Spring 2016

Continuous flow reactor for carbonic acid hydrolysis/pretreatment of microalgal biomass to produce bioethanol

Nicholas Dudenhoeffer

Department: Chemistry

CONTINUOUS FLOW REACTOR FOR CARBONIC ACID HYDROLYSIS/PRETREATMENT OF MICROALGAL BIOMASS TO PRODUCE BIOETHANOL

by

NICHOLAS DUDENHOEFFER

A DISSERTATION

Presented to the Faculty of the Graduate School of

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

2016

Approved by:

Dr. Paul K. Nam, Advisor
Dr. Shubhender Kapila
Dr. Yinfa Ma
Dr. V. Prakash Reddy
Dr. Melanie Mormile

PUBLICATION DISSERTATION OPTION

The dissertation consists of the following three articles that have been published, submitted for publication, or will be submitted for publication as followings:

Paper I, pages 25-42 are intended for submission to BIOFUEL RESEARCH JOURNAL.

Paper II, pages 43-55 are intended for submission to BIORESOURCE TECHNOLOGY.

Paper III, pages 56-75 are intended for submission to FUEL.

This dissertation has been formatted to the specifications prescribed by Missouri University of Science and Technology.

ABSTRACT

In order to maximize bioethanol production an efficient pretreatment method for the hydrolysis of polysaccharides to fermentable sugars is necessary. Commonly used pretreatment methods are the slow enzymatic hydrolysis and the corrosive mineral acid hydrolysis process that requires a post-treatment neutralization step and generates waste stream. We investigated the high temperature water with carbonic acid catalyst as an alternative method. Carbonic acid generated from dissolved CO_2 is inexpensive and environmentally benign, and easily removed by decompression. A high pressure continuous flow reactor that can continuously process the wet biomass stream and perform the carbonic acid hydrolysis in-situ using the pressurized carbon dioxide was designed and successfully tested for the pretreatment of selected biomass feedstocks including microalgae, potato peel, wood cellulose, etc. The reaction conditions such as residence time and temperature greatly influenced the formation of simple sugars and degradation byproducts. The carbonic acid hydrolysis of two different microalgae strains for <10 min at 210°C using 7 MPa CO_2 produced the maximum amount of glucose when the sample was first neutralized with a small quantity (0.05%) of sulfuric acid. The rate of biomass carbohydrate to glucose conversion using the continuous flow reactor was comparable to the conventional dilute mineral acid (5% sulfuric acid) treatment. The amount of byproducts such as 5-HMF and furfural which were produced from the glucose decomposition was approximately one order higher, however, no inhibition was observed during the subsequent fermentation of sugars in the hydrolysate to ethanol using a commercial yeast.

ACKNOWLEDGEMENTS

I would first like to express my gratitude for my advisor Dr. Paul Nam. He has provided financial, guidance, and allowed me to work on a number of different studies throughout my PhD program. I would also like to acknowledge Dr. Keesoo Lee from Lincoln University, who was my undergraduate advisor. Both, Dr. Nam and Dr. Lee played a critical role in my decision to pursue a graduate degree.

I would like to thank the US Department of Agriculture – NIFA and Missouri Life Sciences Research Board for funding.

The members of my graduate committee, Dr. Yinfa Ma, Dr. V. Prakash Reddy, Dr. Dr. Shubhender Kapila, and Dr. Melanie Mormile, have all given their time and assistance and I am forever grateful.

Lastly, I want to thank my family. My parents, brothers, and sister have all been sources of encouragement and inspiration. And most of all my wife, Nicole Dudenhoeffer, without her love and support I would not be the person I am today.

TABLE OF CONTENTS

Page

LIST OF ILLUSTRATIONS

Page

PAPER III

LIST OF TABLES

Page

SECTION

1. INTRODUCTION

1.1 RENEWABLE BIOFUELS

Increasing industrialization and growing transportation needs have led to a high demand of petroleum-based fuels. The global consumption for petroleum is predicted to increase 40% by 2025.[1] Fossil fuels are the main source of energy and are becoming exhausted and also contribute to greenhouse gas (GHG) emissions. These two factors have brought about the pursuit for alternative, renewable, and cost-effective energy sources that are more ecofriendly.[2] Biomass is one of the most encouraging renewable resources that can be used to generate different types of biofuels, such as biodiesel and bioethanol.[3 4]

Biofuels can be liquid, gas, or solid fuels that are generated from biomass. These include ethanol, methanol, biodiesel, hydrogen and methane. Biofuels are portrayed as an ideal source of renewable energy, and can be broadly classified as primary and secondary biofuels.[2] Primary biofuels can be used in their unprocessed form and are most commonly used for heating, cooking, and electrical production; some examples are fuelwood, wood chips and other organic materials. Secondary biofuels are produced after processing the biomass. These secondary biofuels can be further classified based on what type of biomass they were produced from. Figure 1.1. illustrates a common break down of the different types of biofuels.

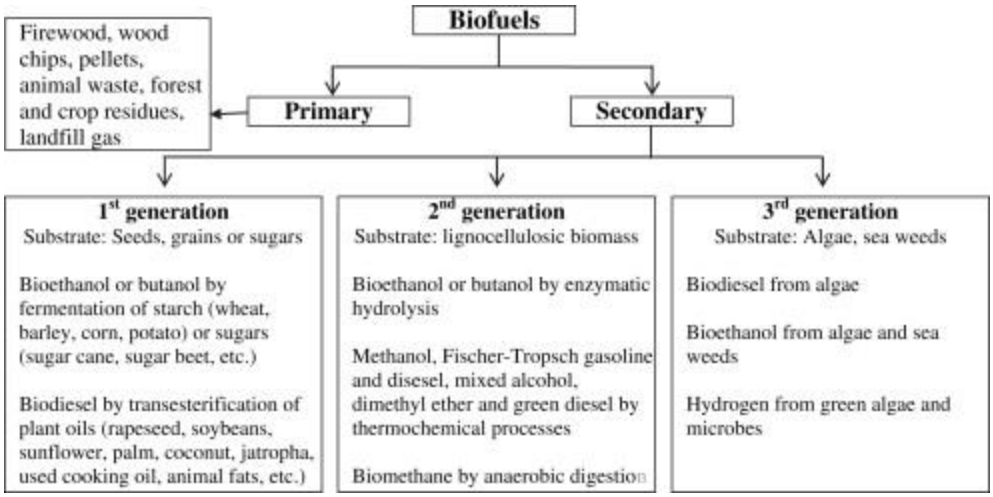

Figure 1.1. Classification of biofuels[2]

First-generation biofuels are generally produced from sugars and vegetable oils. They require very simple processing from the raw material to the finished product. One well known example would be the production of ethanol from corn. The production of biodiesel from vegetable oils by transesterification is also a well-known first-generation biofuel. These first-generation biofuels have been around for some time and are produced at a commercial quantity in a number of countries. However, the raw materials used are also valuable food stocks, which produce a conflict and led to high production costs.

Second-generation biofuels are produced from agricultural lignocellulosic biomass, that are non-edible residues of food crops or non-edible whole plant biomass. Corn stover, corn cobs and wheat straw are all examples of non-edible residues. Non-edible whole plant biomass or energy crops would be switchgrass, sorghum, Miscanthus, or poplar tree. The main advantage to second-generation biofuels is that there is no competition with food supplies like with first-generation biofuels.

Third-generation biofuels are derived from microbes and microalgae. These biofuels are devoid of the major drawbacks of the first and second-generation biofuels. They are not in direct competition with food supplies and do not compete with land use.[5] Third generation biofuel feedstocks have at least 30 times higher energy output per land unit than second generation feedstocks.[1]

Bioethanol can be produced from a variety of feedstocks ranging from sugar or starched based crops (e.g., sugarcane and corn) to lignocellulosic biomass (e.g., rice straw and switchgrass).[2] In Brazil, one of the world's largest bioethanol producers, sugar cane is the main feedstock. The United States primarily relies on corn and the European Union relies on sugar beets.[6] The industrial process for the production of ethanol from these first generation feedstocks, like beet or cane sugars and sugars from grains, are well established. They are also some of the least complex methods for the production of bioethanol. However the cost of the raw materials can be high.

Another major issue, with the use of first generation biofuel feedstocks, is the limitation of sufficient cultivable land on earth. The competition of food verses biofuel based crops could result in an increase of food prices that could greatly affect the poor. With the increasing population this issue will just become greater, raising the question of what to use the limited farm land available on earth for energy or food? Other factors can also be raised from the extensive cultivation of energy crops such as pollution of agricultural land with fertilizers and pesticides, soil erosion, reduced crop biodiversity, biocontrol ecosystem service losses and greenhouse gas emissions.[7 8 9 10 11 12 13]

Second generation feedstocks, lignocellulosic biomass and starchy wastes, that are made of crop residues, grasses, sawdust, woodchips, sludge and livestock manure,

have been looked at as low cost feedstocks. These materials can be enzymatically hydrolyzed to fermentable sugars and converted to bioethanol. There have been several reports of lignocellulosic wastes materials like crop residues[14], municipal solid waste[15], forest products waste[16] [17], leaf and yard waste[18], and a few studies on livestock manures.[19] Even though these feedstocks show some promise because they are noncompeting materials with the food stocks, the low yields and high costs of the conversion to biofuels limits their potential. Furthermore, the lignin in these feedstocks is very difficult to be degraded biologically and cannot be fermented.[20]

1.2 POTENTIAL BIOFUELS FROM MICROALGAE

Algae consist of a large variety of species and are look at as one of the oldest life-forms and are present in all existing earth ecosystems. Microalgae can produce lipids, proteins, and carbohydrates in large amounts over short periods of time. Algae have been proposed as a means of removing carbon dioxide from flue gases from power plants to help reduce GHG emissions because of the efficiency in fixing carbon dioxide. All of the three major components of algae (lipids, carbohydrates, and protein) can be converted into biofuels. Many species have been found to be very rich in fatty acids which can be converted to biodiesel.[21] Some species contain lipids which are hydrocarbons similar to those found in petroleum; others can contain lipids similar to seed oils, which can be converted to synthetic diesel fuel through transesterification. The carbohydrates can be converted into ethanol by fermentation. And all three components can be converted into methane gas by anaerobic digestion. Furthermore, many algae strains can be tailored, genetically or through growth conditions, to produce key components like lipids or

carbohydrates. Table 1.1. lists a several different algal strains and shows their general biomass composition.

