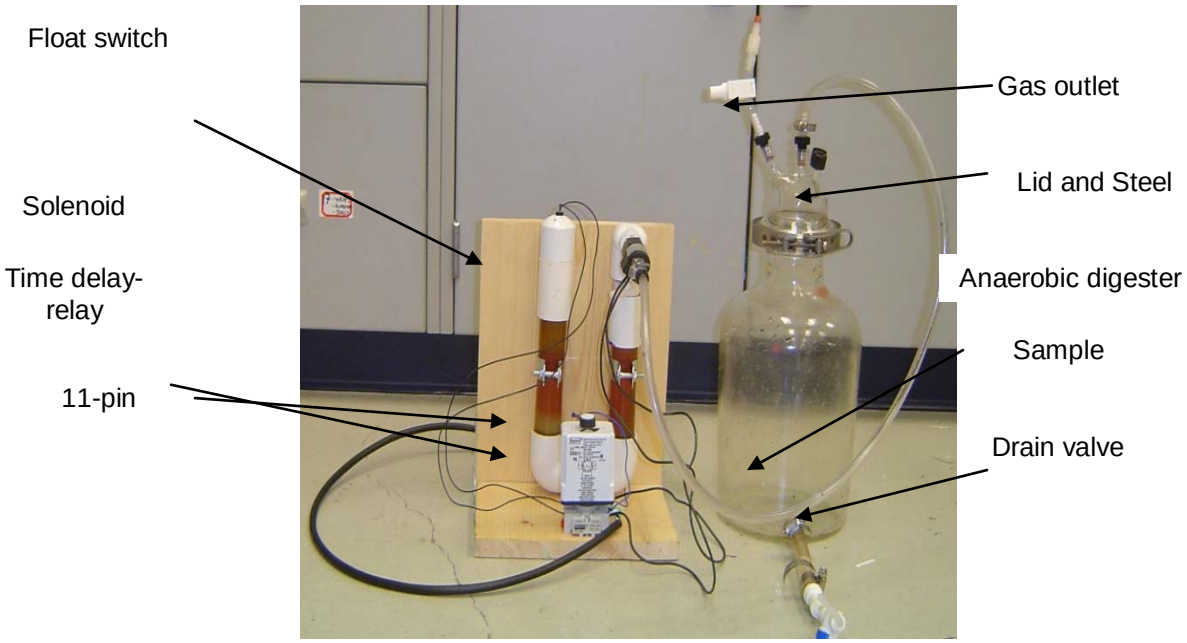


Figure 2-3 Digester setup for biogasification of sugarbeet pulp

Table 2-1 Absorbance readings of reducing sugars - (1.5 mg/ml)

Component	Glucose	Arabinose	Galacturonic acid	Absorbance
A	0.5	0.5	0.5	1.429
B	0.75	0.75	0	1.857
C	0	0.75	0.75	1.449
D	0.75	0	0.75	1.458
E	1	0.2	0.3	1.823
F	0.3	1	0.2	1.968
G	0.2	0.3	1	1.697
H	1	0.5	0	1.973
I	0	1	0.5	1.88
J	0.5	0	1	1.80
K	1.5	0	0	1.715
L	0	1.5	0	1.525
M	0	0	1.5	1.252
		Mean		1.679
		Standard deviation		0.233

Table 2-2 Absorbance readings of reducing sugars - (0.75 mg/ml)

Component	Glucose	Arabinose	Galacturonic acid	Absorbance
A	0.25	0.25	0.25	0.983
B	0.375	0.375	0	1.042
C	0	0.375	0.375	0.843
D	0.375	0	0.375	0.852
E	0.5	0.15	0.1	0.83
F	0.1	0.5	0.15	1.039
G	0.15	0.1	0.5	0.667
H	0.5	0.25	0	0.837
I	0	0.5	0.25	0.529
J	0.25	0	0.5	0.555
K	0.75	0	0	0.853
L	0	0.75	0	0.674
M	0	0	0.75	0.539
		Mean		0.788
		Standard deviation		0.181

Table 2-3 Absorbance readings of reducing sugars - (0.45 mg/ml)

Component	Glucose	Arabinose	Galacturonic acid	Absorbance
A	0.15	0.15	0.15	0.381
B	0.225	0.225	0	0.455
C	0	0.225	0.225	0.372
D	0.225	0	0.225	0.37
E	0.2	0.15	0.1	0.432
F	0.1	0.2	0.15	0.377
G	0.15	0.1	0.2	0.39
H	0.35	0.1	0	0.4
I	0	0.35	0.1	0.423
J	0.1	0	0.35	0.344
K	0.45	0	0	0.441
L	0	0.45	0	0.447

Table 2-3. Continued

M	0	0	0.45	0.311
		Mean		0.396
		Standard deviation		0.0428

Table 2-4 Absorbance readings of reducing sugars - (0.15 mg/ml)

Component	Glucose	Arabinose	Galacturonic acid	Absorbance
A	0.05	0.05	0.05	0.079
B	0.075	0.075	0	0.108
C	0	0.075	0.075	0.087
D	0.075	0	0.075	0.079
E	0.09	0.04	0.02	0.097
F	0.02	0.09	0.04	0.092
G	0.04	0.02	0.09	0.075
H	0.1	0.05	0	0.106
I	0	0.1	0.05	0.097
J	0.05	0	0.1	0.06
K	0.15	0	0	0.112
L	0	0.15	0	0.113
M	0	0	0.150	0.056
		Mean		0.089
		Standard deviation		0.0188

Table 2-5 (%) Sugar recovery after known spike for reducing sugars

Sample	Mixed Liquor	Sugar content (mg/ml)	Sugar spiked into sample (mg/ml)	Expected sugar concentration (mg/ml)	Measured sugar concentration (mg/ml)	% Recovery
A	1 ml	0.167	0.15 mg Glucose	0.317	0.303	95.58
B	1 ml	0.167	0.15 mg Arabinose	0.317	0.290	91.48
C	1 ml	0.167	0.15 mg Galacturonic acid	0.317	0.253	79.81

CHAPTER 3
HYDROTHERMOLYSIS PRETREATMENT AND ENZYMATIC SACCHARIFICATION OF SUGAR BEET PULP

3.1 Introduction

This chapter discusses pretreatment and enzymatic hydrolysis of sugar beet pulp. The aim of this study was to solubilize sugar beet pulp as soluble polysaccharides by hydrothermolysis- pretreatment and maximize the conversion of these polysaccharides into monomeric sugars by enzymatic hydrolysis.

3.2 Background

Sugar beet pulp (SBP) is an important by-product from the sugar refining industry. It is especially rich in polysaccharides, mainly pectic substances (Michel et al., 1985, 1988) and very low in lignin. On a dry weight basis, SBP contains ~66% polysaccharides, consisting of 22.5% cellulose, 21% hemicellulose, and 22.5% pectin (Thibault and Rouau, 1990). Since sugar beet pulp is low in lignin and high in non-starchy polysaccharide content it is an attractive feedstock for production of fuel ethanol.

Typically in biomass cellulose and hemicellulose are potential sources of fermentable sugars (Hinman et al., 1989; Ho et al., 1998; Taherzadeh et al., 1999; Sreenath and Jefferries, 2000). Hemicellulose hydrogen-bonds to cellulose microfibrils, thus forming a network that provides the structural backbone to plant cell wall (Mosier et al., 2005). The structure of hemicellulose which is known as araban, is a group of branched chain compounds with the main chain composed of α-1, 5-linked L-arabinose and the side chain by α-1, 3-linked L-arabinose (Vogel, 1991). Besides arabinose and galactose, hemicellulose also contains xylose and glucose as the main sugar components (Kobayashi et al., 1993). These large amounts of plant cell-wall materials

can be processed to yield fermentable sugars. In addition to cellulose and hemicelluloses, SBP is also rich in pectins.

The conversion of polysaccharides into monomeric compounds is usually obtained by enzymatic hydrolysis and the yield depends on the presence of specific activities and the accessibility of the substrate. The matrix of pectic substances in the cell wall may hinder the degradation of cellulose fibrils, by cellulases (Massiot et al., 1989). Typically cellulosic biomass must be pretreated by heating and/or with chemicals to realize high yields vital to commercial success in biological conversion (Mosier et al., 2005). Pretreatment is among the most costly steps and has a major influence on the cost of both prior (e.g., size reduction) and subsequent (e.g., enzymatic hydrolysis and fermentation) operations (Wooley et al., 1999; Lynd et al., 1996). For example, better pretreatment can reduce use of expensive enzymes (Wyman et al., 2005).

Although several pretreatments are promising, their relative attributes differ, but comparisons have been difficult due to differences in research methodology and substrate used (Wyman et al., 2005). Table 3.1 enlists some of the prominent pretreatment methods and main mechanisms involved. There is an overall consensus that the successful pretreatment should (Holtzapple and Humphrey, 1984):

- Maximize the enzymatic convertibility
- Minimize loss of sugars
- Maximize the production of other valuable by-products
- Not require the addition of chemicals toxic to the enzymes or the fermenting microorganisms
- Avoids the need for particle size reduction of biomass feedstocks
- Preserves the pentose sugar (hemicellulose) fractions

- Limits formation of degradation products that inhibit growth of fermentative microorganisms
- Minimize the use of energy , chemicals and capital equipment
- Be scalable to industrial size

In practical applications it is difficult to accomplish all the above mentioned issues in any process. However, the last two points are important for economical viability of a given industrial process (Jorgensen et al., 2007). The general ideas are to alter or remove hemicelluloses and/or lignin, increase surface area and decrease the crystallinity of cellulose (Wyman et al., 2005; Mosier et al., 2005). Table 3.2 shows comparison of different hydrolysis methods.

Very little information is available on utilization of SBP as a potential feedstock for conversion to ethanol. Three approaches have been investigated for hydrolysis of SBP;

- Enzymic hydrolysis (Thibault and Rouau, 1990)

- Dilute acid hydrolysis (Chamy et al., 1994)

- Fungal hydrolysis (Doran and Foster, 2000)

Thibault and Rouau (1990) conducted studies on enzymic hydrolysis of polysaccharides in sugar beet pulp. The aim was to obtain maximum conversion to sugars and to determine factors limiting degradation. The beet pulp fibers were degraded with three enzymic preparations (SP 249 from *Aspergillus aculeatus*, Driselase from *Irpex lacteus* and Onozuka from *Trichoderma viride*). The overall extent of polysaccharide hydrolysis was ~48% with Onozuka and up to ~80% with SP 249 and Driselase. These values increased to ~90% when chemical pre-treatments such as, persulphate, chlorite or hydrogen peroxide were employed. Chamy et al., (1994) reported on acid hydrolysis of sugar beet pulp as an alternative pretreatment. The

general purpose of this work was to optimize substrate pretreatment by selectively solubilizing the hemicellulose fraction to render a cellulose-enriched fraction for enzyme hydrolysis. Hydrochloric acid and sulfuric acid were used for acid hydrolysis, and *Pichia stipis* was employed for subsequent fermentation of sugars to ethanol. The solids removal, degree of hemicellulose and cellulose hydrolysis was higher for HCl. But higher cellulose hydrolysis was deemed unsuitable because hexose from hydrolyzed cellulose is preferentially fermented by *Pichia stipitis* producing ethanol at high enough concentrations to inhibit pentose metabolism. Hence sulfuric acid was selected as hydrolytic agent, as it produced less hydrolysis of cellulose. The best conditions for sugar beet pulp hydrolysis were; 55 g/l of 32 – 50 mesh sugar beet pulp, 1.1 g sulfuric acid/g sugar beet pulp, reaction temperature of 80 °C, stirring speed of 400 rpm, and reaction time of 2 hours. Under such conditions, 86.3% and 7.8% of hemicellulose and cellulose hydrolysis was obtained. Doran and Foster (2000) carried out enzymatic hydrolysis of beet pulp using fungal cellulases and pectinases. The sugar beet pulp was preincubated with fungal enzymes (0.57% v/v - cellulase and 1% v/v - pectinase) at 42 °C for 24 hours. The hydrolyzed liquid was subsequently fermented to ethanol with an engineered *E.Coli*. The ethanol yield was 0.27 g ethanol/g dw beet pulp. When sugar beet pulp was treated with fungal load of 1% v/v of both cellulase and pectinase, high ethanol yield of 0.328 g ethanol/g dw beet pulp was obtained. This indicated that about 88% of sugars were solubilized. Typically the theoretical yield of ethanol from beet pulp is 0.369 g ethanol/g dw beet pulp.

Based on literature review it is clear that enzymatic hydrolysis is the method of choice because near theoretical yields are possible, but an initial pretreatment of

biomass is required to release polysaccharides from cell-wall. Otherwise a high loading of enzymes together with a long reaction time can achieve high sugar release. Considering the disadvantages of most conventional methods (see Table 3-2), hot compressed water or hydrothermolysis pretreatment could offer high hydrolysis rates and no environmental and corrosion problems. No literature was available on application of this pretreatment method on sugar beet pulp.

Aqueous pretreatments have been used to pretreat lignocellulosic material for more effective enzymatic conversion of the cellulose to glucose. Water pretreatments use pressure to maintain the water in the liquid state at elevated temperatures (Bobleter, 1994; Bobleter et al., 1976, 1981; Bobleter and Concin, 1979; Hormeyer et al., 1988a, b; Walch et al., 1992; Mok and Antal, 1992; Kohlman et al., 1995; Allen et al., 1996; van Walsum et al., 1996). Flow-through processes pass water maintained in the liquid state at elevated temperatures over the cellulosic material. This type of pretreatment has been termed as hydrothermolysis (Bobleter et al., 1976, 1981; Bobleter and Concin, 1979). Hydrothermolysis studies by Hormeyer et al., 1988 and Walch et al., 1992, have shown that hot water removes and solubilizes hemicellulose. Mok and Antal, (1992), found that hemicellulose will dissolve at 200 to 230 °C. van Walsum et al., (1996) pretreated fresh sugarcane bagasse, aspen chips, and mixed hardwood flour using liquid hot water at 220 °C. Whereas hemicellulose solubilization was nearly complete, solubilization of cellulose was less than 10%. Water under pressure can penetrate the cell structure of biomass, hydrate cellulose, and solubilize hemicellulose. The pK_a of water is affected by temperature such that the pH of pure water at 200 °C is nearly 5.0. Water also has a unusually high dielectric constant that

enables ionic substances to dissociate and dissolve hemicellulose, and one half to two thirds of the lignin dissolves from most cellulosic biomass when they are treated at 220 °C for 2 minutes (Wyman et al., 2005). In addition, hot water cleaves hemiacetal linkages and liberates acids that catalyze breakage of ether linkages in biomass (Antal, 1996). The preferred temperature for this approach has been shown to be between 180 and 190 °C for corn stover and 150 and 160 °C for corn fiber. Pretreatment using water at a controlled pH minimizes hydrolysis of the oligosaccharides, while causing hydration of the structure by liquid water at a pressure above the saturation vapor pressure of water at the selected temperature. The primary objective is to avoid the formation of monosaccharides that could degrade further during high temperature pretreatment. Enzymes may be added at lower temperatures for hydrolysis of the resulting cellulose and hemicellulose oligomers (Ladisch et al., 1983; Lynd et al., 1991; Holtzapple, 1993; Mosier et al., 1999). This system has been applied to variety of biomass feedstocks for e.g., corn stover (Mosier et al., 2005, Weil et al., 1997, 1998; Kohlmann et al., 1995).

This chapter addresses the application of hydrothermolysis for sugar beet pulp to solubilize polysaccharides and subsequent enzymatic hydrolysis to produce monosaccharides.

3.3 Methods

The following methods were employed for studies presented in this chapter:

Enzymatic hydrolysis of analytical grade substrates, pectin, arabinogalactan, arabinogalactan using commercial enzymes pectinase (P2611), hemicellulase (H2125)..

1. Enzymatic hydrolysis of sugar beet pulp using commercial enzymes, pectinase (P2611), hemicellulase (H2125) at different loadings.
2. Hydrothermolysis of sugar beet pulp

3. Combination of hydrothermolysis pretreatment and enzymatic hydrolysis of sugar beet pulp using commercial enzymes pectinase (P2611), hemicellulase (H2125).

Saccharification was measured by measuring reducing sugars using DNS method. Details of methods are given in Chapter 2 (Sections 2.2.3, 2.3.2, 2.3.4, 2.3.5, 2.4)

3.4 Results

Comparison of activities of commercial enzyme preparations on pure substrates and sugar beet pulp: Activity of commercial pectinase enzyme, Pectinex, preparation was obtained by determining the amount of reducing sugars released by hydrolysis from analytical grade pectin in 1 h. Pectinase activity was expressed as µmol of reducing sugars released per ml per minute. The manufacturer specified pectinase activity for this enzyme was 9500 µmol/ml/hr or ~158 IU. Two experiments were conducted using analytical grade pectin as substrate. One was loaded with 158 IU of enzyme and in the other the enzyme activity was diluted to a 1000^{th} . In the first experiment an activity of 11040 µmol/ml/hr or ~184 IU was measured over three hours and in the second an activity of 8.64 µmol/ml/hr or ~0.144 IU was measured. These results were in good agreement with manufacturer specifications, validating the methods used in this study for conducting enzyme substrate reactions.

Similarly two experiments were conducted to verify the hemicellulase enzyme (H2125, Sigma-Aldrich) activity. The manufacturer specified activity was 300 µmol/ml/hr or 5 IU. Use of hemicellulase enzyme in the concentrated form showed an activity of 480 µmol/ml/hr or ~8 IU. When diluted 1000 times an activity of 0.56 µmol/ml/hr or ~0.0093 IU was measured. Again the activities measured were in agreement to specifications.

Saccharification of spent sugar beet pulp: Profiles of mean (%) saccharification for enzyme loadings of (9500 µmol/ml/hr – pectinase; 300 µmol/ml/hr – hemicellulase) and (9.5 µmol/ml/hr – pectinase; 0.3 µmol/ml/hr – hemicellulase) are shown in Figure 3-1 A and 1 B, respectively. The plot also shows effect of various combinations of hemicellulase and pectinase in concentrated and dilute forms. It can be seen from Figure 1 A that, after adding only pectinase (9500 µmol/ml/hr), 18.49% (standard deviation = 2.12%) of beet pulp was solubilized in 3 hours. The pulp solubilized rapidly in next 3 hours and reached a peak value of 58.30% (standard deviation = 6.00%), but dropped to 46.34% (standard deviation = 5.19%) after 19 hours from the start. Upon using only hemicellulase (300 µmol/ml/hr), 9.08% (standard deviation = 2.34%) of beet pulp was saccharified in 3 hours, saccharification increased steadily to 31.96% (standard deviation = 4.05%) after 6 hours and increased slightly to 33.28% (standard deviation = 4.96%) in 19 hours. Addition of hemicellulase (300 µmol/ml/hr) 3 hours after initiating reaction with pectinase, solubilization increased slightly to 19.69% (standard deviation = 0.87%) in next 3 hours from 16.25% (standard deviation = 1.38%) and further increased to of 89.51% (standard deviation = 2.81%) in 19 hours.

Profile 1B shows % saccharification on addition of diluted commercial enzyme preparations (pectinase – 9.5 µmol/ml/hr and hemicellulase – 0.3 µmol/ml/hr). After adding pectinase (9.5 µmol/ml/hr) only, 6.70% beet pulp solubilized in 3 hours, increased slightly to 7.23% in next 3 hours, and further increased to 18.03% at the end of 19 hours. Similarly in another run pectinase alone saccharified 12.68% of polysaccharides in 6 hours, no further significant increase in saccharification was seen

when hemicellulase (0.3 µmol/ml/hr) was added to the mixture after 6 hours. When a combination of pectinase (9.5 µmol/ml/hr) and hemicellulase (0.3 µmol/ml/hr) was added to start the run, 6.82% of sugar beet pulp was solubilized in 3 hours, increased to 18.13% in next 3 hours and reached a peak value of 20.54% at the end of 19 hours. If only hemicellulase (0.3 µmol/ml/hr) was added, only 1.24% beet pulp was saccharified in 3 hours, increased slightly to 2.57% at 6 hours, and reached 5.40% at the end of 19 hours. In another run hemicellulase (0.3 µmol/ml/hr) alone saccharified 4.79% of polysaccharides in 6hours, slight increase in saccharification was seen when pectinase (9.5 µmol/ml/hr) was added after 6 hours. The results have been summarized in Table 3-3, 3-4, 3-5, 3-6, 3-7, respectively.

. When a combination of pectinase (9500 µmol/ml/hr) and cellulase (1000 µmol/ml/hr) was added to start the run, 5.86% of sugar beet pulp was solubilized in 3 hours, increased to 21.51% in next 3 hours. Upon addition of hemicellulase (300 µmol/ml/hr) % saccharification reached a peak value of 80.81% at the end of 19 hours. Table 3-5 shows the % saccharification on addition of cellulase to pectinase and hemicellulase.

Profile of mean (%) saccharification after combination of heat and /or acid pre-treatment on sugar beet pulp is shown in Figure 3-2. During hydrothermolysis of sugar beet pulp, 7.09% (standard deviation = 0.47%) of beet pulp was saccharified in 15 minutes. Saccharification further increased to 17.80% (standard deviation = 1.05%) in 30 minutes, 44.95% (standard deviation = 10.47%) in 1 hour, but dropped significantly to 10.95% (standard deviation = 0.85%) at the end of 2 hours. A combination of heat (160 °C) and acid (1% o-phosphoric acid) was tested. 9.51% of sugar beet pulp was

hydrolysed in 15 minutes. Saccharification increased to 29.16% in just 30 minutes, but dropped to 17.11% at 1 hour and increased slightly to 19.01% at the end of 2 hours. The results have been summarized in Table 3-8 and 3-9.

Profile of mean (%) saccharification is shown in Figure 3-3 when hydrothermolysis pre-treatment is followed with enzymatic hydrolysis to enhance saccharification of sugar beet pulp. After adding pectinase (9.5 µmol/ml/hr) only, 55.28% (standard deviation = 2.22%) sugars in hydrolysate were solubilized in 3 hours. Solubilization increased slightly to 56.09% (standard deviation = 2.96%) in next 3 hours, and further increased to 69.99% (standard deviation = 0.94%) at the end of 19 hours.

On adding pectinase (9.5 µmol/ml/hr) at the start, 58.33% (standard deviation = 1.14%) sugars in hydrolysate were solubilized in 3 hours, but % saccharification dropped slightly to 55.14% (standard deviation = 0.81%) in 6 hours. Hemicellulase (0.3 µmol/ml/hr) was added to this mixture at 6 hours; saccharification increased to 88.18% (standard deviation = 3.56%) at the end of 19 hours. When a combination of pectinase (9.5 µmol/ml/hr) and hemicellulase (0.3 µmol/ml/hr) was added to start the run, 70.21% (standard deviation = 0.87%) of sugars in hydrolysate were saccharified in 3 hours, increased to 80.81% (standard deviation = 2.15%) in next 3 hours and reached a peak value of 85.09% (standard deviation = 2.02%) at the end of 19 hours. If only hemicellulase (0.3 µmol/ml/hr) was added, 56.28% (standard deviation = 1.21%) of sugars in hydrolysate were saccharified in 3 hours, increased slightly to 60.70% (standard deviation = 2.22%) at 6 hours, and reached 67.55% (standard deviation = 0.61%) at the end of 19 hours. When hemicellulase (0.3 µmol/ml/hr) was added initially to start the hydrolysis, 58.99% (standard deviation = 1.55%) of sugars in hydrolysate

were solubilized in 3 hours, increased slightly to 59.99% (standard deviation = 1.61%) at 6 hours. Pectinase (9.5 µmol/ml/hr) was added after 6 hours from start, saccharification reached a peak value of 82.86% (standard deviation = 2.22%) after 19 hours. The results have been summarized in Tables 3-10, 3-11, 3-12.

3.5 Discussion

Activities of commercial enzyme preparations on pure substrates and sugar beet pulp: Commercial pectinase enzymes used in the studies here showed activities comparable to that specified by the manufacturer when incubated with analytical grade pectin. It should be noted that the activity specified by the manufacturer was ≥ 9500 (µmol/ml/hr), hence a higher activity obtained in our study is acceptable. The activity of pectinase enzyme was sustained when applied to untreated pulp for a short duration (3 h), indicating that it has mainly pectinolytic activities, including pectinlyase that depolymerizes the highly esterified pectin.

Commercial hemicellulase enzymes used in the studies here showed slightly higher activities than that specified by the manufacturer when incubated with analytical grade arabinogalactan. The activity of hemicellulase enzyme incubated with sugarbeet pulp was sustained for a longer duration (6 h). Slightly higher activities were obtained from hemicellulase enzymes. This can be attributed to the fact that the enzyme mixture could be undefined containing other activities such as, mananase, xylanase and other glycolytic enzymes. It is possible that these activities likely were suppressed when the substrate was pure, but were triggered when applied to sugar beet pulp, which is known to contain ~10% other sugars (rhamnose, galactose, xylose and mannose), leading to additional sugar release from sugar beet pulp.

Enzymatic saccharification of sugar beet pulp: Pectinase (diluted/ undiluted) and hemicellulase (diluted/undiluted) applied singly do not achieve very high saccharification compared to combination of P – H in any order. Pectinase by itself showed high saccharification (58%) than H – alone. Pectinase and Hemicellulase should act together to achieve rapid hydrolysis and that order of enzyme addition may not be crucial in current study. Addition of pectinase had a greater impact on saccharification during the initial period of incubation, whereas addition of hemicellulase was essential during the later stages of hydrolysis to achieve near complete saccharification of sugar beet pulp. Depectination of sugar beet pulp is an essential step to provide access to hemicellulase and cellulase enzymes to saccharify hemicellulose and cellulose fibers. Thibault and Rouau (1990) obtained ~ 80% saccharification from hydrolysis of beet pulp using SP249 enzymes, 84% by Driselase and only 48% by Onozuka after 120 hours. Both Driselase and SP249 had high pectinolytic activities, and also exhibited partial cellulolytic and hemicellulolytic activities. Spagnuolo et al., (1997), achieved a saccharification, that ranged between 16.4% to 44.6% by using pectinase enzyme. These values are comparable to saccharification values obtained from current study ranging between18.5% to 46.3% when, pectinase was used to solubilize sugar beet pulp. Pectinex is able to depolymerize both high and low esterified pectin and also partially hydrolyze cellulose, hemicellulose, starch and protein (Schmitt, 1988). Van der Broke et al., (1997) investigated the macerating effects of Pectinex enzyme preparation and endopectinlyase was found to be the main maceration enzyme. Hence sugar yield was low when sugar beet pulp was solubilized using hemicellulase singly because the pectin layer was not broken down enough to provide access to hemicellulose fibers in

beet pulp. Also, cellulose fibers are encased in the matrix of hemicelluloses and pectin has to be depolymerized and dissolved before cellulase enzymes can access and solubilize cellulose. Therefore, the hydrolysis of pectic substances requires interaction of several enzymes (Stutzenberger, 1992; Sajjanantakul and Pitifier, 1991; Pilnik and Voragen, 1991). Thibault and Rouau, (1990) obtained similar results when SP249 and Onozuka were used as an admixture nearly all polysaccharides were solubilized. Spagnuolo et al., (1997) employed three different combinations of cellulase / hemicellulase/ pectinase to solubilize sugar beet pulp and they achieved a maximal saccharification in the range between 76.6% - 86.5%.

To study the effect of cellulase addition, an experiment was conducted with a combination of pectinase – cellulase – hemicellulase (P-H-C) to hydrolyze sugar beet pulp. Table 3-3, shows the results for this experiment. A combination of cellulase and pectinase was employed to initiate the hydrolysis. After 6 hours only 19.69% polysaccharides were saccharified, this result was close to saccharification obtained after 6hours by pectinase – hemicellulase combination (Table 3-2). Clearly if there was a presence of synergism between cellulase and pectinase, higher values of saccharifcation would be obtained. But in both cases, when hemicellulase enzyme was added after 6 hour sachharification rose rapidly to 80.81% (P-H-C) and 89.57% (P-H-3hr), respectively.

Results from current study indicate a strong presence of synergism when pectinolytic activity is coupled with hemicellulolytic activity. But, this is in contrast to the conclusions made by Spagnuolo et al., (1997), that, there is strong presence of synergism when cellulolytic activity is coupled with pectinolytic activity and the absence

of synergism when hemicellulase is coupled with combined cellulase and pectinase. The absence of synergism between cellulase and pectinase in our experiment could be attributed to presence of cellulase activities in both hemicellulase and pectinase enzyme preparations. Hence addition of cellulase had no significant effect on saccharification in our studies. Himmel et al., (1993), mention that an important difference between the enzymatic hydrolysis of lignocellulosic tissues and sugar rich residues is a small contribution of glucose from hydrolysis of cellulose to the overall yield of soluble sugars. Therefore the emphasis in the current study is on solubilization of cell wall and embedded sugar-rich fibers by pectinolytic and hemicellulolytic enzymes. Hence, synergism observed between these two enzymes in our research supports above facts and reinforces the need for utilizing combination of enzymes to achieve near complete saccharification.