Table 1.1. Algae biomass composition of various strains (% dry wt.)[22]

Alga	Protein	Carbohydrates	Lipids
Anabaena cylindrica	43–56	25–30	4–7
Aphanizomenon flos-aquae	62	23	3
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Euglena gracilis	39–61	14–18	14–20
Porphyridium cruentum	28–39	40–57	9–14
Scenedesmus obliquus	50–56	10–17	12–14
Spirogyra sp.	6–20	33–64	11–21
Arthrospira maxima	60–71	13–16	6–7
Spirulina platensis	46–63	8–14	4–9
Synechococcus sp.	63	15	11

Algae are gaining a large amount of interest as a renewable source of biomass for the production of biodiesel and bioethanol. They fall under the third generation of biofuels, which overcome the major drawbacks of the first and second generation biofuels. As far back as the 1950s algae has been looked at as an energy feedstock[23], with a large effort made in the 1970s with the oil crisis. Since then there has been a continuing effort in the development of algae base biofuels as well as other efforts like the use of algae for CO_2 bioremediation. A great deal of funding has come from the US Department of Energy (DOE) to algae fuel research, with many advances coming from the National Renewable Energy Lab (NREL) in Golden, Colorado.[24]

There are several important features of algae that make them great candidates for renewable fuels. Algae have high photon conversion efficiency and can synthesize and accumulate large amounts of oils or carbohydrates for the production of fuels, and they can do this with inexpensive raw materials.[7] [25] Algae can tolerate high levels of CO_2, which is why they could be used to utilize emitted CO_2 from petroleum based power stations and reduce GHG emission. Aquatic microalgae are buoyant allowing them to not need structural biopolymers such as hemicellulose and lignin, which is a drawback for 2nd generation biofuel feedstocks. Algae belong to a diverse group, comprising of thousands of different species, which allows for many different strains that can be suited for different purposes and environments. Because of the structural differences between algae and terrestrial plants, algae can produce high yields of stored materials when compared to the most productive land plants. One example of this are kelp forests in shallow sub-tidal regions that are among the most productive communities on earth.[26] Marine algae can be utilized for carbohydrate production year round.[27] Algae can be harvested over shorter spans of time when compared to land plants allowing for a better supply to the increasing demand form ethanol feedstocks.[20] Algae have simple growth requirements and can grow to high densities relying on only sunlight, CO_2, and a small amount of other nutrients which they can very effectively utilize.[28]

Algae can be grown in fresh water, saline water or municipal waste water.[29] [30] Because of this algae that is grown in saline water or municipal waste water also offers the advantaged of not competing with the food crops fresh water supply. The algae biomass also contains other components that can be further used like, bio-polymers and proteins.

1.3 ALGAL BIOMASS PRODUCTION

Algae are primitive plants belong to thallophytes. They have no roots, stems and leaves, and have no sterile coverings around their cells. They are photosynthetic life-form that primarily use chlorophyll a as their photosynthetic pigment. Phototropic algae absorb sunlight and assimilate carbon dioxide from the air, discharge gases, and soluble carbonates. They can tolerate and utilize carbon dioxide levels as high as 150,000 ppmv.[31] Algae are present in diverse environments.[32] Algae can be autotrophic, meaning they use photosynthesis, or heterotrophic, these species are able to take up small molecules in the environment and convert them into other compounds. There are also certain species that can utilize inorganic and organic carbon sources and are referred as mixotrophic.

Algae can be generally grouped into two categories, microalgae and macro algae. These two groups are based on the morphology and size of the species. As you would suspect microalgae are microscopic photosynthetic organisms many of which are unicellular but this is not always true. Macro algae one the other hand are larger, comprised of multiple cells that organize together to form structures that resemble higher plant life.[23]

Because of the large variety of algae strains available identifying and selecting the appropriate strain can be a hard task. This is because algae species and strains can vary greatly in terms of growth rate and productivity, light and nutrient requirement, and other factors. Because of this the strain selection is based on the ability to produce that highest ethanol yield either directly or through biomass accumulation. Some desirable characteristics of strains that are being considered for the production of biofuels are (1)

capable of surviving under stressful conditions common in photobioreactors; (2) able to out compete local strains in open pond production systems; (3) high utilization of CO_2; (4) limited nutrient requirements; (5) tolerate a wide range of temperatures resulting from seasonal variations; (6) potentially produce other valuable products; (7) short growth cycle; (8) high photosynthetic efficiency, and (9) display self-flocculation characteristics.[5] Specifically for the production of ethanol strains should have high growth rates and high starch/cellulose content, because the starch and cellulose are the main substrates used in the production of ethanol.

In order to meet the ever growing demand for fuel, algae would need to be produced year round. Since most naturally occurring algae are very low in density, it is necessary for the algae to be mass cultured in controlled environments in order to achieve consistently high productivity and all factors that can impact the biomass yield be optimized and efficiently integrated.[33] The cultivation process can be done in batch, semi-batch, or continuous systems. Batch cultivation consists of a single inoculation of cells which are grown for several days to a desired concentration and then transferred into a larger culture volume and allow to grow until the cultures' stationary phase is reached. The semi-batch system allows a portion of the culture to be harvested and the original culture replenished with fresh media and allowed to continue growing. Continuous systems can be carried out in two ways, turbidostat or chemostat cultures. Turbidostat cultures, media are continuously added to maintain a constant cell density. Chemostat cultures a slow steady flow of fresh media is continually introduced while excess culture is continuously collected.

The most common production systems employed for algal cultivation are outdoor open ponds and enclosed photobioreactors. There is a large variation between the characteristics of these two production types, growth parameter control, contamination, water evaporation, productivity, downstream processing characteristics, capital and operational cost just to name a few.[5]

Open ponds are the most widely used system for larger-scale outdoor microalgae cultivation since they are cheaper, easy to build and operate.[5] These open ponds can be excavated and use lined or unlined with impermeable materials, or be built up with walls. Unlined ponds are not as common as they suffer from several limitation based one algae strain and soil and environmental conditions.[1] Open pond systems are commonly found in three types, raceway pond, circular pond and sloped pond. Raceway ponds have relatively low capital and maintenance costs, circular ponds are less attractive because of expensive concrete construction, high energy consumption of stirring, mechanical complexity of supplying CO_2 and inefficient land use.[23] While the open pond systems are economical, there are several disadvantages such as low productivity, high harvesting cost, water lost through evaporation, and lower CO_2 use efficiency.[34] Another factor is the temperature fluctuations due to diurnal variations are difficult to control in open pond systems.[35] Contamination is also something to deal with in open ponds with other algae and protozoa growing in the cultures. Transgenic algae are particularly a risk because they may be less fit for open cultivation.

The limitations of the open pond systems led to the development of enclosed photobioreactors for the mass cultivation of algae. There are two major types of enclosed photobioreactors, tubular and plate types. There are many advantages to enclosed system

such as narrow light paths, large illumination area and relatively controllable environment, and less contamination issues that all allow for higher dell density in photobioreactors than in open pond systems.[35 36] However there are some short comings, gradients of pH, dissolved oxygen and CO_2 along the tubes, wall growth, fouling, hydrodynamic stress, and high expense to scale up.[23 33 34 36] Because of the higher cell mass productivities harvesting cost can be reduced. But the overall cost of the photobioreactors is still substantially higher than open pond systems.[5]

Recently hybrid systems have been look at which combine the photobioreactors with open pond systems. In these hybrid systems algae strains are first grown in enclosed photobioreactors to densities high enough that strain contamination is not a factor and then they are transferred into open ponds where they are grown to the final product.[5] Once the algae cultures are grown the next task is to harvest the biomass. This is a very tedious task because the algae often have a low specific density and separating and collecting them from the bulk liquid is very hard and expensive.[23] They can be harvested through various means such as physical, chemical and mechanical harvesting methods.

In order to produce a large amount of sugar-rich microalgae biomass for the production of bioethanol at a low cost the carbohydrate productivity for the selective strain must be high. Trigging the accumulation of these high value products like lipids or carbohydrate has gained a great deal of interest worldwide. The sharp increase in the production of lipids and carbohydrates has been show in several studies to be triggered under cultivation in nitrogen deficient environments.[37 38 39] Under nitrogen-depletion conditions the proteins and peptides that are accumulated inside the microalgae cells can be converted into lipids or carbohydrates.[40] The carbon flow in microalgae is allocated to

energy-rich compounds, like lipids and carbohydrates, when under stress and there is a competition between the syntheses of the two. The accumulation of the two under the stressed condition differs from strain to strain.[37]

Microalgae are the most abundant organisms on earth. They are fast growing, can tolerate a wide variety of environments ranging from fresh water, brackish water, sea water, extreme temperatures and pH conditions. These characteristics along with many others make microalgae prime candidates as a feedstock for biofuels. Many microalgae strains are capable of storing lager amounts of carbohydrates making them ideal for the production of bioethanol. *Chlorella, Dunaliella, Chlamydomonas, Scenedesmus,* and *Spirulina* are just a few examples of strains that are capable of accumulating >50% of their dry weight in starch and glycogen.[23] Microalgae can also contain cellulose which can also be converted into ethanol. Also in terms of ethanol production microalgae have several advantages over macro algae. The first advantage being, microalgae have higher sugar productivity because of their faster growth rates. Second, the carbohydrates produced by microalgae are more easily fermented. Macroalgae monosaccharides are primarily alginate and mannitol, which are more difficult to ferment.[41]

1.4 BIOETHANOL FROM MICROALGAL BIOMASS

Bioethanol can be produced from algae through three main methods. Algae can assimilate considerable amounts of biomass in the form of starch/cellulose, which can be converted to fermentable sugars and these sugars con be converted to bioethanol by a suitable ethanol producer. Some algae can act as a mini factory for the production of

ethanol during dark fermentation. Algal strains can be tailored through genetic engineering for the direct production of ethanol.

Algae store mainly starch in their cells and the biomass can be harvested regularly from large cultures and the starch extracted. The starch can be extracted in a number of ways either by mechanical methods or dissolution of the cell walls using enzymes. The starch can then be separated by the extraction with water or an organic solvent and use for the fermentation to yield bioethanol.

Once the starch is extracted from the algal biomass it is processed into ethanol in a similar fashion to other starch-based feedstocks, which involves two processes, saccharification and fermentation.[27 42] These two processes can be carried out in a single or double step process. Before the fermentation can occur the starch needs to be hydrolyzed to simple sugars and this process is called saccharification. Acid or enzymatic hydrolysis can be used for the conversion of the starch into simple sugars. The next step is to ferment the simple sugars into ethanol using a suitable yeast strain. Both of these processes can be done at the same time if the amylase producing strain can be used for the fermentation. The ethanol is then distilled off to remove water and any other impurities from the dilute alcohol product which is normally 10 to 15% ethanol. The concentrated ethanol can then be blended in with fossil fuels or used directly as fuel.[2 5 43]

Zygnemataceace, *Cladophoraceace*, and *Oedogoniales* algal strains have been used for the production of ethanol. These algae strains are starch accumulating and are filament-forming or colony-forming. The algae were grown by aquaculture and were harvested by flocculation, sedimentation, filtration or centrifugation. The biomass was then decayed by a dark and anaerobic aqua environment. The digested biomass was then

fermented with *Saccharomyces cerevisiae* and *Saccharomyces uvarum* to produce ethanol which was then separated from the fermentation broth. This one report offered advantages over similar methods which used single cell free floating algae because of the ease of harvesting.[44]

Biomass that has under gone oil extraction may also be used as a substrate for the production of ethanol. *Chlorococum sp.* was investigated as a potential feedstock for bioethanol by the fermentation using *Saccharomyces bayanus*. The defatted algal biomass yielded 3.8 g/L of ethanol from 10 g/L of the substrate used. This study also showed the potential for this process to be scale to commercial levels.[20]

Algae can also accumulate cellulose in the cell walls and like starch cellulose can also be hydrolyzed into simple sugars which can be fermented into ethanol. Green algal biomass which produces both reserve starch and wall material cellulose can be used for the production of bioethanol. Unlike higher plant life which contain cellulose that is accompanied by lignin, algae have no need for the structural purposes of lignin making complex and expensive pretreatments processes and digestion processes unnecessary.