Effect of enzyme loading: Figure 3-1 A, B, shows the profile of effect of enzyme loading on the % saccharification of sugar beet pulp. Only 20.53% saccharification was obtained by P (9.5 µmol/ml/hr) – H (0.3 µmol/ml/hr) combination in 19 hours. Saccharification reached 81% when P (9500 µmol/ml/hr) – H (300 µmol/ml/hr) was used for hydrolysis. The results indicate that higher enzyme loading is necessary for complete conversion of sugar-rich fibers to monosaccharides. Most importantly the rate of saccharification was significantly higher in P (µmol/ml/hr) – H (300 µmol/ml/hr), as 88% saccharification was achieved in just 6 hours as compared to just 18% by P (9.5 µmol/ml/hr) – H (0.3 µmol/ml/hr). Mussatto et al., (2008) have shown that enzyme loading has a strong influence on hydrolysis of cellulose. They found that an increase in enzyme loading from 5 to 45 FPU/g resulted in an average increase in the glucose yield

and cellulose conversion of 53.1% and 39.4%, respectively. Further evaluation revealed that glucose yield from cellulose increased up to loading of 45 FPU/g substrate. Glucose yield remained constant for enzyme loading values ranging between 45 and 85 FPU/g. Similar conclusions were drawn by Gan et al., 2003; Pan et al., 2005; Eklund et al., 1990; Manonmani and Sreekantiah, 1987; Yáñez et al., 2006; Kaur et al., 1998; Vlasenko et al., 1997and Liao et al., 2005). It is possible that there is an optimum enzyme loading beyond which there is no further increment in rate of saccharification. Enzyme loading is an important parameter because enzyme costs are critical for successful hydrolysis process. Different enzyme concentrations and substrate concentrations can be tested to optimize enzyme loading and develop a strategy for efficient enzymatic hydrolysis.

Effect of pre-treatment on saccharification: In the current study to solubilize the pectin-rich layer and to reduce the enzyme loading, sugar beet pulp was pretreated by thermochemical means. High temperature (160 °C) and water/acid was utilized to solubilize pectins/ hemicellulose in beet pulp fibers. ~44% saccharification was obtained when water (hydrothermolysis) was used as a medium, in 1 hour, whereas, when thermhydrolysis was coupled with 1% o-phosphoric acid, ~ 29% saccharification was obtained in just 30 minutes. But the performance of hydrothermolysis pretreatment dropped significantly after 2 hours. It is possible that extended heat treatment caused side reactions that consumed soluble sugars or formed inhibitory products leading to loss of total sugars. In fact, Weil et al., (2002) confirmed this when pretreating 19.4% (w/w) of corn fiber at 150 °C for 2 h using hydrothermolysis. They detected 0.5% (w/o) furfural in the hydrolysate. They attributed this to the production of aldehydes, when

hexoses and pentoses in an aqueous solution are exposed to high temperatures above 150°C under acidic conditions common to acid-catalyzed lignocellulosic biomass pretreatment. Further explanation to this lies in the mechanism of aqueous pretreatment process at high temperatures. Water can act as a hydrating agent, diffusing inside the crystalline regions of the cellulose and swelling it to disrupt its crystallinity, thus making the cellulose more reactive (Weil et al., 2002). When pH is not controlled, water acts like a weak acid and promotes rapid acid-catalyzed hydrolysis of polysaccharides to monosaccharides which subsequently degrade to furfural, hydroxymethyl furfural (HMF) and other inhibitors (Weil et al., 2002). Furthermore, acid hydrolysis of xylan is more troublesome because it contains acetyl groups that upon hydrolysis form acetic acid (Frazer et al., 1989; Beall et al., 1991). Importantly, xylan hydrolyzes more readily to xylose which leads to formation of furfural (Frazer et al., 1989; Beall et al., 1991). Weil et al., (1998) showed that formation of inhibitory products can be prevented by controlling the pH during hydrothermolysis. They added KOH to maintain the pH, this addition did not affect the solubilization of corn fiber, but improved subsequent enzymatic hydrolysis of pretreated hydrolysate. In current study, pH was not controlled during hydrothermolysis, but no inhibitory product formation was observed during 1 h pretreatment of sugar beet pulp. This was confirmed by high values of saccharification obtained from enzymatic hydrolysis of 1 h hydrolysate from pretreatment. But the drop in sugars in hydrolysate after 2 h of pretreatment, confirms that pH control might be needed to sustain sugar release and suppress side reactions if the pretreatment needs to be extended beyond 1 h.

Sugar beet pulp contains ~66% sugars (glucose, galacturonic acid and arabinose)on dry weight basis. Hydrothermolysis pretreatment was able to successfully recover 44% of these sugars. Schaffeld et al., 1987; Chamy et al., 1994; Spagnuolo et al., (1997), achieved complete saccharification using 12 M sulfuric acid hydrolysis, though duration of acid pre-treatment varied from 1h – 7h. Particularly, hemicellulose was hydrolyzed ~90%. Ali et al., (1984) obtained 80% solubilization of pectin using heat treatment. Duration of heat treatment and temperature range was not specified for this work. Foster et al., (2001) used a novel ammonia pressurization depressurization (APD) process to pre-treat sugar beet pulp to enhance enzymatic hydrolysis. However APD was found to be effective in degrading cellulose but had no effect on hydrolysis of pectin and hemicellulose fractions. Effectiveness of hydrothermolysis pre-treatment can be enhanced by increasing the temperature to ~200 °C, but may not be required since the lignin content in beet pulp is less than 4%. Considering all the pre-treatment methods hydrothermolysis is effective in saccharifying both pectins and hemicellulose completely and duration of 1 hr was sufficient to achieve maximum release of sugars.

Sequential hydrothermolysis and enzymatic hydrolysis of sugar beet pulp: The hydrolysate obtained from hydrothermolysis of sugar beet pulp was further saccharified by using combinations of diluted pectinase / hemicellulase enzymes [P(9.5 µmol/ml/hr– H (0.3 µmol/ml/hr)] combination attained ~81% saccharification in just 6 hours of enzymatic hydrolysis. This shows that hydrothermolysis by solubilizing pectins and hemicellulase enhances saccharification and high sugar yields are possible using very low enzyme loadings. Cost of enzymes are an important economic consideration in development of pre-treatment process. Spagnuolo et al., (1997) achieved high

saccharification of 87.7% after complete acid hydrolysis of beet pulp. But significant time (7 h acid pretreatment and 7 day incubation for enzyme hydrolysis) was required to achieve ~80% saccharification. In contrast, hydrothermolysis pre-treatment achieved similar results in just 7 h.

It is clear from above discussion that neither heat treatment / acid hydrolysis is able to achieve complete saccharification of sugar beet pulp when used singly. Acid hydrolysis achieved high yield (~95%) of fermentable sugars at high reaction rates, but conditions used are severe and may produce undesirable by-products, such as phenolic compounds and furfurals that derive primarily from xylose and five carbon sugars in pectin-rich biomass (Schaffeld et al., 1987). Other disadvantages are: a) high cost of acid b) sugar decomposition at elevated temperatures c) high operating cost for acid consumption. Enzyme hydrolysis eliminates some of the disadvantages of acid hydrolysis but pre-treatment of biomass, high cost of enzymes and low hydrolysis rates are major hurdles in implementation of enzyme hydrolysis. Application of hydrothermolysis to solubilization of sugar beet pulp offers significant improvements over acid hydrolysis and APD reported in literature. Hydrothermolysis utilizes the properties of water at elevated temperature and pressure to effectively solubilize hemicellulose and pectins in beet pulp to release monomeric sugars. This pretreatment method is non-corrosive, no associated environmental problems and reasonably high reaction rates make this pretreatment method an attractive option for beet pulp hydrolysis. As is evident a combination of hydrothermolysis followed by enzymatic hydrolysis achieves high saccharification in shorter durations and low enzyme costs.

3.6 Conclusions

Fermentable sugars were obtained by sequential hydrothermolysis and enzymatic hydrolysis of spent sugar beet pulp. In the hydrothermolysis stage 44% saccharification was obtained in just 1 h. Hemicellulose and pectins were solubilized to its constituent monomeric sugars during hydrothermolysis. In the enzyme hydrolysis stage, the hydrolysate achieved almost complete saccharification (88%) in just 6 h by using a combination of pectinase (9.5 IU) and hemicellulase (0.3 IU). That is the total time to achieve complete saccharification was just 7 h. It was found that there exists a strong synergism between pectinase and hemicellulase enzymes to achieve complete saccharification and pectinase was an important enzyme. The combined hydrothermolysis pre-treatment and enzymatic hydrolysis method investigated here offers significant benefits over other saccharification methods: (1) Faster reaction rates (2) no environmental problems (3) Complete saccharification of pectin and hemicellulose in shorter duration (4) reduced enzyme loading to achieve complete saccharification (5) no size reduction of feedstock required. (6) no chemical addition

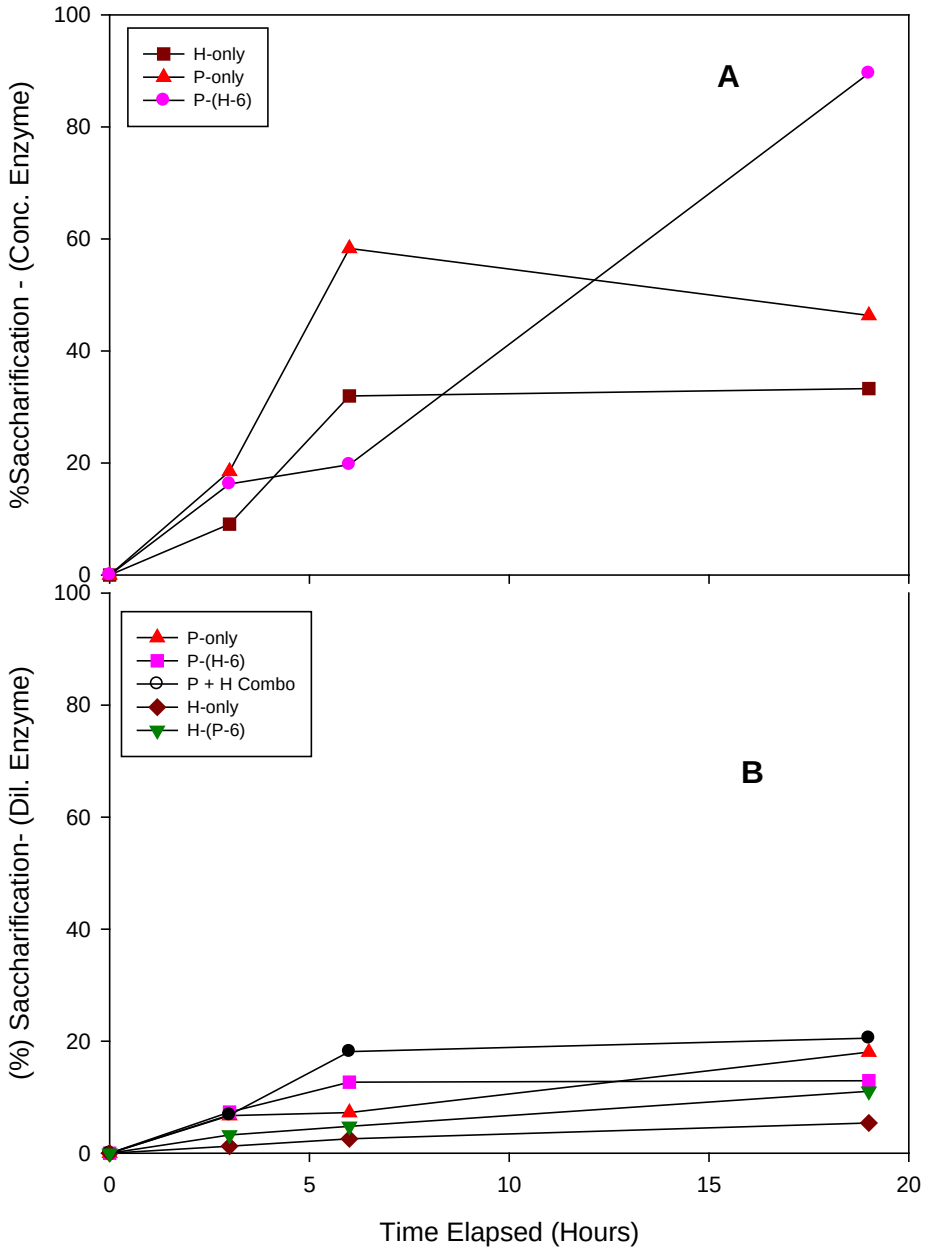


Figure 3-1 (%) Saccharification - Effect of enzyme loading : A)– Concentrated enzyme loading (P – 9500 µmol/ml/hr , H – 300 µmol/ml/hr), B)– Dilute enzyme loading (P- 9.5 µmol/ml/hr, 0.3 µmol/ml/hr)

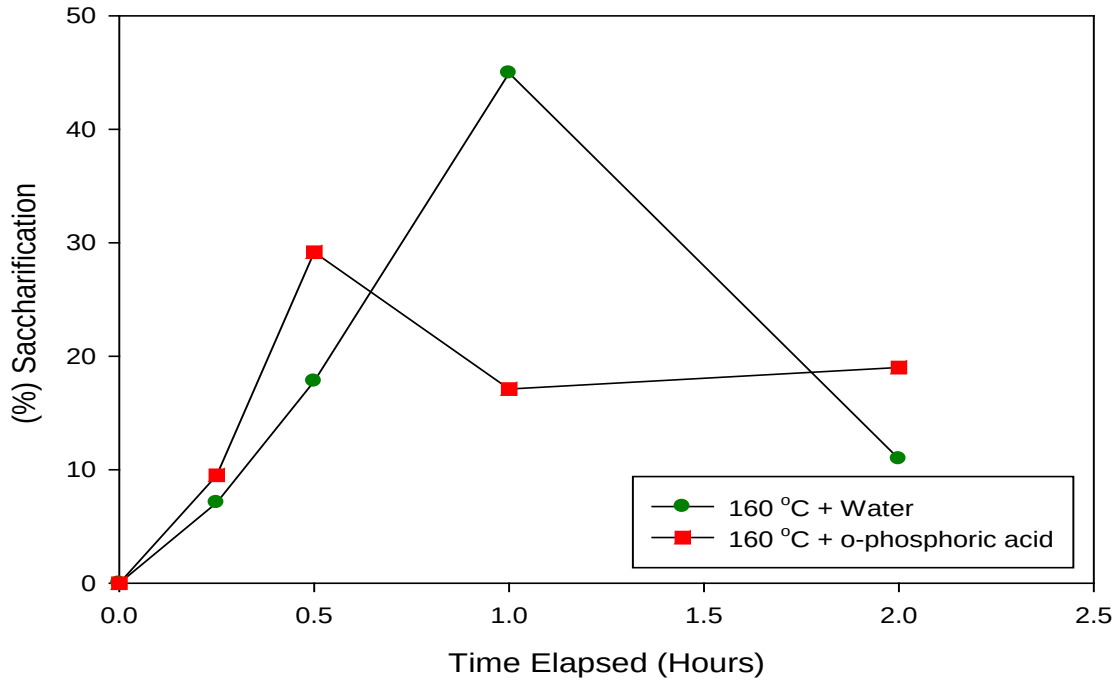


Figure 3-2 (%) Saccharification - Effect after pre-treatment (heat and acid/water)

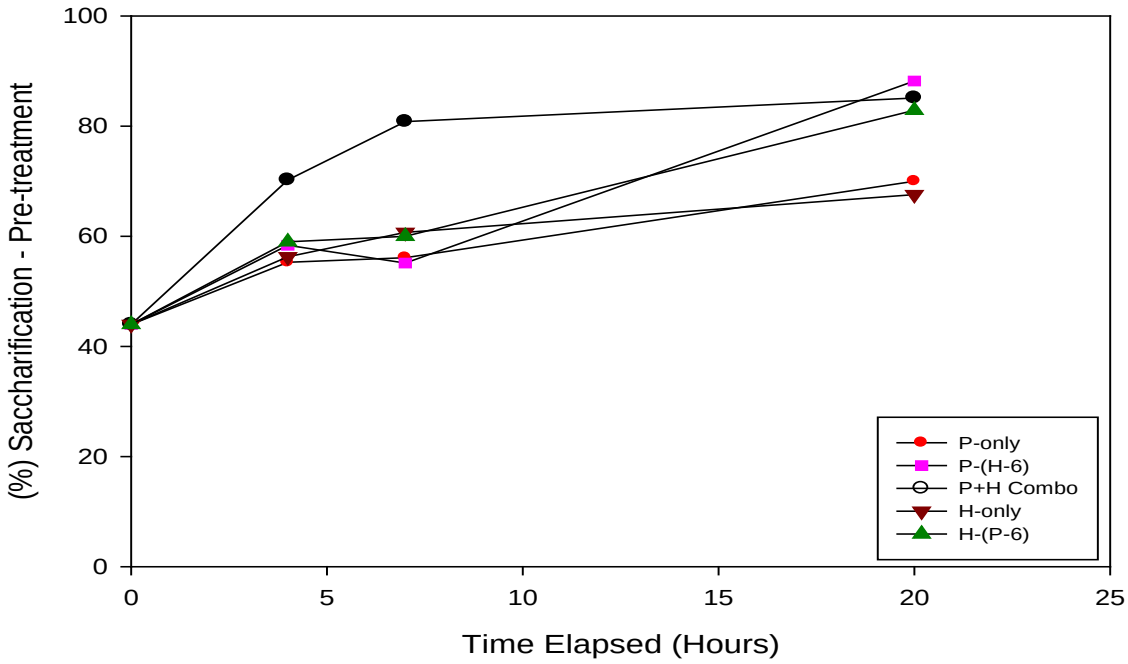


Figure 3-3 (%) Saccharification - Effect of pretreatment after enzyme addition

Table 3-1 List of pretreatment methods and main mechanisms involved (Jorgensen et al., 2007)

Pretreatment Method	Main Principle
Dilute acid Steam explosion (auto hydrolysis) Acid-catalyzed steam explosion	Partial hydrolysis and solubilization of hemicelluloses, redistribution of lignin on fiber surfaces, fractionation of fibers
Hot water flow through	Removal of hemicelluloses and lignin
Lime	Removal of lignin
Wet oxidation Wet explosion	Removal and partial degradation of lignin, solubilization and oxidation of some hemicelluloses
AFEX	Cleavage of lignin and partial depolymerization of hemicelluloses and cellulose
Organisolv/Alcell	Removal of lignin and some hemicelluloses

Table 3-2 Comparison of different hydrolysis methods[a]

Hydrolysis Method	Conditions	Glucose Yield (%)[b]	Advantages and Disadvantages (A and D)
Concentrated acid	30-70% H_2SO_4 40 °C, 2-6 hours	90	A: high sugar recovery, high reaction rate D: environmental and corrosion problems, high cost for acid recovery
Dilute acid	<1% H_2SO_4 215 °C, 3 minutes	50 – 70	A: high sugar recovery, very high reaction rate D: environmental and corrosion problems, sugar decomposition at elevated temperature, high utility cost, high operating cost for acid consumption
Alkaline	18% NaOH 100 °C, 1 hour	30	A: high reaction rate D: sugar decomposition by alkali attack, low sugar yield
Enzymatic	Cellulase 70 °C, 1.5 days	75 – 95	A: high yield of relatively pure sugar, mild environmental conditions, no environmental and corrosion problems D: pretreatment of biomass required, high cost of cellulase enzymes, low hydrolysis rate
Hot compressed water	30-70% H_2SO_4 40 °C, 2-6 hours	<40	A: no environmental and corrosion problems, low maintenance cost, relatively high reaction rate D: relatively low sugar yield

[a]Adapted from Bobleter, 1994
[b]Source from Hamelinck et al., 2005

Table 3-3 (%) Saccharification - Conc. enzyme hydrolysis – Hemicellulase – Raw SBP

Time	Mean	Standard Dev.	Range
	% Saccharification		
3	9.08	2.34	9.08 ± 2.34
6	31.96	4.05	31.96 ± 4.05
19	33.28	4.96	33.28 ± 4.96

Table 3-4 (%) Saccharification - Conc. enzyme hydrolysis - Pectinase - Raw SBP

Time	Mean	Standard Dev.	Range
	% Saccharification		
3	18.49	2.12	18.49 ± 2.12
6	58.30	6.00	58.30 ± 6.00
19	46.34	5.19	46.34 ± 5.19

Table 3-5 (%) Saccharification - Conc. enzyme hydrolysis - (Pectinase - Hemicellulase) - Raw SBP

Time	Mean	Standard Dev.	Range
	% Saccharification		
3	16.25	1.38	16.25 ± 1.38
6	19.69	0.87	19.69 ± 0.87
19	89.57	2.81	89.57 ± 2.81

Table 3-6 (%) Saccharification - Conc. enzyme hydrolysis - (Pectinase – Cellulase – Hemicellulase 6h) - Raw SBP

Time	Mean	Standard Dev.	Range
	% Saccharification		
3	5.86	1.22	5.86 ± 1.22
6	21.51	1.44	21.51 ± 1.44
19	80.81	8.53	80.81 ± 8.53

Table 3-7 (%) Saccharification - Dilute enzyme hydrolysis – Raw SBP

Time	P-only	P-(H-6hrs)	P + H Combination	H-only	H-(P-6hrs)
			% Saccharification		
3	6.70	7.33	6.82	1.24	3.23
6	7.23	12.68	18.13	2.57	4.79
19	18.03	12.93	20.53	5.40	11.05

Table 3-8 (%) Saccharification - Hydrothermolysis of sugarbeet pulp

Time	Trial 1	Trial 2	Mean	Standard Dev.	Range
			% Saccharification		
0.25	6.75	7.42	7.09	0.47	7.09 ± 0.47
0.5	18.54	17.05	17.80	1.05	17.80 ± 1.05
1	37.55	52.35	44.95	10.47	44.95 ± 10.47
2	10.36	11.60	10.95	0.88	10.95 ± 0.88

Table 3-9 (%) Saccharification - (Thermolysis + Acid addition)

Time	% Saccharification
0.25	9.51
0.5	29.16
1	17.11
2	19.01

Table 3-10 Mean - (%) Saccharification (Enzymatic Hydrolysis only)

Time	P-only	P-(H-6hrs)	P + H Combination	H-only	H-(P-6hrs)
			% Saccharification		
3	6.15	12.54	37.48	8.25	13.93
6	7.85	5.85	59.73	17.53	16.03
19	36.98	75.19	68.71	31.89	64.02

Table 3-11Range - (%) Saccharification (Enzymatic Hydrolysis only)

Time	P-only	P-(H-6hrs)	P + H Combination	H-only	H-(P-6hrs)
			% Saccharification		
3	6.15 ± 4.66	12.54 ± 2.40	37.48 ± 1.83	8.25 ± 2.54	13.93± 3.25
6	7.85 ± 6.21	5.85 ± 1.69	59.73 ± 4.51	17.53 ± 4.66	16.03 ± 3.39
19	36.98 ± 1.98	75.19± 7.48	68.71 ± 4.23	31.89 ± 1.27	64.02 ± 4.66

Table 3-12 Mean - (%) Total Saccharification (Hydrothermolysis + Enzymatic addition)

Time	P-only	P-(H-6hrs)	P + H Combination	H-only	H-(P-6hrs)
			% Saccharification		
4	55.28	58.37	70.21	56.28	58.99
7	56.09	55.14	80.81	60.70	59.99
20	69.99	88.18	85.09	67.55	82.86

CHAPTER 4
COMPARISON OF THERMOPHILIC AND MESOPHILIC SINGLE-STAGE, UNMIXED, BATCH ANAEROBIC DIGESTION OF SPENT SUGAR BEET PULP[†]

4.1 Introduction

The effect of thermophilic and mesophilic temperatures on biogasification of SBP without any pretreatment was compared and quantified using identical laboratory scale designs and operating conditions. A single stage leach-bed design was employed for biogasification. This chapter presents results from these investigations and further discusses the effectiveness of employing a single stage leach-bed process to biogasify spent sugarbeet pulp. The performance of the process in terms of its kinetics, methane yield and extent of degradation was evaluated and then compared to other processes that have been developed for spent sugarbeet pulp.

4.2 Background

Anaerobic digestion or biogasification studies have been carried out using whole beets (Demirel and Scherer, 2008), beet tops (Seppälä et al., 2008; Bukic et al., 2008; Svensson et al., 2007; Lehtomäki et al., 2007), beet leaves (Parawira et al., 2008 and 2004), beet pulp (Brooks et al., 2008; Koppar and Pullammanappallil, 2008) and beet tailings (Liu et al., 2008). Of these, beet pulp and tailings would be the priority feedstocks for biogasification as these are byproducts and wastes, produced and collected in large quantities at one site namely in the processing facility.

Biogas potential studies have shown that if all the organic by products and waste streams produced in a sugarbeet processing facility were to be biogasified, pressed

[†] This chapter contains material taken from the following published journal articles: 1) Koppar, A.K., Pullammanappallil, P.C., 2009. "Comparison of thermophilic and mesophilic single-stage, unmixed, batch anaerobic digestion of sugarbeet pulp". Sugar Industry Journal, 134 (9), pp 586-591 and 2) Koppar, A.K. and Pullammanappallil, P.C., 2008. "Single-stage, batch, leach-bed, thermophilic anaerobic digestion of spent sugarbeet pulp". Bioresource Technology, Volume 99, Issue (8), 2831 – 2839.

sugar beet pulp (SBP) would contribute to a major fraction (52%) of this biogas (Polematidis et al., 2008). Hence biogasification of SBP has received much attention (Lane, 1983; Frostell et al., 1984; Garcia et al., 1984; Stoppok and Buchholz, 1985; Ghanem et al., 1992; Weiland, 1993; Hutnan et al., 2000, 2001; Koppar and Pullammanappallil, 2008; Brooks et al., 2008). Biogasification processes are principally carried out in two temperature regimes, mesophilic (32 – 40 ^{o}C) and thermophilic (50 – 57 ^{o}C). SBP has been biogasified at both these temperature regimes. Laboratory scale and pilot scale systems have digested beet pulp at thermophilic temperature (Frostell et al., 1984; Koppar and Pullammanappallil, 2008), however mesophilic temperature appears to be the preferred temperature of operation (Lane, 1983; Garcia et al., 1984; Stoppok and Buchholz, 1985; Ghanem et al., 1992; Weiland, 1993; Hutnan et al., 2000, 2001; Brooks et al., 2008) including the temperature regime for the only large scale anaerobic digester in Hungary (Brooks et al., 2008). Thermophilic digestion may offer advantages like increased rate of degradation, improved methane yield and greater solubilization (Hegde and Pullammanappallil, 2007; Koppar and Pullammanappallil, 2008) but on the other hand it is perceived that the process at this temperature may be unstable and could also result in higher accumulation of volatile organic acids. Benefits of one operating temperature over another are not conclusive. It was difficult to compare the benefits of digesting SBP at mesophilic or thermophilic temperature using data from literature as studies were carried out using different digester designs with or without pretreatment. Several processes have been developed to effectively biogasify spent sugarbeet pulp. These processes typically involve size reduction (Frostell et al., 1984; Lane, 1983; Weiland, 1993) and/or pretreatment by hydrolyzing enzymes (Garcia

et al., 1984) before being anaerobically digested in continuously agitated single stage (Weiland, 1993; Frostell, 1984; Lane, 1983; Ghanem et al., 1992) or two stage (Stoppok and Buchholz, 1985; Hutnan et al., 2000; Hutnan et al., 2001) systems. A biogasification technology that has been recently used for the treatment of solid wastes is a batch, leach-bed process. This technology has been successfully applied to digest feedstocks like, municipal waste, yard wastes, biosolids and vegetable and fruit wastes (Chynoweth et al., 1992; Chugh et al., 1999; Hegde and Pullammanappallil, 2007). The process offers several advantages as it does not require fine shredding of waste, does not require mixing or agitation of digester contents, does not require bulky, expensive, high-pressure vessels as it can be operated at low (ambient) pressures and can be operated stably at both mesophilic and thermophilic temperatures (Pullammanappallil et al., 2005).

The leach-bed process can be operated in a single-stage or dual-stage mode depending on the characteristics of the biomass feedstock. Originally it was devised to be operated in the dual stage mode (Chynoweth et al., 1992) where fresh biomass as received (or after coarse shredding) is loaded into a vessel and wetted. The leachate that is produced during the wetting process is flushed through another vessel containing previously stabilized anaerobically digested residue. The effluent generated in this vessel during the flushing operation is returned back to the fresh biomass. This mode of leachate recirculation is called sequencing. Sequencing is repeated until methanogenesis

is initiated in the fresh biomass bed and pH of this bed is close to neutral, after which sequencing was terminated and the leachate recirculated directly within the bed.