1.5 CONVENTIONAL HYDROLYSIS/PRETREATMENT OF BIOMASS

The hydrolysis of biomass is necessary to convert the storage carbohydrates into monomeric sugar which are suitable for fermentation into ethanol. There are two common methods of hydrolysis, enzymatic and chemical.

Enzymes are produced in microbial bioreactors for commercial use. Enzymatic hydrolysis is much more expensive and slower than acid hydrolysis.[45] Enzymatic hydrolysis is an environmentally benign process and can obtain high yields without the

production of inhibiting byproducts due to the mild reaction conditions. Another drawback to enzymatic hydrolysis is that it often needs physical or chemical pretreatments which themselves can be costly or energy consuming. The composition of hydrolytic enzymes can have significant effects on the efficiency of the hydrolysis of algal biomass, due to specific structures and proportions of cellulose and starch in different strains.[40] These enzyme mixtures commonly contain endoglucanase, B-glucosidase, and amylases.[40] Another key factor into the performance of enzymatic hydrolysis is the substrate to enzyme loading, this must be optimized to obtain high glucose production and rate to reduce the sugar production cost.[40]

In general chemical hydrolysis whether it be acid or alkaline conditions, is faster, easier and cheaper than other types of hydrolyses. However the acidic conditions may lead to decomposition of the sugars into unwanted compounds that can inhibit the fermentation process, hydroxymethly furfural (HMF) is the main degradation product.[46] [20] [47] Acid catalysis unfortunately comes with undesired environmental burdens and economic costs.[48] Never the less the use of strong acids is still commonly used. Dilute acid hydrolysis often uses sulfuric acid concentrations ranging from 0.1% to 5.0% with temperatures and reaction time of 120°C and 20 – 120 min, respectively.[40] Surprisingly high sugar yields can be obtain through dilute acid hydrolysis of algae biomass compared to terrestrial plants where dilute acid hydrolysis is often used as a pretreatment. The algal biomass contains simpler carbohydrates compared to the terrestrial plants which often contain lignin.[4] This is one of the advantages of using microalgae biomass over other lignocellulosic materials.

1.6 CARBONIC ACID CATALYZED HYDROLYSIS/PRETREATMENT OF BIOMASS

For the hydrolysis of lignocellulosic feedstock strong acid are often used even if enzymatic hydrolysis is the primary method. In order to eliminate the need for strong acids in the hydrolysis process and reduce the operation, construction, and environmental costs associated with the commercial biomass conversion, studies were conducted on the use of carbonic acid. There has been great interest in the use of carbonic acid in industrial processes, but little is known about its fundamental behavior in the range of 150-250°C. Whether carbonic acid can serve as a hydrolysis catalyst is not clear because of its weak acidity and poorly understood behavior at high temperatures and pressures. CO_2 has been used for steam explosion treatments for biomass feedstocks. There have been previous studies investigating the use of carbonic acid catalyzed reaction for the dehydration of cyclohexanol to cyclohexene and for the alkylation of p-cresol with tert-butyl alcohol to form 2-tert-butyl-4-methylphenol.[48] There have also been reports of the carbonic acid hydrolysis of starch and cellulose. Carbonic acid, if effective, could offer some advantages compared to conventional strong acid. It is less corrosive and has lower neutralization requirements. The pH of carbonic acid is determined by the partial pressure of CO_2 in contact with water, and thus it can be neutralized simply by releasing the pressure from the reactor. The CO_2 used to produce the carbonic acid may be available at low cost, because CO_2 is a byproduct of the fermentation process converting biomass to ethanol.

There have been several studies showing the potential use of carbonic acid as a catalyst for the hydrolysis of lignocellulosic biomass. In 2001, Van Walsum conducted tests using carbonic acid to hydrolyze purified xylan obtained from beech wood. The

study showed that charging a small reaction vessel with a solution of xylan with CO_2 and then heating the vessel to temperature of 190°C for 16 min would result in the hydrolysis of the carbohydrate. The yields however were relatively low about 30% conversion of the xylan to xylose.[49]

In 2002, another study conducted on the hydrolysis of aspen wood with the used of carbonic acid did not show a difference with a pure water system. Being that the majority of the biomass was comprised of hemi-cellulose and has a relatively high concentration of acetyl groups, it is possible that the application of carbonic acid to a more acidic substrate may counter act any catalytic properties.[50]

In 2004 van Walsum, reported the hydrolysis of corn stover with the use of carbonic acid. The experiments were able to obtain 22.5% conversion of xylan to xylose. The differences in the chemical composition of the cover stover compared to the aspen wood used in the previous study where suggested to be the cause of the different out comes. The corn stover contains less endogenous acid producing groups when compared to the aspen wood. The aspen wood was subject to autocatalysis resulting from the release of acetyl groups, this obscured the effect of the carbonic acid.[51]

One counterintuitive observation from these studies was that the final pHs of the hydrolysates were higher for the treatments that used carbonic acid. Because the treatments that used carbonic acid show higher conversion of the corn stover to simple sugars one would expect their pH to be lower but this was not the case. Researchers developed an analytical procedure to monitor the concentrations of organic acids and other degradation products produced during the treatments. While they observed

different concentrations in these compound from the treatments with and without carbonic acid, the amounts did not offer an explanation to the pH difference.[52]

Rogalinski and colleagues in the Brunner group, have also explored the hydrolysis of corn starch, pure cellulose, and lignocellulosic biomass (rye and rice straw) in water under elevated temperatures and pressures, and applied high pressure CO_2, above its saturation limit, to achieve hydrolysis of oligomeric sugars. They found that the addition CO_2 significantly increased the glucose yields from the hydrolysis of corn starch and cellulose.[53 54 55]

Jerry W. King, et al. tested samples of corn stover, corn cob, and switchgrass. The study showed that for corn substrates the carbonic acid pre-treatment sufficiently reduced the carbohydrate polymers to lower oligomers and monomers. The optimized conditions for the corn substrates were found to be 300 bar pressure of CO_2, 170 °C, 90 min residence time and 75-106 um particles size. To obtain good yields for the switchgrass higher pressures were need.

1.7 REACTION MECHANISMS

Cellulose and starch are the two most common storage carbohydrates found in biomass feedstocks.[56] Cellulose is comprised of D-glucose connected by β-1,4-glycosidic bonds, while starch uses α-1,4-glycosidic bonds to connect the D-glucose units. During hydrolysis these glycosidic bonds are broken by the addition of water. Figure 1.2. shows the mechanism for the acid catalyzed hydrolysis of cellulose.

The main degradation product that is produced from the hydrolysis of starch and cellulose is hydroxymethyl furfural (HMF). There are several proposed pathways for the

formation of HMF, the most common and widely accepted in the acid catalyzed dehydration of hexoses. Two other common routes are through Maillard reactions or aldol reactions.[58] There are two proposed pathways for the acid catalyzed formation of HMF, an acyclic pathway and a cyclic pathway. However there is no true consensus on which is the correct pathway.

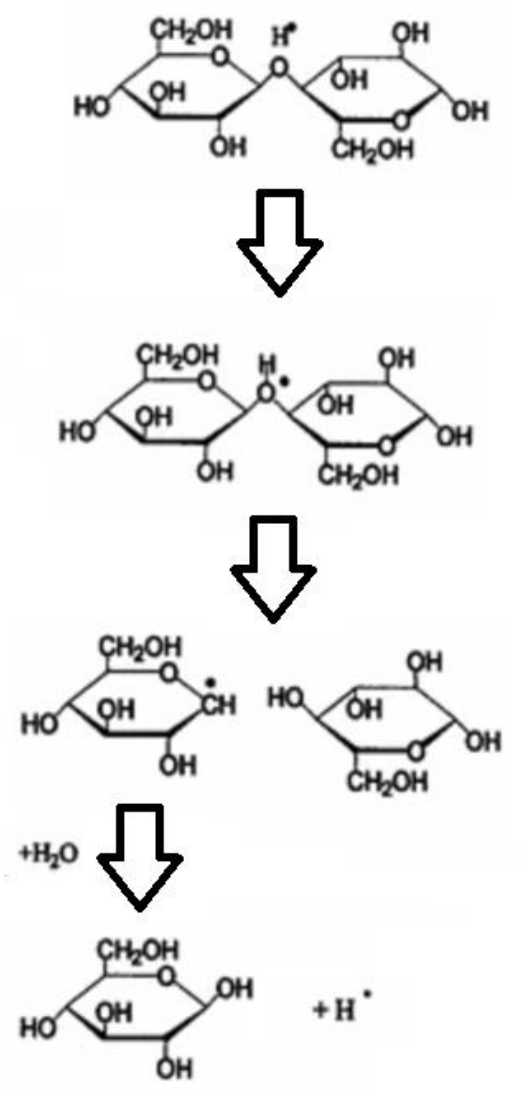

Figure 1.2. Acid catalyzed hydrolysis of cellulose.[57]

The acyclic pathway has a rate-limiting step of the formation of a linear 1,2-enediol. This intermediate molecule then under goes two β-dehydrations, a ring closure, and a final water elimination to create HMF (Figure 1.3.). The cyclic pathways all begin with the isomerization of hexoses to fructose rings. The fructose ring will undergoes a dehydration at the C_2 forming a carbenium cation. This is then followed by two more β-dehydrations to final form HMF (Figure 1.4.).[58]