Degradation of biomass feedstocks like municipal solid wastes, yard trimmings, water hyacinth and sorghum were achieved within 20 to 30 days in this process (Chynoweth et al., 1992; Chugh et al., 1999). This process was modified to a single stage design by flooding the fresh biomass bed with liquid drained from the previous digestion of the feedstock (Hegde and Pullammanappallil, 2007). It was shown that wastes from fruit and vegetable markets can be digested within ten days using such a single stage process.

4.3 Method

The following methods were employed for studies in this chapter:

1. Thermophilic and Mesophilic anaerobic digestion of sugar beet pulp using single-stage, leach-bed digester.
2. Three runs were conducted for both mesophilic and thermophilic digestion serially.
3. The performance of the system was analyzed by monitoring pH, volatile fatty acids (VFA), gas composition, gas production, VS (volatile solids) and DM (dry matter) reduction.
4. The results obtained from the experiments were compared with other anaerobic digestion processes biogasifying sugar beet pulp.

Details of the methods are given in Chapter 2 (Sections 2.2.1, 2.2.2, 2.3.1, 2.4)

4.4 Results

The dry matter content of the spent beet pulp as received was 22% and 96% of the dry matter was volatile. Table 4-1 lists the quantities of pulp (in terms of wet, dry and volatile matter) loaded into the digesters for each run and the loading characteristics. Even though 99 g (0.099 kg) dry matter was loaded into the digester, the packing density was only 25 kg dry matter/m^3 due to the addition of lava rocks as bulking agent. At the beginning of each run 450 g (0.45 kg) wet weight of spent pulp was added without removing solids residue from the previous run. At the end of Run 5

the dry matter remaining in the digester was measured to be 39.6 g (0.0396 kg) and 54% of the dry matter was volatile. The dry matter and volatile solids reduction achieved by biogasification was 92% and 96% respectively.

Figure 4-1 shows the specific volumetric methane production rate (m^3 of methane at STP / m^3 digester operating volume / day) from the digesters for all runs. This parameter signifies reactor productivity. In Run 1, three days after start up the methane production rate was at 0.86 $m^3\ m^{-3}\ d^{-1}$ in thermophilic digester and 0.83 $m^3 m^{-3}\ d^{-1}$ in mesophilic digester, indicating an early onset of methanogenesis in both cases. After 6 days the methane rate peaked at 1.49 $m^3\ m^{-3}\ d^{-1}$ in thermophilic digester whereas the highest rate attained was only 0.83 $m^3\ m^{-3}\ d^{-1}$ in mesophilic digester. In thermophilic digester, Run 1 reached completion in 12 days when the daily methane production rate dropped to 0.07 $m^3 m^{-3}\ d^{-1}$. In mesophilic digester, Run 1 reached completion in 17 days when the daily methane production rate dropped to 0.15 $m^3 m^{-3}\ d^{-1}$. The next digestion run (Run 2) in both cases was initiated by flooding with liquor from Run 1. No further alkalinity was added to the inoculum. It can be seen from Figure 1 that methanogenesis was initiated quicker in Run 2 when compared to Run 1 in thermophilic digester where the methane production rate substantially increased peaking at 2.89 $m^3\ m^{-3}\ d^{-1}$ within 3 days. The methane production rate decreased to 0.15 $m^3\ m^{-3}\ d^{-1}$ in 9 days indicating that the SBP was almost completely degraded. However, in the mesophilic digester, only a slight improvement over Run 1 was observed. The methane production rate peaked at 0.93 $m^3\ m^{-3}\ d^{-1}$ by day 6 and decreased to 0.13 $m^3\ m^{-3}\ d^{-1}$ in 26 days. Specific volumetric methane production rate in Run 3 under thermophilic conditions reached a peak value of 2.25 $m^3\ m^{-3}\ d^{-1}$ in 3 days and by day 14 it had decreased to

0.13 m^3 m^{-3} d^{-1}. The methane production rate in Run 3 under mesophilic conditions peaked at 1.12 m^3 m^{-3} d^{-1} in 7 days and deceased to 0.1 m^3 m^{-3} d^{-1} in 26 days.

Figure 4-2 shows the average methane content (along with the associated error) in the biogas from the digesters for 3 runs under thermophilic conditions and 3 runs under mesophilic conditions. The error band is one standard deviation above and below the mean. Biogas produced from the digester is likely to be used on site as a fuel and this parameter influences the calorific vale of the biogas. As the run progresses methane content in the biogas increases from 0% to about 60% within 3 days for thermophilic digestion. The methane content fluctuates around this value for the rest of the duration of run and increases to 67.5% towards the end of the run. In the case of mesophilic digestion the methane content increases to 76% in 9 days and maintains this value for the rest of the duration of the run.

The cumulative methane produced at standard temperature (273 K) and pressure (1 atm), STP, was calculated for each run and divided by the amount of SBP loaded (in terms of volatile solids) at the beginning of the run to yield a cumulative methane yield. The mean, standard error and standard deviation of cumulative methane yield from thermophilic and mesophilic digestion is also determined. The average methane yield from thermophilic digestion, 0.353 m^3/ kg VS, was higher than that from mesophilic digestion experiments (0.314 m^3/ kg VS). Further quantification of the performance of the two temperature regimes was carried out using the cumulative methane yield data. The Gompertz equation for batch bacterial growth that was modified for a batch anaerobic digestion process was used to evaluate performance. This modified equation calculates the lag time, ultimate methane yield and the

maximum methane production rate. These parameters for each run was computed using the solver feature of MS-Excel and is listed in Table 4-2. The mean lag time for the three runs under thermophilic conditions was about 1 day and at mesophilic condition was 1.2 days. The ultimate methane yield for thermophilic digestions was on average 0.359 m^3/ kg VS in close agreement with the experimental value. For mesophilic digestion mean ultimate methane yield was 0.307 m^3/ kg VS which was once again in agreement with the experimental value. The table also lists the duration of digestion required to produce 95% of the ultimate methane yield in each case. For thermophilic digestion this duration was only 9.2 days whereas mesophilic digestion took twice as long, about 17. 2 days. Figure 3 shows the plot of the Gompertz fit for both temperature regimes. The plot was generated using the mean values of lag time, ultimate methane yield and maximum methane rate listed in Table 4-2. A 95% confidence interval for the data set is also shown. This band was generated by plotting the Gompertz fit using the values of parameters one standard deviation above and below the mean value.

In thermophilic digester during Run 1 the pH dropped from 8.2 to about 7.0 on the second day of digestion. The pH increased gradually and stabilized between 7.5 - 8.0 at the end of Run 1. In Run 2 the pH decreased to 7.77, but increased slightly to 7.92 by end of third run in thermophilic digester. The pH in mesophilic digester at the end of Run 1 was 8.19, decreased to 7.44 by end Run 2, but again increased to 8.56 at the end of Run 3. Alkalinity addition was not required because pH values were maintained around 8. The pH was not corrected in these experiments but allowed to evolve.

Profile for volatile fatty acids (VFA) in thermophilic and mesophilic digesters is shown in Figure 4-4. Only volatile fatty acid measurements from Run 2 in both cases are shown here. Acetic, propionic and butyric acids were the primary volatile fatty acids measured in the digester. The concentrations of acetic acid, propionic acid and butyric acid in thermophilic digester at the end of day 3 were, 438 mg L^{-1}, 261 mg L^{-1}, 474 mg L^{-1}. Concentrations of acetic acid and butyric acid increased to 1581 mg L^{-1} and 713 mg L^{-1}, respectively, whereas, propionic acid concentration decreased to 39 mg L^{-1} at the end of Run 2. The concentrations of acetic acid, propionic acid and butyric acid in mesophilic digester at the end of day 3 were, 2285 mg L^{-1}, 662 mg L^{-1}, 300 mgL^{-1}. Concentrations of acetic acid and butyric acid decreased to 896 mg L^{-1} and 112 mg L^{-1}, whereas propionic acid concentration increased to 1009 mg L^{-1} at the end of Run 2.

Ammonia-nitrogen concentrations was measured in all runs. The total ammonia-nitrogen concentration in thermophilic digester at the end of Run 1 was 105 mg L^{-1}, increased to 846 mg L^{-1} at the end of Run 2 and to 2509 mg L^{-1} at the end of Run 3. The total ammonia-nitrogen concentration in mesophilic digester at the end of Run 1 was 93.2 mg L^{-1}, increased to 204.5 mg L^{-1} at the end of Run 2 and to 1179.4 mg L^{-1} at the end of Run 3.

4.5 Discussion

SBP was readily digested under both thermophilic and mesophilic conditions. However, for the duration of digestion runs the methane yield from these temperature regimes differed. At thermophilic temperature the methane yield was a statistically significant 17% higher than at mesophilic temperature, indicating that a higher proportion of the SBP was biogasified at 55 ^{o}C. Methane yields obtained here is

comparable to that obtained by Frostell et al (1984), Stoppok and Buchholz (1985) and Hutnan et al. (2000).

Biogasification efficiency can be calculated as a percentage of the feed chemical oxygen demand (COD) converted to methane. Using a specific COD value of 1.295 kg COD/kg dry matter (Hutnan et al., 2000) for SBP and knowing that 1 kg COD of substrate when completely degraded would yield 0.35 m^3 of methane at STP, then 1 kg VS of SBP should theoretically produce a maximum of 0.472 m^3 of methane at STP (VS is 96% dry matter). By comparing the mean ultimate methane yield for the SBP to the theoretical maximum, biogasification efficiencies was determined to be 76% and 65% at thermophilic and mesophilic conditions respectively.

The maximum rate of methane production in the thermophilic digestion process was more than twice as much as that produced from mesophilic digestion. Concomitantly duration of thermophilic digestion was half as that taken for mesophilic digestion. As a rule of thumb it is generally believed that the specific growth of bacteria and hence the reaction rate doubles for every 10 ^oC rise in temperature. According to this, the degradation rate of SBP (or the methane production rate) should have increased by about 3.6 times in the thermophilic digester compared to that in the mesophilic digester. Even though such a dramatic increase was not seen here the rate did double.

The methane content of the biogas produced in the mesophilic digester was higher than that produced in the thermophilic digester. This would result in the biogas from the mesophilic digester having a higher calorific value than that produced in the thermophilic digester. At 76% methane content, biogas from mesophilic digester will

have a high heating value (HHV) of 28 MJ/m^3 whereas that from a thermophilic digester will have a HHV of 22 MJ/m^3. The other component in the biogas is carbon dioxide. Typically in dry biogas, other than methane the rest of the biogas is made up of carbon dioxide, therefore a lower methane content means a higher carbon dioxide content. Anaerobic digestion of carbohydrate feedstocks produce equimolar quantities of methane and carbon dioxide, which means biogas should contain methane and carbon dioxide at 50% each. Since carbon dioxide is readily soluble in water and methane is sparingly soluble, some of carbon dioxide produced during digestion remains dissolved in water. This leads to carbon dioxide in the biogas being less than 50%. Since solubility of gases is inversely related to temperature, the higher temperatures in a thermophilic digester may cause lower amounts of carbon dioxide to dissolve which in turns results in a higher carbon dioxide content (consequently lower methane content) in biogas.

The process of mineralization of macromolecules in SBP to methane and carbon dioxide occurs first through fermentation of these compounds to volatile organic acids (like acetic, propionic and butyric). Higher chain organic acids like propionic and butyric acid are converted to acetic acid. Acetic acid serves as the substrate for methanogenesis. It is typical to observe an initial accumulation of volatile organic acids during batch anaerobic digestion. It can be seen from Figure 4 that acetic acid accumulated rapidly in the thermophilic digester indicating high acidogenic fermentation activity. However, it was also degraded quickly. This was reflected in the in a quick increase in methane production rate values. Propionic and butyric acid did not build up to high levels. In the mesophilic digester accumulation of acetic acid was much slower

taking about 6 days to build up to 4000 mg L^{-1}. Within about the same duration propionic and butyric acids also accumulated to over 1000 mg L^{-1}. These acids were subsequently degraded. It appears the lower temperature of operation under mesophilic conditions also slows down acidogenic fermentation step in addition to methanogenesis. The level of volatile organic acids in mesophilic and thermophilic digesters were similar.

During anaerobic fermentation as nitrogen containing organic compounds (mainly proteins) are degraded ammonia is released. Total ammonia concentration was higher in the thermophilic digester at the end of each run. Since the digested liquor from one run was reused in a subsequent run the ammonia concentration kept accumulating. However, the change in ammonia concentration between successive runs increases from Run 2 to Run 3 in both cases. Change in ammonia-nitrogen concentration between Run 2 and Run 1 was 741 mg L^{-1} in thermophilic digester and 111.3 mg L^{-1} in mesophilic digester. The change between Run 3 and Run 2 was 1663 mg L^{-1} for thermophilic digestion and 974.9 mg L^{-1} mesophilic digestion. This indicated a greater break down of nitrogen containing compounds in the thermophilic digester and could have resulted in a higher extent of solubilization of the SBP. Since the solids were not removed from the digester prior to subsequent run, the retention time of the solids in the digester was very long (about 39 days for thermophilic and 78 days for mesophilic operation) which allowed these to undergo a large extent of degraded. The protein content of SBP is about 11.4% of dry weight (Frostell et al., 1984). Assuming 12% nitrogen in the protein, then ammonia-nitrogen that will be released from 0.450 kg wet SBP (0.099 kg dry weight) loaded into the digesters would be 1.44 g resulting in a

total ammonia-nitrogen concentration of 719 mg L^{-1}. At the end of three runs this concentration should be 2156 mg L^{-1} neglecting any uptake of nitrogen by cells or any volatilization of ammonia. It should be noted that the concentration of ammonia-nitrogen at the end of Run 3 in thermophilic digester was 2509 mg L^{-1} indicating almost complete breakdown of organic nitrogen compounds in SBP. Concentration of ammonia in mesophilic digester at the end of Run 3 was only 1179 mg L^{-1} indicating only a 50% breakdown of organic nitrogen compounds in SBP. Therefore, if an appropriate design of a thermophilic digester can be developed that will retain solids for over 30 days then most of the organic nitrogen could be solubilized. Such a design would have a lower hydraulic retention time for liquids. On the contrary as organic nitrogen is released as ammonia further treatment of effluent may be required for nutrient removal prior to its disposal.

Comparisons of single stage leach bed other processes: Table 4-3 compares the biogasification performance of the thermophilic digestion in present study for sugar beet pulp anaerobic digestion to that reported in literature. Much of the research concerning biogasification of spent sugarbeet pulp has been carried out in two-stage systems (i.e., separate acidogenic and methanogenic stages) under mesophilic conditions (Weiland, 1993; Stoppok and Buchholz, 1985; Hutnan et al., 2000; Hutnan et al., 2001). Sugarbeet pulp was first hydrolyzed and acidified in a stirred tank reactor and then only the liquid portion from this acidification stage was pumped to the methanogenic reactor. The methane yield for two-stage processes (Stoppok and Buchholz, 1985; Hutnan et al., 2000) was about 0.350 m^3CH_4 kg VS^{-1}. In the work done by Hunan et al. (2001) at pilot scale, a combination of STR (stirred tank hydrolysis

reactor)-UASB (upflow anaerobic sludge blanket methanogenesis reactor) recorded a lower methane yield of about 0.235 m^3CH_4 kg VS^{-1}. They attributed this to the unstable conditions (possibly build up of volatile organic acids at higher loading rates) in the operation of pilot plant. The organic loading rates (OLR) achieved were between 0.9-2.7 kg COD m^{-3} d^{-1} (Stoppok and Buchholz, 1985) and 2-6.7 kg COD m^{-3} d^{-1} (Hutnan et al., 2000 and 2001). The significant difference between these two studies was the mode of feeding. Stoppok and Buchholz (1985) fed the system in a continuous mode at hydraulic retention time between 2.5 and 7 days (HRT) whereas Hutnan et al (2000, 2001) opted for a semi-continuous mode of feeding at HRT between 13 and 17 days. Weiland (1993) reported a methane yield of 0.298 m^3CH_4 kg VS^{-1} for two-stage systems under mesophilic conditions. The system was operated at OLR of 10 kg COD m^{-3} d^{-1} in the hydrolysis/acidification stage and 6 kg COD m^{-3} d^{-1} in the methanogenesis stage at an overall HRT of 13 days. Importantly, pretreatment was achieved by maceration (80% of solids were less than 0.63 mm) of beet pulp before feeding to the hydrolysis reactor. Other studies deal with one-stage process under thermophilic conditions (Frostell et al., 1984) and mesophilic conditions (Lane, 1983; Garcia et al., 1984; Ghanem et al., 1992; Weiland, 1993). Frostell et al., (1984) operated a one-stage stirred tank reactor under thermophilic conditions at pilot scale. The system was fed in a semi-continuous mode and size reduction was done using a milling operation before feeding to the digester. OLR was 5.7±1.7 kg COD m^{-3} d^{-1} and the HRT of the system was between 27±8 days. Lane (1984) operated a one-stage, semi-continuously fed stirred tank reactor under mesophilic conditions (36±1°C) that was fed hammer-milled feedstock (12mm particle size) and obtained a methane yield of 0.263 m^3CH_4 $(kg\ VS)^{-1}$ at OLR of 3.05 kg COD m^{-}

^{3} d^{-1}. Weiland (1993) reported a methane yield of 0.272 m^3CH_4 (kg VS)$^{-1}$ when working with a one-stage mesophilic digester. Pretreatment was achieved by maceration (80% of solids were less than 0.63 mm) of beet pulp before feeding to the digester. The HRT and OLR for the system were 10 days and 8 kg COD m^{-3} d^{-1}, respectively.

In comparison, the biogasification system investigated in this study offers significant improvements over one-stage and two-stage systems reported in literature. It is a one-stage system where the feedstock is not size reduced and mixing is not required in the digester. Also, requirement of an additional vessel for acidification/hydrolysis is completely eliminated in this design. Values of methane yield (0.336 m^3 at STP (kg VS^{-1})), retention time (7 days) to achieve the methane yield and equivalent loading rate (4 kg COD m^{-3} d^{-1}) obtained from this system was comparable with the one-stage and two-stage systems (Table 2). Another important feature of leach-bed system is the reuse of inoculum from previous run. This allows the growth of robust microbial population in digester. The time required for building the inoculum is approximately 15 days and is significantly lower than most systems mentioned in the literature. For example, it took up to one year for Frostell et al. (1984) to fully adapt inoculum. On the downside the effluent released from one-stage process here has high organic matter content as it contains all undigested suspended solids. An additional solids separation and aerobic treatment step might be required to further polish effluent from anaerobic stage to meet local regulations for water discharge. Perhaps due to milling of feedstock Frostell et al., (1984) produced an effluent that was much higher in soluble COD (0.33 g COD g VS^{-1} compared to 0.114 g COD g VS^{-1} produced here) and therefore indicated the need for a combined anaerobic-aerobic treatment system to treat

effluent because of high COD and BOD concentration (25.2±1.9 kg COD m^{-3} and 7.35 ±2.33 kg BOD m^{-3}). The effluent quality after treatment was 5.14±1.47 kg COD m^{-3} and 2.00±0.59 kg BOD m^{-3}).

The process developed here can be scaled up either to a semi-continuous system which is fed once daily or to a modular batch system that will receive one day production of spent pulp. The modular system will contain eight digesters, inoculum holding tank, a lamella separator and a screw conveyor. The beet pulp from the plant will be fed to the digesters via screw conveyor. One digester will be charged daily. Once the digester is charged, the inoculum from holding tank will flooded into the digester and digestion allowed to proceed. This procedure will be repeated everyday till all the digesters are filled. Each digester will be emptied after every eight days. The residue along with the liquid will pass through a lamellar separator to separate the solids. The solids can be land-applied. The liquid will be taken to the inoculum holding tank. Since liquid will be lost with the solids, there will be a need for adding make up water to the inoculum which will dilute any inhibitory agents like ammonia.

4.6 Conclusions

The following conclusions were drawn from this study:

- SBP is readily degraded at both mesophilic and thermophilic temperatures.
- For both mesophilic and thermophilic digestion the ultimate methane potential was more than 0.3 m3 at STP / kg VS much higher than typical biomass feedstocks.
- Average ultimate methane potential was about 17% higher at thermophilic temperature than mesophilic temperature.
- Average duration of digestion to yield 95% of respective ultimate methane potential was twice for mesophilic temperature (17.2 days) compared to thermophilic temperature (9.2 days).

- By calculating chemical oxygen demand (COD) recovered as methane as a fraction of COD fed in SBP, a biogasification efficiency for thermophilic digestion was 75% and for mesophilic digestion it was 62%.

- Average methane composition was higher in biogas produced in mesophilic digester at 75% compared to 60% in that produced from thermophilic digester.

- Thermophilic digestion resulted in a maximum specific volumetric methane productivity of 2.89 m3 at STP/ m3 digester volume/day which was about threefold higher than that achieved in mesophilic digestion of 1.1 m3 at STP/ m3 digester volume/day.

- Levels of volatile organic acids were similar in both thermophilic and mesophilic digesters.

- Over a 39 day period (the retention time of the solids) in the digester almost all the organic nitrogen was solubilized under thermophilic conditions whereas only 50% of the organic nitrogen was solubilized over a 78 day period (retention time) in the mesophilic digester.

- The leach-bed system investigated here offers significant benefits with comparable performance over other systems as it eliminated the need for 1) separate hydrolysis/acidification and methanogenesis reactors, 2) pretreatment of solids and 3) mixing within the digester.

Based on the conclusions obtained from thermophilic temperature range was chosen for preparation of enzyme extracts.