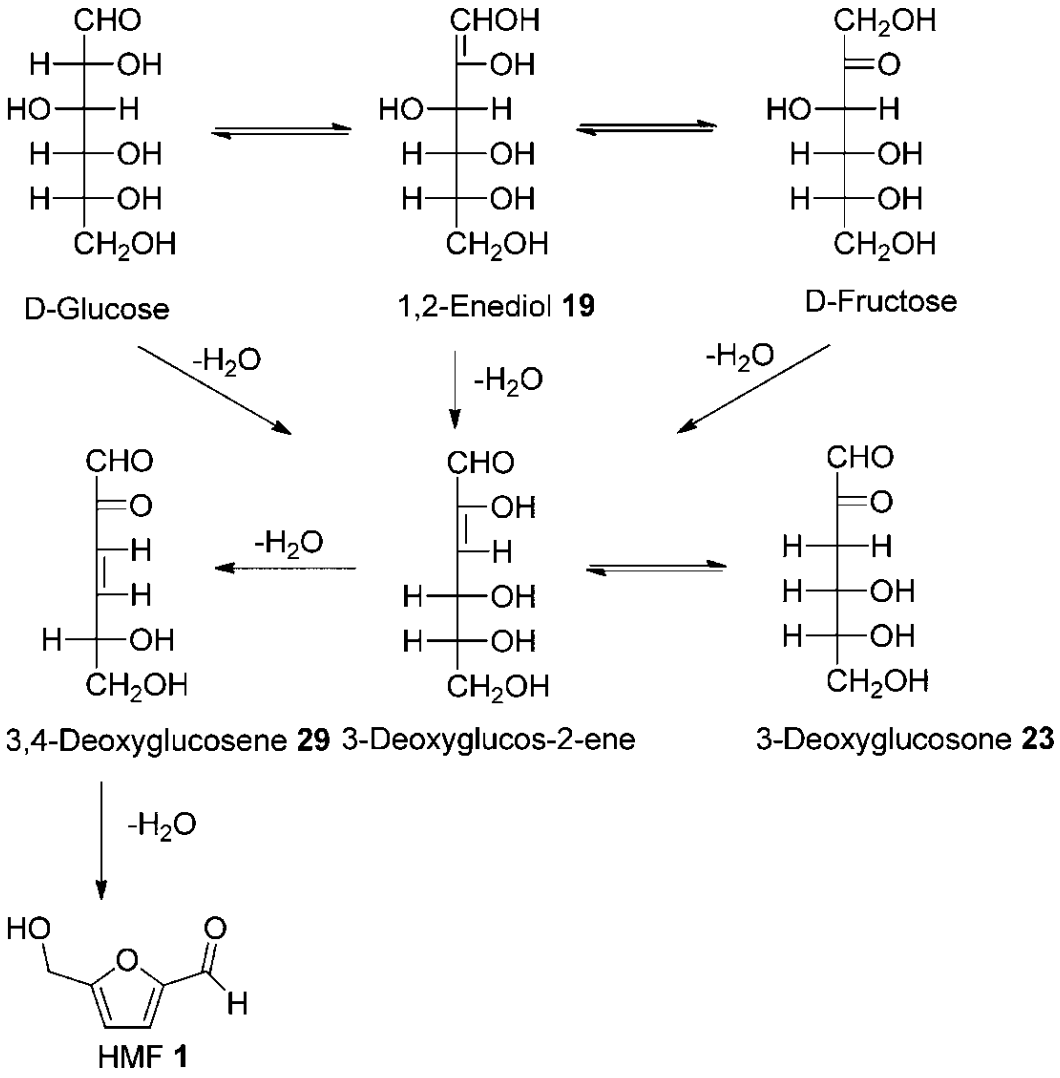

Figure 1.3. Acyclic pathway for the formation of HMF.[58]

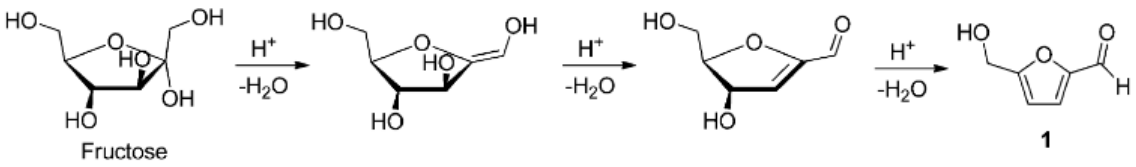

Figure 1.4. Cyclic pathway for the formation of HMF.[58]

1.8 CARBON DIOXIDE SOLUBILITY AND CARBONIC ACID FORMATION

Carbonic acid is produced when CO_2 dissolves into an aqueous solution. The CO_2 reacts with water to form carbonic acid in the following reaction.

$$CO_2(aq) + H_2O(l) \leftrightarrow H_2CO_3(aq) \quad (1)$$

The carbonic acid will then deprotonate generating the bicarbonate ion. The bicarbonate ion can also deprotonate to generate the carbonate ion. The degree to which the species will deprotonate depends on the chemical equilibrium constant for both reactions, K_{a1} and K_{a2} respectively.

$$H_2CO_3(aq) \leftrightarrow HCO_3^-(aq) + H^+(aq) \quad (2)$$

$$HCO_3^-(aq) \leftrightarrow CO_3^{2-}(aq) + H^+(aq) \quad (3)$$

In order to understand how CO_2 can affect the pH of a solution the solubility and the acid ionization constant (K_{a1}) must be studied under a range of temperatures and CO_2 pressures.

Several investigators have measured the solubility of CO_2 in water at a range of temperatures and CO_2 partial pressures.[56] [57] [58] These studies indicate that up to 100°C, CO_2 solubility decreases with increasing temperatures and increases with increasing CO_2

partial pressure. However the measurements of CO_2 solubility in water above its boiling point by Sabirzyano et al. indicates that with increasing CO_2 partial pressure and water temperature above 150°C, the solubility of CO_2 increases.[59] Hunter and Savage compared this trend in the K_H (solubility of CO_2) with the K_{a1} of carbonic acid at different temperatures.[60] Figure 1.5. shows a minimum in the solubility of CO_2, that subsequently increases with temperature over 150°C. However, increasing temperatures cause the Ka1 of carbonic acid to decrease. These two trends show a clear trade off with increasing temperatures causing increasing solubility but decreasing acidic strength. The bicarbonate ion is generally assumed to have little effect on the generation of H^+ and the K_{a2} is omitted from consideration. Table 1.2. lists the pH and concentrations of dissolved CO_2, carbonic acid, bicarbonate ion, and carbonate ion, in solution with varying CO_2 partial pressure at 25°C.

Table 1.2. Concentrations of CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-} in aqueous solution at 25°C at various CO_2 partial pressures.

$P(CO_2)$ atm	pH	$[CO_2]$	$[H_2CO_3]$	$[HCO_3^-]$	$[CO_3^{2-}]$
10^{-8}	7	3.36×10^{-10}	5.71×10^{-13}	1.42×10^{-9}	7.90×10^{-13}
10^{-7}	6.94	3.36×10^{-9}	5.71×10^{-12}	5.90×10^{-9}	1.90×10^{-12}
10^{-6}	6.81	3.36×10^{-8}	5.71×10^{-11}	9.16×10^{-8}	3.30×10^{-11}
10^{-4}	5.92	3.36×10^{-6}	5.71×10^{-9}	1.19×10^{-6}	5.57×10^{-11}
3.5×10^{-4}	5.65	1.18×10^{-5}	2.00×10^{-8}	2.23×10^{-6}	5.60×10^{-11}
10^{-3}	5.42	3.36×10^{-5}	5.71×10^{-8}	3.78×10^{-6}	5.61×10^{-11}
10^{-2}	4.92	3.36×10^{-4}	5.71×10^{-7}	1.19×10^{-5}	5.61×10^{-11}
10^{-1}	4.42	3.36×10^{-3}	5.71×10^{-6}	3.78×10^{-5}	5.61×10^{-11}
1	3.92	3.36×10^{-2}	5.71×10^{-5}	1.20×10^{-4}	5.61×10^{-11}
2.5	3.72	8.40×10^{-2}	1.43×10^{-4}	1.89×10^{-4}	5.61×10^{-11}
100	3.42	3.36×10^{-1}	5.71×10^{-4}	3.78×10^{-4}	5.61×10^{-11}

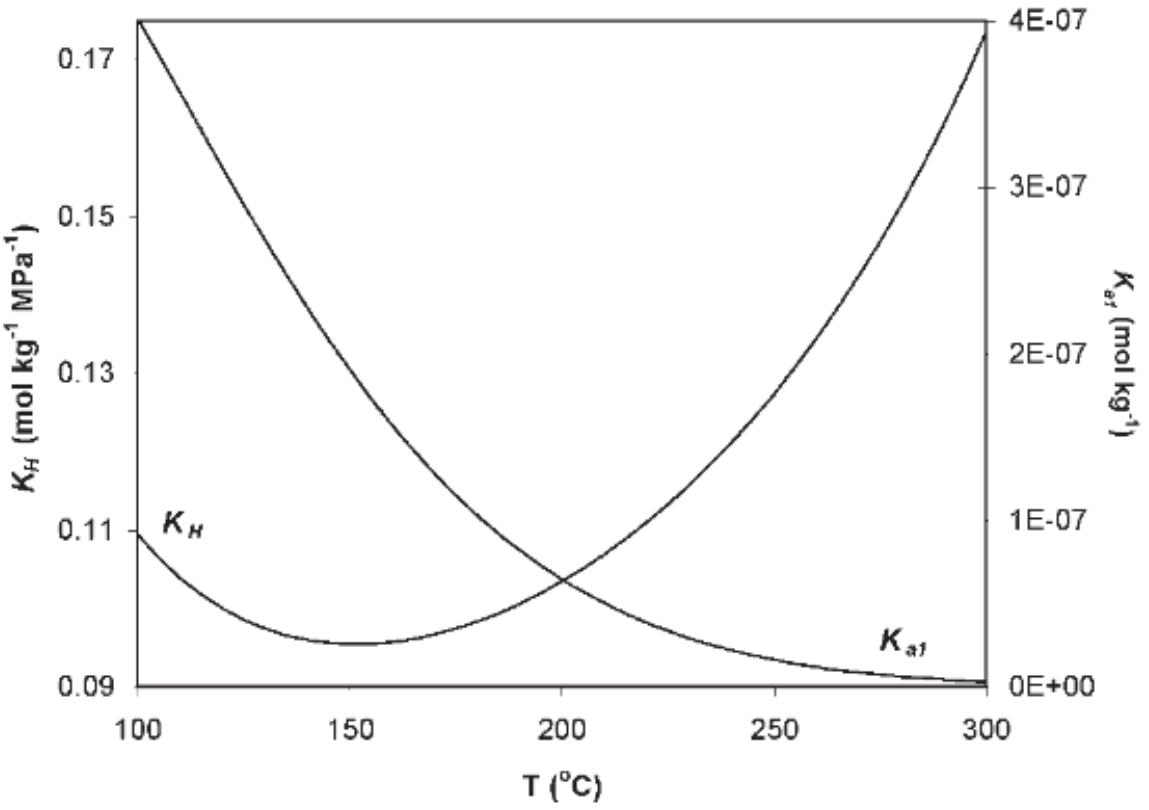

Figure 1.5. The solubility (K_H) of CO_2 and the acid ionization constant of carbonic acid (K_{a1}) at various temperatures.

To better understand the solubility of CO_2 at high temperatures and pressure Van Walsum (2001) compiled past reports of solubility relating Henry's constant to temperature and pKa1 values for carbonic acid. Using these past studies, a method for predicting the pH at various CO_2 pressures up to 150bar and temperatures ranging from 100 to 250°C was developed.

$$pH = (8.00x10^{-6})T^2 + 0.00209T - 0.216\ln(P_{CO_2}) + 3.92 \quad (4)$$

It should be noted, there appeared to be a discrepancy between the theoretical prediction and observed reactivity of their carbonic acid system. Figure 1.6. shows pH verses CO_2 partial pressure, as calculated and plotted by Dr. Monika Johanseen of TUHH

in Hamburg, Germany. Again, there we can see that although increasing temperature should increase the CO_2 solubility at higher temperatures the predicted pH values increase due to the decreasing acidic strength of carbonic acid.