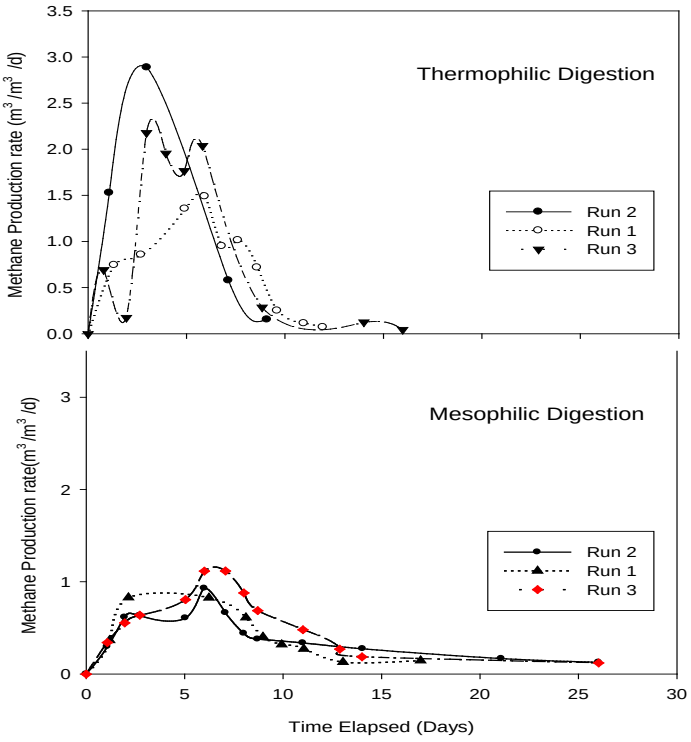


Figure 4-1. Specific volumetric methane production rate in digesters

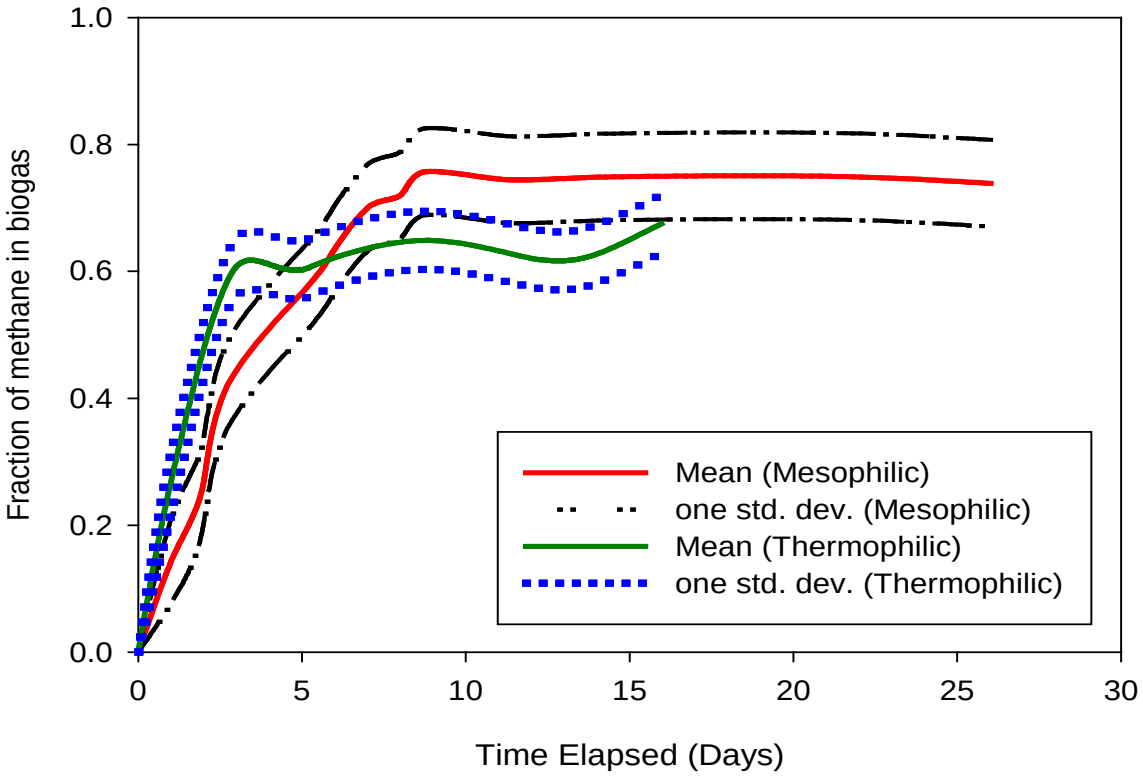


Figure 4-2: Average methane gas content in biogas

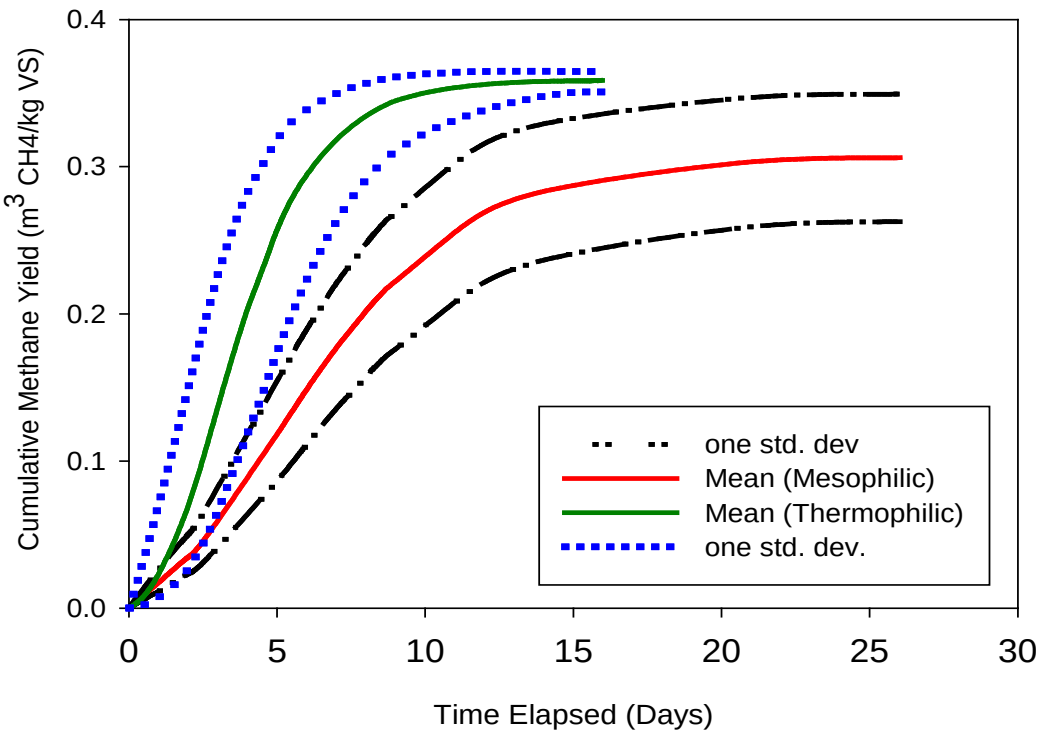


Figure 4-3: Average methane yield

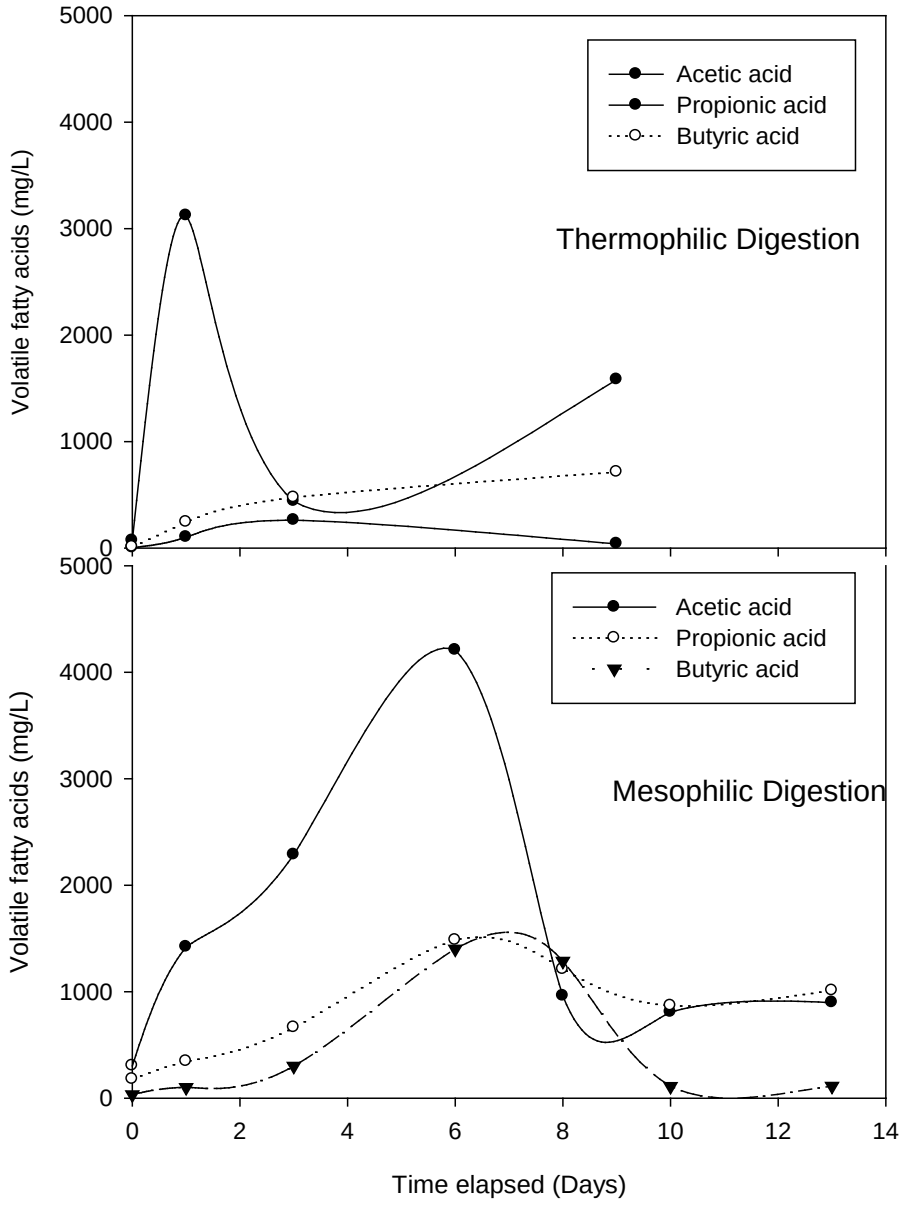


Figure 4-4: Profile of volatile fatty acids for Run 2

Table 4-1 Loading and unloading data for digesters

Loading	Wet weight (kg)	0.45
	Dry matter (kg)	0.099
	Volatile matter (kg)	0.093
	Inoculum added (L)	2.0
	Packing Density (kg/m^3), (wet weight basis)	225
	Packing Density (kg/m^3), (dry weight basis)	25
	Digestion Temperature (^{o}C)	55
Unloading	Dry matter (kg)	0.0396
	Volatile matter (kg)	0.0214
	Dry matter reduction (%)	92
	Volatile matter reduction (%)	96

Table 4-2 Summary of performance of digesters

Digester Runs	Temp	Final pH	Cumulative Methane Yield at STP (Experimental)	Gompertz Equation Parameters: Ultimate Methane Potential at STP (P)	Maximum Methane Rate at STP (R_m)	Lag time (λ)	Duration to produce 95% ultimate methane potential
	^{o}C		m^3/kg VS	m^3/kg VS	m^3/kg VS/day	days	days
				THERMOPHILIC			
Run 1		7.99	0.344	0.364	0.054	1.27	11.79
Run 2	55	7.92	0.352	0.353	0.073	0.12	7.23
Run 3		7.77	0.364	0.360	0.082	1.73	8.51
		Mean	0.353	0.359	0.070	1.04	9.17
	Standard Error		0.010	0.0032	0.0083	0.48	1.36
	Standard Deviation		0.0059	0.0056	0.014	0.83	2.35
		Range	0.353 ± 0.0059	0.359 ± 0.0056	0.070 ± 0.014	1.04 ± 0.83	9.17 ± 2.35
	Confidence Interval				95%		
				MESOPHILIC			
Run 1		8.19	0.273	0.271	0.033	1.06	14.30
Run 2	37	7.44	0.305	0.294	0.024	0.91	19.73
Run 3		8.56	0.365	0.355	0.036	1.59	17.59

Table 4-2. Continued

Mean	0.314	0.307	0.031	1.19	17.2
Standard Error	0.027	0.025	0.0036	0.21	1.58
Standard Deviation	0.047	0.043	0.0062	0.36	2.74
Range	0.31 ± 0.047	0.307 ± 0.043	0.31 ± 0.006	1.19 ± 0.36	17.2 ± 2.74
Confidence Interval			95%		

Table 4-3 Comparison of performance of present biogasification study to that reported in literature

Parameters	Frostell, B.et al (1984)	E.Stoppok and K.Buchholz (1985)	Hutnan, M. et al (2000)	Hutnan, M. et al (2001)	Present Work (2007)
Reactor Configuration	One-stage with aerobic reactor downstream	Two-stage	Two-stage	Two-stage	One-stage
Reactor Type	STR	STR	Non-stirrred tanks	STR-UASB	Non-stirred
Scale of operation	Pilot	Lab-scale	Lab-scale	Pilot	Lab-scale
Feeding mode	Semi-continuous	Continuous	Semi-continuous	Semi-continuous	Batch
Temperature °C	55	35	35	35	55
Pretreatment	Size reduction-Milling	No	No	No	No
Methane Yield m^3 CH4/ kg VS	0.358	0.346-0.355	0.352	0.235	*0.336
Biogasification Efficiency (%)	82	81	81	54	77
Solids/Hydraulic Retention Time (Days)	27±8	2.4 -7	13-17	13	7

Table 4-3. Continued

Organic loading rate kg COD/m^3/d	5.7±1.7	0.9-2.7	2.5-6.7	2	4
Residual SCOD g COD/g VS	0.33	NA	NA	NA	0.114
VS reduction (%)	81±2	NA	NA	92	96

"NA" denotes: Not available
*** Denotes: Value reported at STP**

CHAPTER 5
SACCHARIFICATION OF SUGAR BEET PULP USING ENZYME EXTRACTS FROM ANAEROBIC DIGESTER

5.1 Introduction

This chapter discusses the various enzyme activities found in anaerobic leach-bed digester, location of these activities in the digester and enzymatic hydrolysis of sugar beet pulp hydrolysate using biofilm-associated enzyme extract. The aim of this study was to saccharify sugar beet pulp using anaerobic extract from the leach-bed digester.

5.2 Background

Anaerobic of solid organic waste has gained increased attention as a means of producing energy and reducing problems associated with the disposal of organic waste (Sonakya et al., 2001). Anaerobic digestion is a multiple-stage process in which hydrolysis is one of the main steps. During hydrolysis complex insoluble organic materials are hydrolyzed by extracellular enzymes excreted by hydrolytic microorganisms (Zhang et al., 2007). Typically polysaccharides are converted to simple sugars; hydrolysis of cellulose by the cellulase enzyme complex yields glucose; hemicellulose degradation results in monosaccharides such as hexoses (xylose, galactose) and pentoses (arabinose, mannose); pectins degradation yields galacturonic acid. The secretion of extracellular enzymes represents represents a widespread physiological property of bacteria and fungi (Priest, 1992). The term “extracellular enzyme” is broadly used for any enzyme that has been transported outside the cell membrane , either up in the extracellular polymer matrix or dissolved into bulk liquid (Zhang et al., 2007). Chen and Wang (2009) categorized extracellular enzymes into two subgroups according to their locations in reference to the producer cells. Any enzyme that remains associated with its producer either in the periplasmic space or bound with

extracellular polymer is termed as a ”cell-associated enzyme”, whereas “cell-free enzyme” denotes any enzyme that has lost contact with its producer cell and dissolved into the bulk environment. During hydrolysis, organic solids function as biofilm support as well as substrate (Chanakya et al., 1999). Therefore, microbial cells could attach to the surface of these organic wastes to carry out degradation using cell-associated enzymes (Chanakya et al., 1999).

Moloney et al., (1984) and Coughlan et al., (1988) have shown that solid-state cultures of the fungus such as *T reesei* and *T. emersonii* grown on sugar beet pulp exhibit cellulolytic , hemicelluloytic and pectinolytic activities and has been used to saccharify sugar beet pulp. But considering the complex structure of sugar beet pulp pectinolytic, hemicellulolytic and cellulolytic enzyme systems need to act synergistically to effect complete saccharification of sugar beet pulp. For example, extracts of solid – state cultures of *T. emersonii or T. reesei* MCG77 grown on sugar beet pulp contain adequate amounts of cellulase and hemicellulase activities but lack full complement of enzymes needed for hydrolysis of pectins (Coughlan et al., 1984). *P. capsulatum* has been found to produce all three activities, though Fpase (Filter paper lyase) activity was found to be lower than *T. emersonii or T. reesei* MCG7. Arntz and Buchholz (1988) showed that multiple mechanisms of enzyme production are certainly involved in polysaccharide hydrolysis by mixed cultures of anaerobic digestion bacteria. Notably, they found high activities of extracellular enzymes, particularly, pectin, araban, galactan and glucan hydrolyzing enzymes in anaerobic reactor digesting sugar beet pulp under mesophilic conditions. Furthermore, Arntz and Buchholz (1988) concluded that high levels of activities obtained in the digester makes isolation and further concentration

technically feasible. It is possible to that high extracellular activities are present in a single-stage, thermophilic leach-bed digester based on peak activities obtained in just two days in a recent study on thermophilic anaerobic digestion of sugar beet pulp conducted by Koppar and Pullammanappallil (2008). The extraction of these extracellular enzymes and utilization of these enzymes for saccharification of treated/untreated sugar pulp offers a novel approach to reduce the impact of enzyme cost on pre-treatment towards ethanol production. This approach will provide on-site enzymes for saccharification of sugar beet pulp, making hydrolysis process less susceptible to prices of commercial enzymes.

5.3 Methods

The following methods were employed for studies presented in this chapter:

- Detection and extraction of hemicellulolytic and pectinolytic activities in the anaerobic digester.
- Sugar beet pulp hydrolysate was prepared by hydrothermolysis of pulp in water at 160 °C for 1 hr.
- Enzymatic hydrolysis of sugar beet pulp hydrolysate using biofilm-associated enzymes.

Saccharification was measured by measuring reducing sugars using DNS method. Details of methods are given in Chapter 2 (Sections 2.2.1, 2.3.3, 2.3.4, 2.3.5 and 2.4)

5.4 Results

Enzyme activities in anaerobic digester: Table 5-1 shows cell-free, cell-bound (or cell-associated) and biofilm-associated activities from the anaerobic digester on day 14 (i.e. 14 days) after the start of digestion. Cell bound pectinolytic and hemicellulolytic activities were 0.016 IU and 0.008 IU, respectively. Cell-free pectinolytic and hemicellulolytic activities were higher at 0.024 IU and 0.038 IU, respectively. Biofilm-

associated pectinolytic and hemicellulolytic activities showed lower activities were 0.012 IU and 0.010 IU, respectively. Further work was focused on biofilm-associated activity only. Table 5-6 summarizes the pectinolytic and hemicellulolytic activities on day 6, 9 and 14 after the statrt of digestion. Pectinolytic activity decreases from 0.070 IU (Day 6) to 0.032 IU (Day 9) and further to 0.012 IU (Day 14). Similar trend was noted for hemicellulolytic activity. The hemicellulolytic activity dropped from 0.128 IU (Day 6) to 0.036 IU (Day 9) and further to 0.01 IU (Day 14). Table 5-2 shows the effect of increase in solids loading activities. “Solids loading” is defined as the amount of “solids residue” withdrawn from the digester used for extraction of biofilm-associated activities. Three solids (10 g, 20 g, 30 g) loadings were studied. The activities were measured 9 days after the start of digestion. The pectinolytic and hemicelluloytic activities for 10 g solids loading were 0.026 IU and 0.034 IU. Pectinolytic activity showed a slight increase to 0.032 IU when solids loading was 20 g, but dropped slightly to 0.030 IU when solids loading was 30 g. Hemicelluloytic activity showed a slight increase to 0.036 IU when the solids loading was 20 g, the activity further increased to 0.042 IU when the solids loading was 30 g. Table 5-3 shows the effect of increase on time for extraction on activities. The extraction time was increased from 2 hr to 15 hours to assess the effect. The pectinolytic and hemicelluloytic activities for 10 g solids loading were 0.040 IU and 0.040 IU. Pectinolytic activity showed a slight increase to 0.048 IU when solids loading was 20 g, increased further to 0.064 IU when solids loading was 30 g. Hemicelluloytic activity showed a slight increase to 0.048 IU when the solids loading was 20 g, the activity further increased to 0.052 IU when the solids loading was 30 g. Table 5-4 shows the hemicelluloytic and pectinolytic activities in the biofilm-associated extract for day 6

(i.e. 6 days after start of digestion). The solids loading for this run was 20g. The assays for measuring activities were carried at pH 4.8 by adding citrate buffer to the reaction mixture. Value of pH 4 – 5 was found optimum for both hemicelluloytic and pectinolytic enzymes (Jayani et al., 2005). The pectinolytic activity after 2 hr of extraction was 0.070 IU and hemicellulolytic activity was 0.128 IU.

Saccharification of sugar beet pulp hydrolysate: Saccharification was initiated by adding biofilm-associated enzyme extract to sugar beet pulp hydrolysate. The results have been summarized in Table 5-6. Saccharification increased to 49.53% in 3 hrs from 44% at the start, increased further to 52.67% in 6 hrs and further showed a marginal increase to 58.28% at the end of 24 hours.