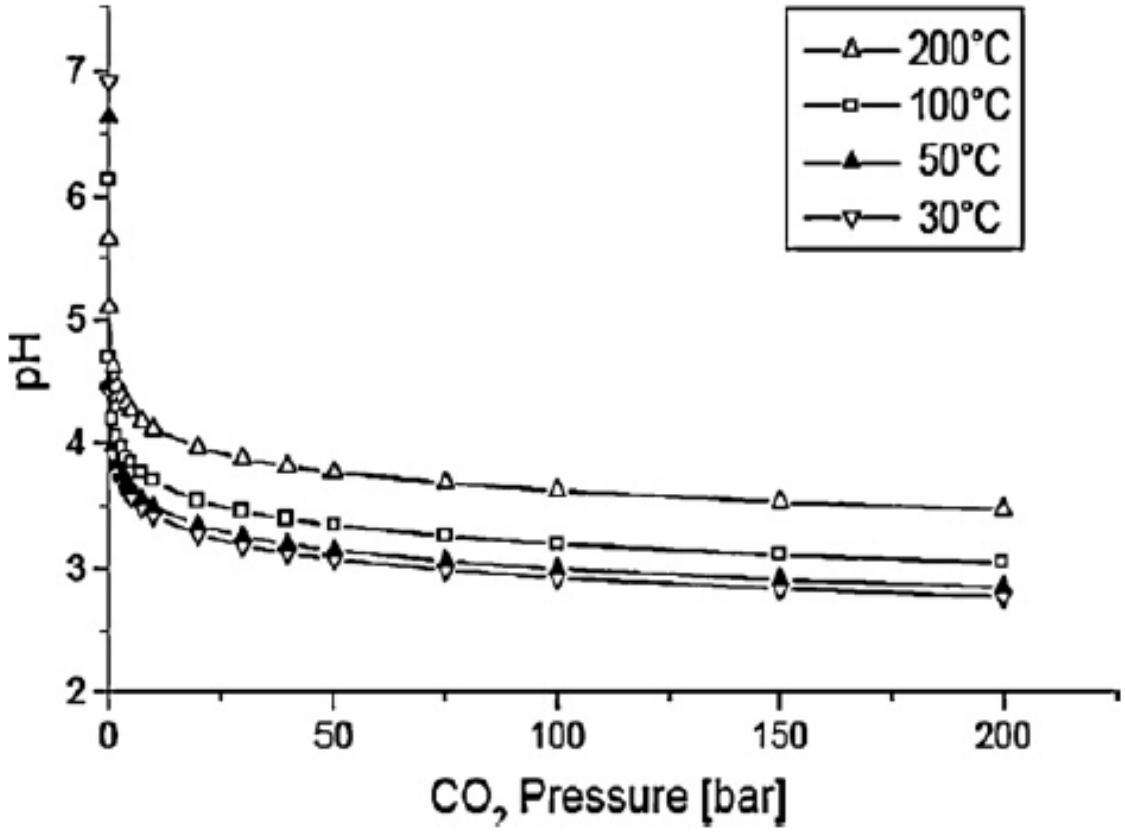

Figure 1.6. pH verses CO_2 partial pressure, as calculated and plotted by Dr. Monika Johanseen of TUHH in Harburg, Germany.

2. RESEARCH OBJECTIVES

Bioethanol is one of the main biofuels that is used as a substitute for petroleum fuels. Bioethanol can be produced from a wide variety of biomass sources such as corn, sugarcane, switch grass, corn stover, and algae. The carbohydrates in the biomass feedstock are converted into simple sugars and then fermented to produce ethanol. Mineral acid and enzymatic hydrolysis are the two most common methods for the conversion of carbohydrates to fermentable sugars. However, these two methods have drawbacks and limitations. Mineral acid hydrolysis requires harsher reaction conditions that can led to the production of unwanted degradation products. Strong acids are toxic and corrosive, and require neutralization that generates downstream waste. Enzymatic hydrolysis is a slower process and costlier. It can also require pretreatment steps to breakdown the biomass substrate prior to the hydrolysis itself. Carbonic acid has been identified as a potential replacement for strong acid catalysts in the hydrolysis process. Carbonic acid is a weak acid that is produced by pressurizing CO_2 and water. It is less toxic and corrosive compared to the mineral acid commonly used, and can be easily removed by depressurizing the system, which eliminates the need for neutralization after hydrolysis. The main objective for this work is to develop and evaluate a high pressure carbonic acid hydrolysis for algal biomass.

PAPER

I. HYDROLYSIS AND DECOMPOSITION PRODUCTS FROM MICROCRYSTALLINE CELLULOSE TREATED WITH HOT CARBONIC ACID

ABSTRACT

Cellulosic biomass is an abundant photosynthetic product and has been receiving great interest as renewable resource for the production of fermentable sugars that can be converted to combustible biofuel. The current study was designed to investigate the carbonic acid induced hydrolysis of microcrystalline cellulose (MCC) in order to understand the hydrolytic behavior of carbonic acid catalyst and find the efficient conditions that can be applied to the pretreatment of other cellulosic feedstocks having irregular composition and structure. Carbonic acid hydrolysis is advantageous to commonly used mineral acid hydrolysis because of its ease of production, less toxic and corrosive nature, and lack of neutralization or acid recovery steps. MCC was exposed to carbonic acid at elevated temperatures of 150–210°C with a reaction time ranging from 60-180 min. The hydrolysis reaction duration of 90-180 min was necessary in order to achieve greater yield of reducing sugar at lower temperatures of 150-170 °C; whereas the 60 min reaction required higher temperatures of 190-210 °C to produce higher amount of fermentable saccharides. The amount of 5-hydroxymethylfurfural (HMF) and furfural from decomposition of glucose also increased with increasing reaction time and temperatures. These decomposition products reduce the available sugar and can inhibit

the enzyme activities during the fermentation process. The highest glucose conversion along with low HMF and furfural formation was achieved for 60 min reaction at 210 °C with carbonic acid catalysis. Therefore, it appears that shorter reaction time at higher temperatures will provide the maximum conversion of cellulosic material to fermentable sugars.

Keywords: Microcrystalline cellulose, glucose, hydroxymethylfurfural, furfural, carbonic acid, hydrolysis

1. INTRODUCTION

The search to find an alternate source of fuel to replace fossil fuels has become a major area of research.[1] Particularly the conversion of cellulose and lignocellulosic biomass into biofuels is one of the most favorable and desirable solutions because they are the most abundant nonfood biomass resources produced via photosynthesis.[2] Hydrolysis of cellulosic biomass into glucose is typically accomplished by employing mineral acid, enzymes or microorganisms. Once converted into glucose, the material can be further processed into various specialty chemicals or fermented into ethanol. However, many obstacles are presented when producing biofuels from cellulosic biomass because of great variations in size, crystallinity, and complexity of cellulosic feedstock.

Two most common methods for the hydrolytic treatment of cellulosic materials are acid hydrolysis or enzyme hydrolysis. Acid hydrolysis can be performed with concentrated or diluted mineral acids; the most widely used acids for this process are phosphoric acid, sulfuric acid, and hydrochloric acid. The use of concentrated acids for cellulose hydrolysis has produced high glucose yield with fewer degradation products, however, these concentrated acids are very toxic, corrosive to processing equipment, and have high neutralization costs, which make them less appealing.[3] Dilute acids, on the other hand, are less hazardous and corrosive, and have shown high hydrolysis yield. These acid hydrolysis techniques are advantageous compared to other cellulose hydrolysis techniques because other methods often require additional pretreatment step when processing biomass in order to completely liberate the cellulose from lignin.[4] Some of these pretreatment techniques are mechanical commutation, carbon dioxide or steam

explosion, ammonia fiber explosion, ozonolysis, acid hydrolysis, alkaline hydrolysis, organosolvation, and pulsed electrical field.[5] Many of these pretreatments processes require large power consumptions or expensive materials. Enzymatic hydrolysis produce high glucose yield, but the reaction rate is often slow and product yield can be affected by contaminants present in the biomass.[6] These enzymatic hydrolysis treatments are typically paired with a pretreatment technique in order to enhance the hydrolysis abilities of the enzyme, which is a step not necessary in acid hydrolysis.

Carbonic acid treatments have recently been investigated as an alternative method to the commonly used mineral acid treatments. Carbonic acid treatments are less severe than mineral acid treatments and do not require corrosive resistant materials and hardware. They also do not require neutralization or produce the same resulting waste that mineral acid treatments do. Since carbonic acid is easily produced by dissolving pressurized carbon dioxide in water, large quantities of carbonic acid can be readily available in contrast to mineral acids which would require an acid recovery system after cellulose hydrolysis and processing in order to be practical.[7] [8] Carbonic acid does not provide the same level of hydrolytic capabilities as sulfuric acid, but according to studies[9] [10], they have demonstrated the enhanced catalytic effect of carbonic acid on hydrolysis at higher temperatures. Van Walsum (2001) has demonstrated that at temperatures of 200 °C, carbonic acid exhibits a catalytic effect on hydrolysis.[9]

The goal is to investigate the effects of carbonic acid hydrolysis on pure microcrystalline cellulose. Previous studies using carbonic acid have primarily focused on biomass substrates like switch grass, corn stover, and aspen wood.[5, 11] These biomass substrates contain varying amounts of cellulose, xylan, hemicellulose and lignin that

coexist and show different behavior in the presence of carbonic acid. By studying a simpler more homogeneous substrate we hope to gain a better understanding of the conditions need during and carbonic acid hydrolysis/pretreatment. Once these base experiments are preformed then more complex biomass substrates can be tested.

2. METHODS

2.1 MATERIALS

Microcrystalline cellulose (MCC) with an average particle size of 50um was obtained from Acros Organic (Waltham, MA). Siphon tube CO_2 cylinder was supplied by Ozarc Gas (Rolla, MO). Pure standards used for HPLC analysis including glucose, 5-hydroxymethylfurfural (HMF), and furfural were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were reagent grade.

2.2 CARBONIC ACID HYDROLYSIS OF MICROCRYSTALLINE CELLULOSE

Batch treatments of cellulose samples with carbonic acid catalysis were carried out using a 170 mL stainless steel pressure vessel heated by a temperature controlled heating mantle. Schematic diagram of the CO_2 pressurized reaction vessel setup is shown in Figure 2.1. Microcrystalline cellulose (500 mg) was soak overnight with 25 mL of distilled water and placed in the reaction vessel with additional 25 mL of distilled water. The initial pH was recorded. The pressure vessel was sealed, connected to the siphon tube CO_2 cylinder using a quick-connect, and pressurized until the cylinder pressure (~800 psi) was reached. After the shutoff values were closed, the vessel was detached from the CO_2 cylinder and placed inside a temperature controlled heating mantle and covered with ceramic wool blanket insulations. The vessel was then heated and the internal temperature was monitored using a thermocouple placed inside the vessel. The

vessel was heated to a set temperatures which ranged from 150 to 210 °C for a specific amount of reaction time ranging 60-180 min. As seen in Figure 2.2, the inside temperature of the reaction vessel increased slowly due to the large mass and the set temperature was reached only after 30 min or more heating time.

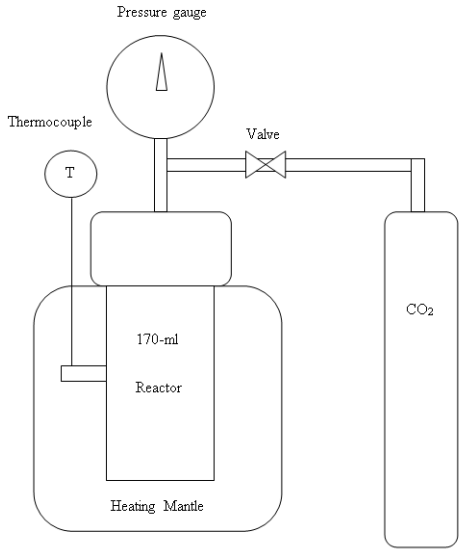

Figure 2.1. Schematic diagram of the CO_2 pressurized reaction vessel setup

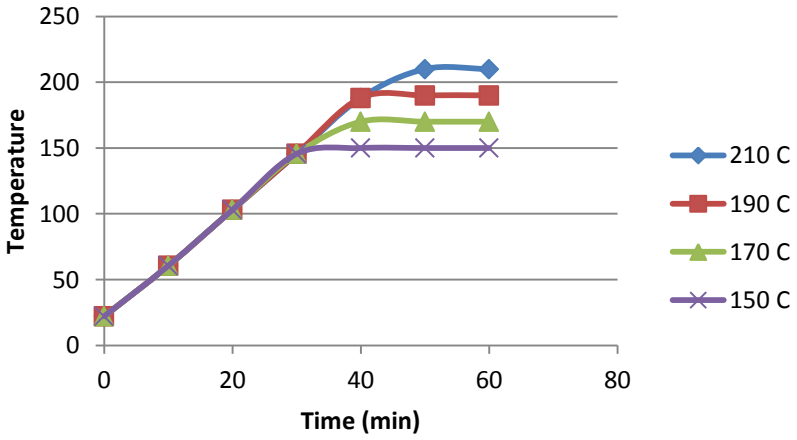

Figure 2.2. Inside temperature profile of the high pressure reaction vessel heated with a temperature controlled heating mantle

Once the desired reaction time had reached, the vessel was removed from the heating mantle and placed into a cold water bath to stop the reaction. The reaction vessel was depressurized by opening the inlet valve and venting the CO_2 gas. After measuring the pH, the treated samples were analyzed for the hydrolysis and decomposition product contents.

2.3 DNS ASSAY FOR TOTAL REDUCING SUGARS

The 3,5-dinitrosalicylic acid (DNS) method was used to determine the total reducing sugar contents from the microcrystalline cellulose. The hydrolysates were neutralized using Na_2CO_3, filtered with a 0.22 um nylon membrane filter and then treated with DNS reagent. The intensity of the color developed was measured at 540 nm using a Thermo Genesis 10 spectrophotometer (Madison, WI).

2.4 HPLC ANALYSIS FOR GLUCOSE AND DECOMPOSITION PRODUCTS

Hydrolysis products and decomposition byproducts were determined using the National Renewable Energy Laboratory biomass laboratory analytical procedure (NREL/TP-510-42623). Analysis was carried out using Hitachi (Tokyo, Japan) Elite LaChrom HPLC system equipped with refractive index and UV-vis detectors. A Bio-Rad (Hercules, CA) Aminex HPX-87H column (250×4.8 mm) at 60 °C was used with 0.001 N sulfuric acid mobile phase at a flow rate of 0.6 ml/min. Glucose, HMF and furfural were quantified using calibration curves generated with authentic standards. All

samples were filtered prior to injection using a 0.22 um nylon membrane filter and a 25 uL volume was introduced.

2.5 STATISTICAL ANALYSIS

All tests were conducted in triplicates. All data are presented as means ± standard deviation. Microsoft Excel was used for the analysis of variance at 95% confidence, differences with P values of 0.05 or less were considered significant.

3. RESULTS AND DISCUSSION

Reaction temperature and time greatly influenced the hydrolysis of crystalline cellulose into simple sugars and formation of decomposition products. Figure 3.1. shows the significant increase in fermentable sugars with the addition of carbon dioxide. Furthermore, the increase in reaction temperature resulted in increased yield of reducing sugars. At lower temperatures of 150, 170 and 190°C the yield of total reducing sugars were progressively increased with increasing residence time. In contrast, increasing the residence time above 90 min at 210°C had a negative effect on the yield of total reducing sugars. The catalytic effect of carbonic acid below 170°C was substantially low as seen in low reducing sugar yields (1.7% to 9.1% on weight basis) when compared with significantly higher yields (16.9% to 38.4%) achieved with high temperatures of 190°C and 210°C. Increasing catalytic activity of carbonic acid at high temperatures were also reported by Kumar and Gupta (2008) who observed the higher hydrolysis product yields for temperature increase from 302 to 405°C.[12]

For the control samples processed in same conditions except the carbon dioxide pressurization, a slight but proportional increase in the yield of reducing sugars was observed with the increases in both temperatures and residence time. The catalytic effect of carbon dioxide induced carbonic acid at high temperature and pressure was very clear. Previous studies conducted on hydrolysis of polysaccharides such as starch, agar, xylan and guar gum also showed an increase in the production of monosaccharaides with the introduction of carbon dioxide.[9, 13] It's been reported that under high temperatures and

pressure, carbon dioxide in water results in large formation of carbonic acid, which is a weak acid and can effectively catalyze biomass hydrolysis.[14]

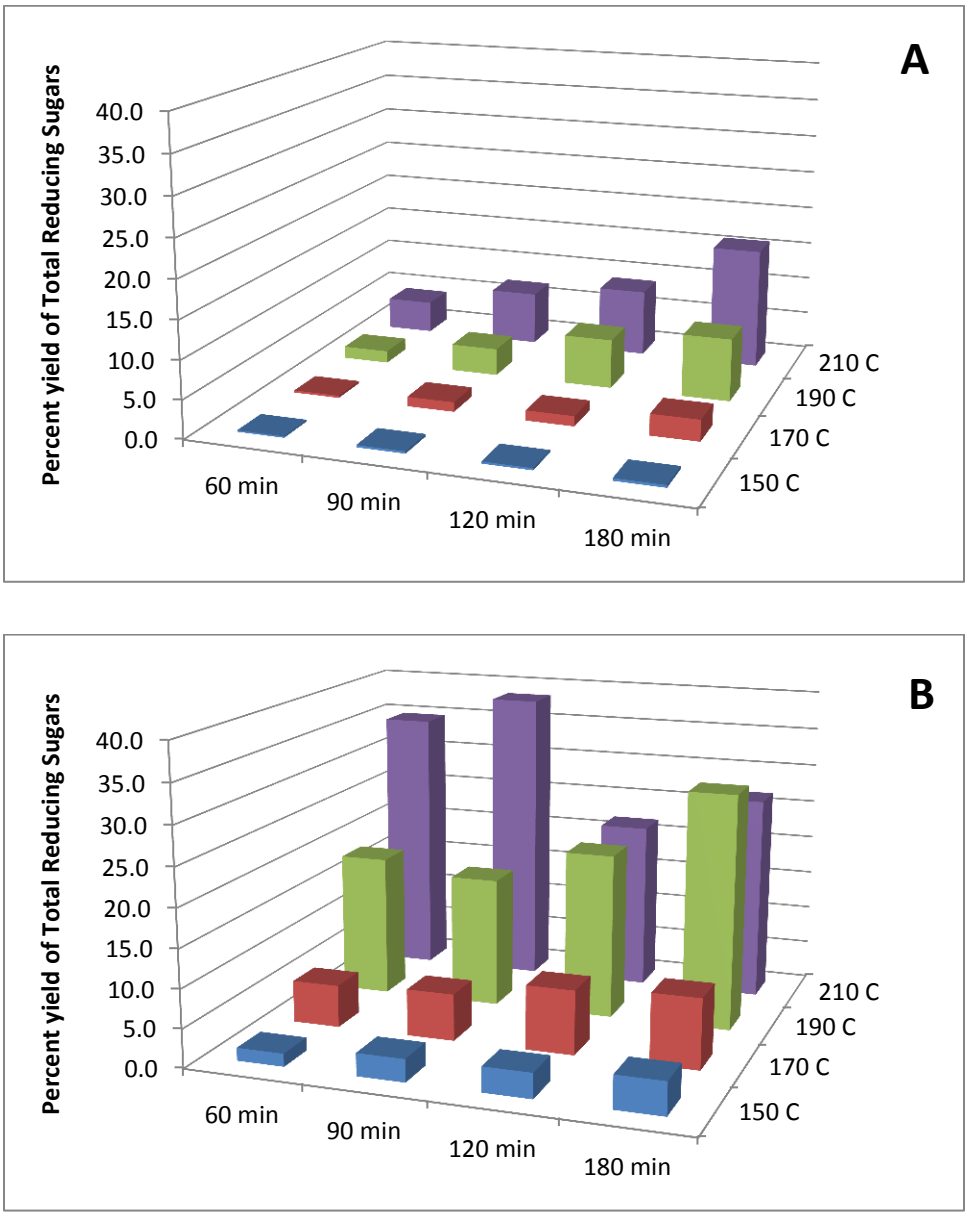

Figure 3.1. Reducing Sugars produced from crystalline cellulose treated (A) without and (B) with pressurized CO_2 induced carbonic acid at different residence times and temperatures

Chemistry and mechanism of hydrothermal degradation of cellulose at elevated temperatures is proposed in the sequential order of cellulose to glucose to decomposition products, in which hydrolysis to glucose is an important step.[15-19] Therefore the degree of degeneration of cellulose into glucose is given in Figure 3.2. From the Figure 3.2. it is appearing that increase in temperature and residence time will show an increase in glucose yield from 150°C to 190°C. While at 210°C, glucose yield fall significantly with increasing residence time. However at low residence time of 60 minutes at 210°C showed highest average yield of glucose (20% w/w) simultaneously 90, 120 and 180 minutes residence time resulted in drastically lower yield of glucose from 17, 4 and 2% respectively. Correspondingly, researchers studying the effects of a CO_2 pretreatment on various cellulosic biomasses found an increase in reducing sugars from enzymatic hydrolysis with a CO_2 pretreatment process.[5, 20-21]