5.5 Discussion

Enzyme activities in anaerobic digester: The pectinolytic and hemicelluloytic activities of cell-bound and biofilm-associated activities in digester were lower than the cell-free activities. Increase of solid loading to extract biofilm-associated activties increased the concentration of activities in biofilm enzyme extract. Optimum pectinase activity was obtained from solids loading of 20g. Highest hemicellulase activity was obtained from 30g solids loading. Both activities increased almost 1.5 times when the extraction duration was increased from 2 hr to 15 hr. Pectinolytic (0.07 IU) and hemicelluloytic (0.128 IU) activities were measured on day 6 and these activities were comparable to the diluted (1000^{th}) activities of commercial enzymes. For example diluted commercial pectinolytic activity was 0.158 IU and the corresponding pectinolytic activity in anaerobic extract was about half (0.07 IU). Similarly it was found that the hemicellulolytic activity in anaerobic extract was found to be 5 times higher than the diluted hemicellulolytic activity. Hemicelluloytic activity was higher than pectinolytic

activity. Zhang et al., (2007), reported that cell-bound activities were lower than cell-free and biofilm-associated activities during the hydrolysis of vegetable waste. Parawira et al., (2005), measured 0.006 IU of pectinolytic activity at day 30 in a two-stage mesophilic anaerobic digestion of potato waste. Furthermore, higher cell-free activities were found over cell-bound activities in the digester. Morgenroth et al., 2002, attributed this to the efficiency of excreted enzyme to degrade suspended particulate matter, which is higher than the cell-bound activity. Kosugi et al.,(2001) reported similar results for xylanase activity of *Clostridium cellulovorans*, an anaerobic, mesophilic, cellulolytic bacteria. Free-amylase enzyme activity was also reported to be greater than the cell-bound enzyme activity during starch hydrolysis under anaerobic conditions by Sharp and Macfarlane (2000). It is possible that in current study, that, different groups of microorganisms were supported in the digester at different times leading to fluctuations of hemicelluloytic and pectinolytic activities. Similar observations were noted by Parawira et al., (2005). The results from our study suggest presence of high activities of polysaccharide degrading enzymes within leach-bed digester. Hutnan et al., (2001) detected an activity of 0.0015 IU during the anaerobic digestion of sugar beet pulp in a pilot-scale two-stage system under mesophilic conditions. Arntz and Buchholz (1988) found peak hemicellulolytic and pectinolytic activities of 0.0059 IU and 0.0037 IU in a stirred tank reactor digesting sugar beet pulp under mesophilic conditions. They further concluded that it was essential to have an adapted mixed culture for one or two fermentations on the substrate without pH control so that growth of methanogens would be inhibited. Activities obtained in current study are 10 times higher than that achieved by Arntz and Buchholz (1988). This can be attributed to thermophilic temperature of

operation, which likely produces highly active enzymes and perhaps leach-bed mode of operation. In present study, even though the biofilm-associated enzyme activities were slightly lower than the cell-free activity, it was possible to achieve higher biofilm-associated activity equivalent to or more than cell-free activity by increasing the concentration of solids loading. Arntz and Buchholz (1988), found that factors important for concentration of enzymes are fermentation mode and substrate concentration. Based on current studies it was found that duration of enzyme extraction is another important parameter as higher activities were obtained by prolonging the duration of extraction. Coughlan et al., (1988) found very high pectinolytic (60.8 IU), hemicellulolytic (0.312 IU) and cellulolytic (50.53 IU) in extracts of solid-state cultures of *Penicillium capsulatum*, grown on sugar beet pulp. Similar activities were detected on *Trichoderma Reesei* MCG77 by Moloney et al., (1984) and Coughlan et al., (1988). These are certainly higher activities than those obtained from anaerobic extracts. But just like aerobic fungus, anaerobic extracts do possess wide array of extracellular enzymes that can solubilize sugar beet pulp.

Saccharification of sugar beet pulp hydrolysate: Pretreated sugar beet pulp (hydrolysate) attained a saccharification of 58% in 19 hrs after incubating with anaerobic digester enzyme extracts (from Day 6) at 50°C. The reduced saccharification (%) in sugar beet pulp could be due lower pectinolytic activitity. The anaerobic digester extract can be supplemented with diluted commercial pectinase to enhance the saccharification of sugar beet pulp. Moloney et al., (1984) achieved a polysaccharides hydrolysis of 25.2% - 34.5% after 4 days, when the beet pulp (size reduced by ball-milling or grinding) was incubated in a culture filtrate of *Talaromyces emersonii* UCG 208.at pH 5

at 60°C. Furthermore, when a combination of size reduction and peracetic acid treatment was used, 76.7% saccharification was achieved after 4 days of incubation. Moloney et al., (1984) found that neither *Trichoderma reesei* MCG 77 nor *Talaromyces emersonii* UCG 208 filtrates had full range of enzymes necessary for pectin degradation. Moloney et al., (1984) pretreated sugar beet pulp with pectinase enzyme (*Aspergillus niger*) and digested with unconcentrated filtrate of *Talaromyces emersonii* UCG 208 for 4 days. 69% of total polysaccharides were solubilized whereas, only 32.6% beet pulp was solubilized when only filtrate was used. Coughlan et al., (1988) achieved 76% saccharification by *Penicillium capsulatum* enzyme preparation at pH 5, 50°C and 2.5 days. It was difficult to compare the (%) saccharification of spent sugar beet pulp obtained in current research to that in literature because of the variability of sugar beet pulp composition. For example, the (%) saccharification calculations by Moloney et al., (1988) and Coughlan et. al., (1988) were based on 85%(dry weight of cellulose, hemicellulose and pectin) whereas, basis for (%) saccharification calculation for current research was 66% (dry weight of cellulose, hemicellulose and pectin). Irrespective of the differences in calculation, saccharification obtained from anaerobic digester enzyme extract here was comparable to aerobic fungus and diluted (1000^{th}) commercial enzymes.

The use of microbial extracellular enzymes from the anaerobic digester for saccharification of sugar beet pulp is a novel concept and offers a few advantages over fungal and commercial enzymes such as 1) On-site production of highly active microbial enzymes is possible and this approach can possibly improve the economics of pretreatment, as the costs of enzymes are independent of market fluctuations 2) The

anaerobic extracts possess both hemicelluloytic and pectinolytic activities besides other activities important for degradation of pentoses in sugar beet pulp, possibly reducing the costs of using commercial enzymes 3) The method for enzyme extraction developed here can be scaled up to a continuous or a semi-continuous operation.

5.6 Conclusions

Extracellular enzymes are indeed secreted during thermophilic anaerobic digestion of sugar beet pulp in leach bed digester, particularly, pectin and hemicellulose hydrolyzing enzymes. The maximum activity of both hemicelluloytic and pectinolytic enzymes were found in cell-free (leachate) portion of the digester, followed by biofilm-associated and cell-bound activities. It is possible to obtain highly concentrated activities from biofilm (solids residue). It was found that 20 g of solids residue was optimum loading to achieve high biofilm-associated activities. Also by prolonging the duration of extraction to 15 hrs the activities increased almost 1.5 times over activities obtained after 2 hr. The hemicelluloytic activity from the extract was 5 times higher that the diluted hemicellulase commercial enzyme preparation, whereas the pectinolytic activity in extract was ½ of commercial pectinase preparation. The activities in thermophilic digester were approximately 10 times higher than mesophilic digesters. 58% saccharification was obtained in just 24 hrs by microbial extracts from anaerobic digester after hydrothermolysis pretreatment as compared to 76% obtained from *P. capsulatum* fungus in 2.5 days.

Table 5-1. Pectinolytic and hemicellulolytic activities in anaerobic digester – Day 14

Time,hrs	Cell-bound		Cell-free		Biofilm-associated	
	Pec (IU)	Hemi (IU)	Pec (IU)	Hemi (IU)	Pec (IU)	Hemi (IU)
4	0.016	0.008	0.024	0.038	0.012	0.010

Table 5-2 Biofilm - associated activities at different solid residue loading – 2 hr extraction – Day 9

Time,hrs	10 g		20 g		30 g	
	Pec (IU)	Hemi (IU)	Pec (IU)	Hemi (IU)	Pec (IU)	Hemi (IU)
4	0.026	0.034	0.032	0.036	0.030	0.042

Table 5-3 Biofilm-associated activities at different solids residue loading - 15 hr extraction - Day 9

Time,hrs	10 g		20 g		30 g	
	Pec (IU)	Hemi (IU)	Pec (IU)	Hemi (IU)	Pec (IU)	Hemi (IU)
4	0.040	0.040	0.048	0.048	0.064	0.052

Table 5-4 Biofilm-associated activities - 2 hr extraction - Day 6

Time, hrs	Biofilm-associated	
	Pec (IU)	Hemi (IU)
2	0.070	0.128

Table 5-5 Biofilm-associated activities - 2 hr extraction

Time, days	Biofilm-associated	
	Pec (IU)	Hemi (IU)
6	0.070	0.128
9	0.032	0.036
14	0.012	0.010

Table 5-6 (%) Saccharification - using extracted anaerobic enzyme extract (biofilm-associated)

Time, hrs	% Saccharification
3	49.53
6	52.67
24	58.28

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The conclusions of this research work were based on the findings from experimental studies conducted on:

- Thermophilic and mesophilic anaerobic digestion of spent sugar beet pulp using leach-bed reactor.
- Hydrothermolysis pretreatment of spent sugar beet pulp
- Saccharification of spent sugar beet pulp using commercial enzyme preparations and enzyme extracts from anaerobic digester

This research provides a comprehensive understanding of location of extracellular enzymes activities in the anaerobic digester and effects of pretreatment on saccharification. All the specific objectives of this research were met successfully.

Important critical findings were:

- Dinitro-salicyclic method can be used to quantify sugar concentration in enzyme catalyzed reaction with minimum error based on comprehensive calibration charts developed for reducing sugar analysis.
- Fermentable sugars were obtained by sequential hydrothermolysis and enzymatic hydrolysis of spent sugar beet pulp. In the hydrothermolysis stage 44% saccharification was obtained in just 1 h.
- Hemicellulose and pectins were solubilized to its constituent monomeric sugars during hydrothermolysis. In the enzyme hydrolysis stage, the hydrolysate achieved almost complete saccharification (88%) in just 6 h by using a combination of pectinase (9.5 IU) and hemicellulase (0.3 IU).That is the total time to achieve complete saccharification was just 7 h.
- It was found that there exists a strong synergism between pectinase and hemicellulase enzymes to achieve complete saccharification and pectinase was an important enzyme.
- The combined hydrothermolysis pre-treatment and enzymatic hydrolysis method investigated here offers significant benefits over other saccharification methods: (1) Faster reaction rates (2) no environmental problems (3) Complete saccharification of pectinase and hemicellulase in shorter duration (4) reduced enzyme loading to

achieve complete saccharification (5) no size reduction of feedstock required. (6) no chemical addition

- Sugar beet pulp is readily degraded at both mesophilic and thermophilic temperatures.

- For both mesophilic and thermophilic digestion the ultimate methane potential was more than 0.3 m3 at STP / kg VS much higher than typical biomass feedstocks.

- Average duration of digestion to yield 95% of respective ultimate methane potential was twice for mesophilic temperature (17.2 days) compared to thermophilic temperature (9.2 days).

- Thermophilic digestion resulted in a maximum specific volumetric methane productivity of 2.89 m3 at STP/ m3 digester volume/day which was about threefold higher than that achieved in mesophilic digestion of 1.1 m3 at STP/ m3 digester volume/day.

- The leach-bed system investigated here offers significant benefits with comparable performance over other systems as it eliminated the need for 1) separate hydrolysis/acidification and methanogenesis reactors, 2) pretreatment of solids and 3) mixing within the digester.

- Extracellular enzymes are indeed secreted during thermophilic anaerobic digestion of sugar beet pulp in leach bed digester, particularly, pectin and hemicellulose hydrolyzing enzymes.

- The maximum activity of both hemicelluloytic and pectinolytic enzymes were found in cell-free (leachate) portion of the digester, followed by biofilm- associated and cell-bound activities. It is possible to obtain highly concentrated activities from biofilm (solids residue).

- It was found that 20 g of solids residue was optimum loading to achieve high biofilm-associated activities. Also by prolonging the duration of extraction to 15 hrs the activities increased almost 1.5 times over activities obtained after 2 hr.

- The hemicelluloytic activity from the anaerobic extract was 5 times higher that the diluted hemicellulase commercial enzyme preparation, whereas the pectinolytic activity in extract was ½ of commercial pectinase preparation.

- The activities in thermophilic digester were approximately 10 times higher than mesophilic digesters.

- 58% saccharification was obtained in just 24 hrs by microbial extracts from anaerobic digester after hydrothermolysis pretreatment as compared to 76% obtained from P. capsulatum fungus in 2.5 days.

6.2 Future Work

The research studies presented here opens exciting avenues to explore and deepen our understanding in developing cost-effective pretreatment methods to saccharify pectin-rich biomass residues:

- Develop strategies to optimize the temperature of operation for hydrothermolysis pretreatment and minimize side reaction, understanding effect of enzyme loading on saccharification and develop techniques for extraction of extracellular enzymes from the anaerobic digester.
- Study various combinations of commercial enzyme combinations and optimize enzyme loadings to attain maximum saccharification of sugar beet pulp.
- Develop strategies to find the “zone of optimum extracellular activity” in the anaerobic digester.
- Develop better methods to extract cell-free and biofilm activities from anaerobic digester.
- Develop a better understanding of cell-free, cell-bound and biofilm-associated activities to potentially enhance anaerobic digestion and use extracellular activity monitoring to assess the performance of AD system.
- Develop a close-loop system that can simultaneously produce enzymes for saccharification and generate valuable biogas (methane) from the digester.
- Develop a economic model to assess the benefits of sequential hydrothermolysis and saccharification using anaerobic extracts compared to commercial processes.

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BIOGRAPHICAL SKETCH

Abhay Krishna Koppar was born in Pune, India in the year 1977. He received his Bachelor of Engineering in Chemical Engineering from the University of Pune, India in July 2000. He then, traveled to the United States of America to pursue further studies and enrolled in the Masters program in College of Tropical Agriculture and Human Resources at the University of Hawaii at Manoa in year 2003. He obtained a Master of Science in Bioengineering in August 2005. Thereafter, he enrolled at the University of Florida to pursue Doctoral studies. He obtained the Doctor of Philosophy (PhD) in Agricultural and Biological Engineering in December 2009. After finished his studies, he wishes to pursue a career in academia and contribute meaningfully to the field of science and engineering by solving problems that touch lives of common people.