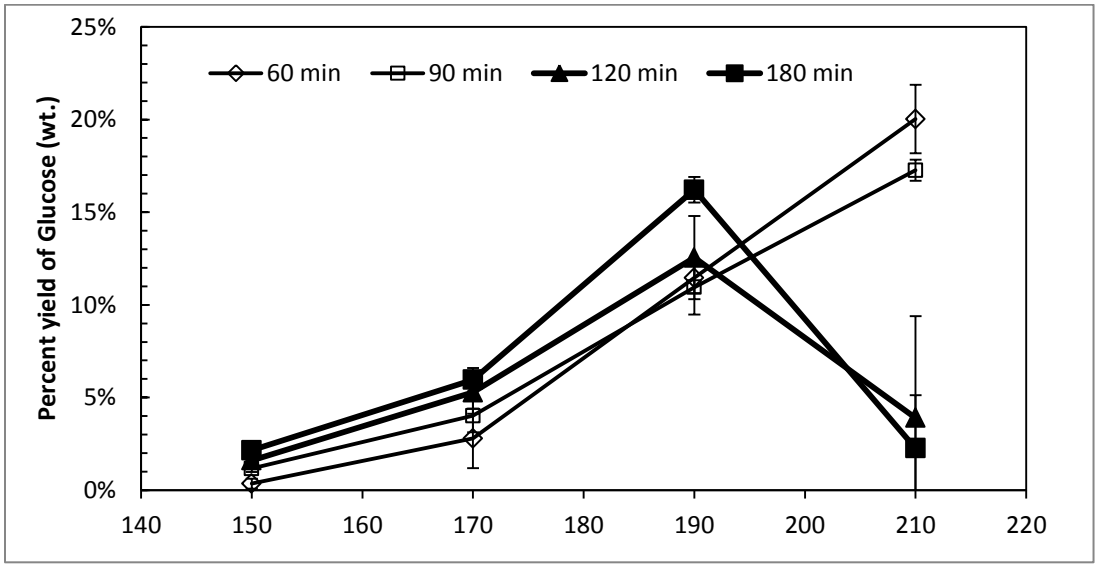

Figure 3.2. Percent yield of glucose measured by HPLC for the CO_2 treatments at different residence times and temperatures

This decrease in glucose yield could be attributed to the increase in degradation products as proposed by Antal and co-workers[15-19], the sequence of degeneration of cellulose in the order of cellulose to glucose to decomposition products. Therefore the yield of decomposition products like HMF and furfural are shown in Figures 3.3. & 3.4. respectively. As anticipated the yield of furfural and HMF showed their increasing levels with increase in both resistances times as well as with increase in temperatures. Due to the catalytic effect of carbonic acid on the thermal hydrolysis of cellulose the elevated levels of HMF and furfural were recorded at all the tested temperature and residence time regimes than their respective control counterparts. However, this is not true for the 210°C reaction temperature with a 180 min reaction, which produces 722 ppm and 708 ppm of HMF for the control and CO_2 respectively, this could be attributed to the temperature and residence time induced decomposition of glucose into HMF and furfural. Incidences of increased yield of decomposition products like HMF were also observed by Kumar and Gupta (2008)[12] with increasing temperatures in the thermal hydrolysis of cellulose. The higher production of HMF and furfural when using carbonic acid hydrolysis pretreatments can also be seen in other studies on corn stover and switch grass as well.[5, 22] Subsequently several studies have demonstrated that the higher levels of decomposition products like HMF and furfural have their negative effect on microbial fermentation of cellulosic hydrolysate into bioethanol.[23-26]

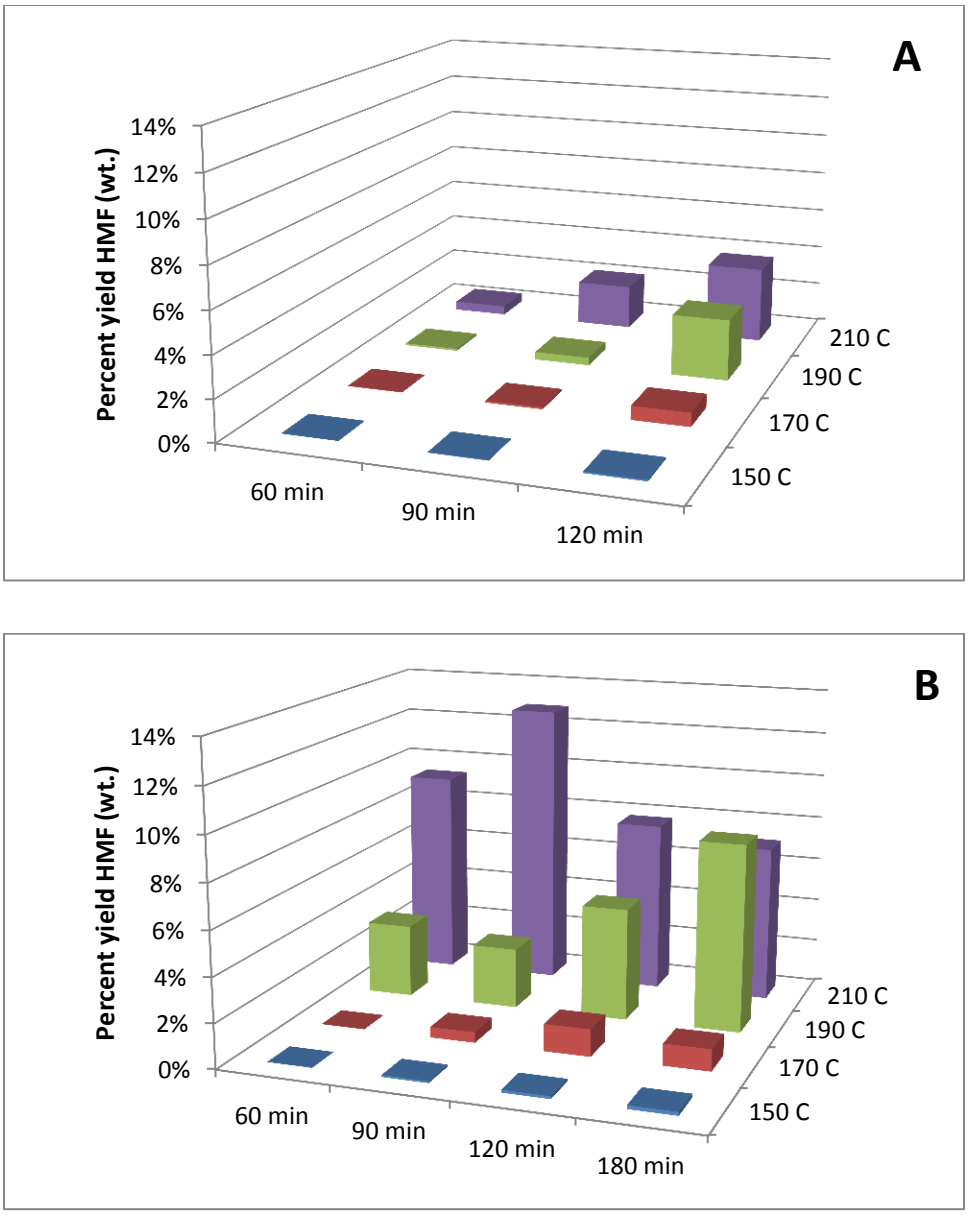

Figure 3.3. Percent yield of HMF measured by HPLC for without CO_2 (A) and CO_2 treatments (B) at different residence times and temperatures

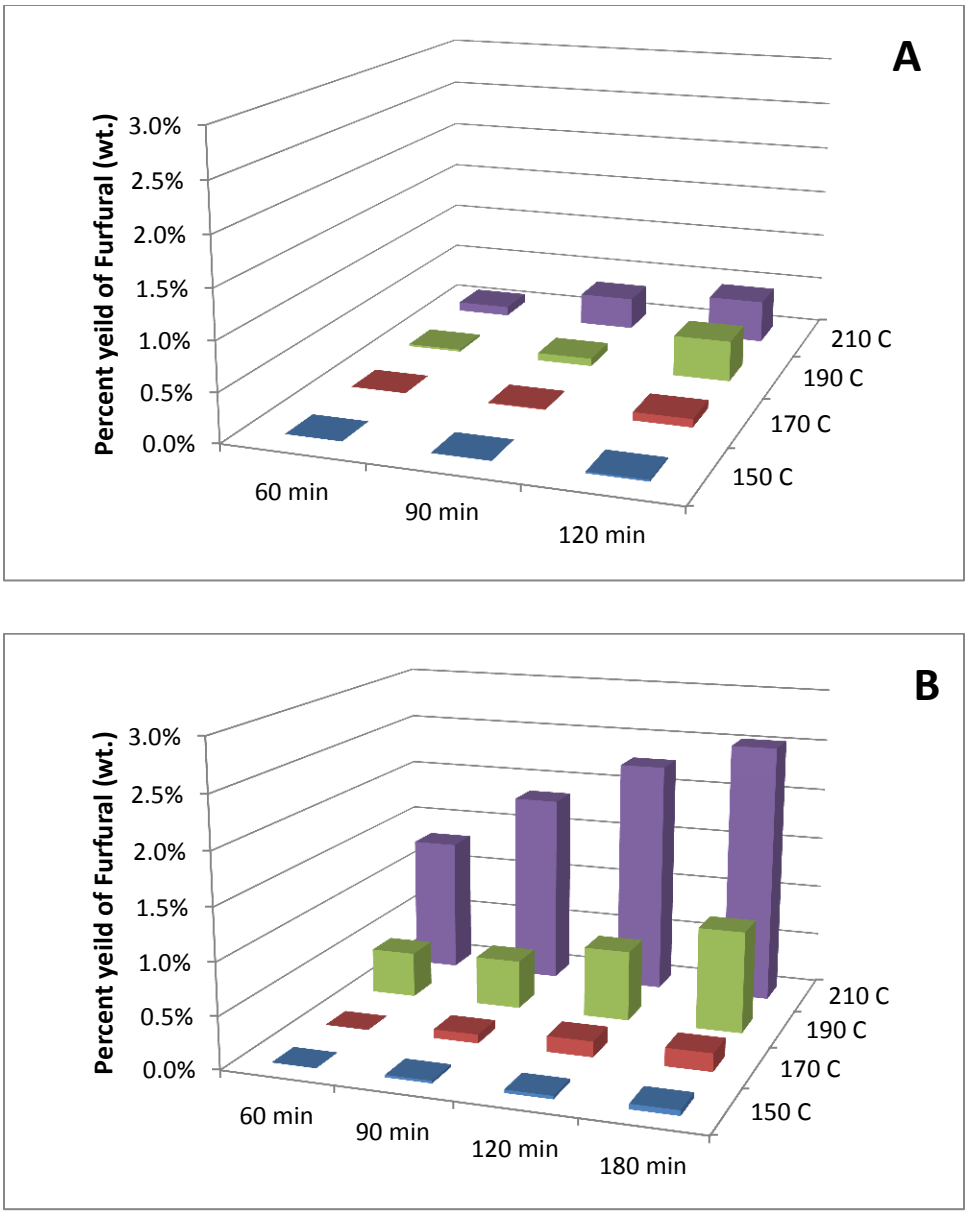

Figure 3.4. Percent yield of furfural measured by HPLC for without CO_2 (A) treatments and CO_2 treatments (B).

4. CONCLUSION

Microcrystalline cellulose can be effectively converted into monosaccharaides by thermal hydrolysis. However most of the studies that are conducted on thermal hydrolysis of cellulose are in continuous flow reactors. The continuous-flow reactor allows a continuous process that is basically favored but at the same time have some disadvantages of relatively low substrate concentrations feedings and the high energy demand for the commination process. Therefore it is being suggested to develop fixed bed reactors for the thermal hydrolysis of cellulosic substance for marginal profit making bioethanol production industrial use.[21] In order to develop a fixed bed reactor for thermal hydrolysis of cellulosic substances, thermal hydrolysis of cellulose in fixed batch reactor at varied temperatures and residence times were conducted and have showed that the glucose yield increased with increasing temperatures followed by the shorter reaction times and similarly lower yield of HMF and furfural produced at high temperatures with decrease in reaction times. Therefore, from this study it can be concluded that the shorter run times of 60 min at high temperatures of 210°C provide the high conversion of cellulosic material to fermentable sugars with the considerably reduced levels of decomposition products like HMF and furfural. The experimental results also showed that the fixed batch reactors needed a significant time period to heat up the reactor to reach the targeted temperature. So, the future needs to be conducted to minimize the time that takes to heat up the reaction vessel as well as to maximize the product yield using both bed reactor and semi continuous flow reactor designing.

5. REFERENCES

(1) Goldemberg, J. *Science* **2007**, *315*, 808.

(2) Klemm, D.; Heublein, B.; Fink, H.-P.; Bohn, A. *Angewandte Chemie International Edition* **2005**, *44*, 3358.

(3) Iranmahboob, J.; Nadim, F.; Monemi, S. *Biomass and Bioenergy* **2002**, *22*, 401.

(4) Chang, V.; Holtzapple, M. In *Twenty-First Symposium on Biotechnology for Fuels and Chemicals*; Finkelstein, M., Davison, B., Eds.; Humana Press, 2000.

(5) King, J. W.; Srinivas, K.; Guevara, O.; Lu, Y.-W.; Zhang, D.; Wang, Y.-J. *The Journal of Supercritical Fluids* **2012**, *66*, 221.

(6) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. *Bioresource Technology* **2005**, *96*, 673.

(7) Sun, Y.; Cheng, J. *Bioresource Technology* **2002**, *83*, 1.

(8) von Sivers, M.; Zacchi, G. *Bioresource Technology* **1995**, *51*, 43.

(9) van Walsu, G. P. *Appl Biochem Biotechnol* **2001**, *91-93*, 317.

(10) Puri, V. P.; Mamers, H. *Biotechnology and Bioengineering* **1983**, *25*, 3149.

(11) McWilliams, R.; van Walsum, G. P. *Appl Biochem Biotechnol* **2002**, *98-100*, 109.

(12) Kumar, S.; Gupta, R. B. *Industrial & Engineering Chemistry Research* **2008**, *47*, 9321.

(13) Miyazawa, T.; Funazukuri, T. *Biotechnology Progress* **2005**, *21*, 1782.

(14) Hunter, S. E.; Savage, P. E. *Industrial & Engineering Chemistry Research* **2003**, *42*, 290.

(15) Bobleter, O. *Progress in Polymer Science* **1994**, *19*, 797.

(16) Schwald, W.; Bobleter, O. *Journal of Carbohydrate Chemistry* **1989**, *8*, 565.

(17) Antal Jr, M. J.; Mok, W. S. L.; Richards, G. N. *Carbohydrate Research* **1990**, *199*, 91.

(18) Antal Jr, M. J.; Leesomboon, T.; Mok, W. S.; Richards, G. N. *Carbohydrate Research* **1991**, *217*, 71.

(19) Mok, W. S.; Antal, M. J.; Varhegyi, G. *Industrial & Engineering Chemistry Research* **1992**, *31*, 94.

(20) Kim, K. H.; Hong, J. *Bioresource Technology* **2001**, *77*, 139.

(21) Rogalinski, T.; Ingram, T.; Brunner, G. *The Journal of Supercritical Fluids* **2008**, *47*, 54.

(22) Peter Van Walsum, G.; Garcia-Gil, M.; Chen, S.-F.; Chambliss, K. *Appl Biochem Biotechnol* **2007**, *137-140*, 301.

(23) Alves, L.; Felipe, M. A.; Silva, J. A.; Silva, S.; Prata, A. R. *Appl Biochem Biotechnol* **1998**, *70-72*, 89.

(24) Palmqvist, E.; Almeida, J. S.; Hahn-Hägerdal, B. *Biotechnology and Bioengineering* **1999**, *62*, 447.

(25) Taherzadeh, M. J.; Gustafsson, L.; Niklasson, C.; Lid´n, G. *Applied Microbiology & Biotechnology* **2000**, *53*, 701.

(26) Rachma Wikandari, R. M., Siti Syamsiyah, Ririn Muriana, and Yuliana Ayuningsih *Agricultural Journal* **2010**, *5*, 105.

II. CONTINUOUS FLOW CARBONIC ACID HYDROLYSIS OF STARCH FOR ETHANOL PRODUCTION

ABSTRACT

Starch is an abundant renewable resource on earth and hence is gaining importance in biofuel industry for the production of bioethanol. The continuous flow hydrolysis of starch using carbonic acid catalyst from pressurized carbon dioxide were evaluated for different reaction times and temperatures. The maximum yield of 90.5% weight hydrolysis products that consisted of 81.3% weight simple sugars (2.4 % maltose, 67.9% glucose and 1.1 % sucrose) and 9.1% weight of glucose degradation products (6.3% 5-hydroxymethylfurfural, 0.5% furfural, 2.5% 1,6-anhydro-β-glucose and 0.3% glycoaldehyde dimmers) was achieved for the 10 min reaction at 220°C. The significant effect of treatment temperature and duration on the starch depolymerization and glucose decomposition in the presence of carbonic acid catalyst revealed the need for optimization of reaction conditions for different substrates.

Keywords: Starch, Thermal hydrolysis, Glucose, hydroxymethylfurfural, furfural, carbonic acid

1. INTRODUCTION

Water is an inexpensive eco-friendly reaction solvent due to its nontoxicity, inflammability, and availability. It has unique properties that enable a wide variety of reactions without catalysts and make it an ideal reaction solvent for various commercial applications.[1-2] Hydrolysis is one of the most important reactions performed under hydrothermal conditions. The most abundant components of biomass produced via photosynthesis are carbohydrates. For the economical utilization of carbohydrate-rich biomass, an efficient hydrolysis of polysaccharides is necessary to produce alternative chemical substrates.[1-3] Conventional hydrolysis method utilized for the bioethanol production is conducted in the presence of enzymes or mineral acids, which requires pH control and additional process such as neutralization, desalination or purification. Therefore, continuous efforts are being made to find the low cost as well as an ecofriendly process to convert polysaccharides to monosaccharaides. Previous studies on the carbonic acid enhanced hydrolysis of polysaccharides like starch and cellulosic substances have shown that the complex carbohydrates can be readily converted to simple reducing sugars under the high temperature and pressure reaction conditions. This utilization of weak acid also eliminated the neutralization and desalination steps.

Most of the studies were performed in batch reactors to investigate the scope of thermal hydrolysis with and without the influence of carbon dioxide.[4-6] Difficulties were encountered during the optimization of the reaction conditions to avoid the decomposition or deterioration of mono- and oligo-saccharides to byproducts like furfural and 5-hydroxymethylfurfural (HMF) at elevated temperatures. HMF and furfural are

known to be toxic to the microbial culture and inhibit the fermentation process.[7-9] Glucose is the major hydrolysis product of starch and has many commercial applications in pharmaceuticals, food and fuel.[4]

In current scenario the development of economical renewable fuels such as ethanol, hydrogen, methane, etc. is considered as one of the most effective ways to achieve the sustainable energy supply. Fossil fuel reserves are rapidly depleting due to the increasing demand and are also associated with the environmental and ecological issues. Increasing the use of biofuels at global level can prolong the existing fossil fuel reserves and reduce environmental impact.[10-12]

Algae based biofuels are gaining importance because of their versatile capacities. Algae biofuels are not without limitations especially when it comes to high cost of production. Therefore combined bio-refinery approach is being proposed where multiple biofuels are produced from one biomass source to lower the cost of production and make the algae based biofuels more economical and sustainable.[13] Several microalgae species that are being produced commercially for various applications from pharmaceuticals to food & fuel, showed noticeably very high levels of starch contents.[14-15] Additionally, starch is present in a wide variety of agricultural crops and food wastes.[6] In order to utilize these starchy substances as the feedstock for bioethanol production, a continuous flow carbonic acid reactor designed and tested for the hydrolysis of starch. Hydrolytic behavior under the influence of carbon dioxide at high temperature conditions as well as to produce reducing sugars that can be successfully utilized for microbial fermentation to produce bioethanol or bio-hydrogen.

2. MATERIALS AND METHODS

2.1 MATERIALS

Reagent grade soluble starch and HPLC grade acetonitrile were obtained from Fisher Scientific (Waltham, MA, USA). Siphon tube CO_2 cylinder was supplied by Ozarc Gas (Rolla, MO, USA). Pure glucose, maltose, galactose, xylose, fructose, cellobiose, 5-hydroymethyl-2-furaldehyde (HMF), and furfural standards were obtained from Sigma (St. Louis, MO, USA). Ultrapure deionized (DI) water was produced from a Milli-Q Gradient water purification system (Millipore, Bellerica, MA, USA).

2.2 CONTINUOUS FLOW CARBONIC ACID HYDROLYSIS OF STARCH

A schematic of the continuous flow reactor is shown in Figure 2.1. The reactor consisted of a Jasco (Easton, MD) PU-2080 HPLC pump for starch solution delivery and a Jasco PU-1580-CO_2 pump for the CO_2 delivery. A Jasco CO-1580 column thermostat oven was used to heat the preheating coil for the starch solution and was set to 80 °C. CO_2 was pumped into a preheating coil located inside a Dionex (Sunnyvale, CA) series 600 SFC/GC oven which also housed the reactor coil set to temperature range of 210-230 °C. The reactor coil consisted of a 454.8 cm long stainless steel tubing with an internal diameter of 0.212 cm. The pressure inside of the reactor was controlled by a Jasco BP-1580-81 back pressure regulator which was set to a 7 MPa and a temperature of 80 °C. The reactor was first pressurized with CO_2 and heated to the experimental temperatures and allowed to stabilize with a CO_2 flow rate of 0.2 ml/min. Starch solution of 1 g/L concentration was continuously pumped into the reactor at a specific flow rates to

achieve the desired residence times. Treated product streams were passed through a cooling coil and collected at the outlet of the back pressure regulator and immediately analyzed with the HPLC. All experiments were conducted in triplicate.

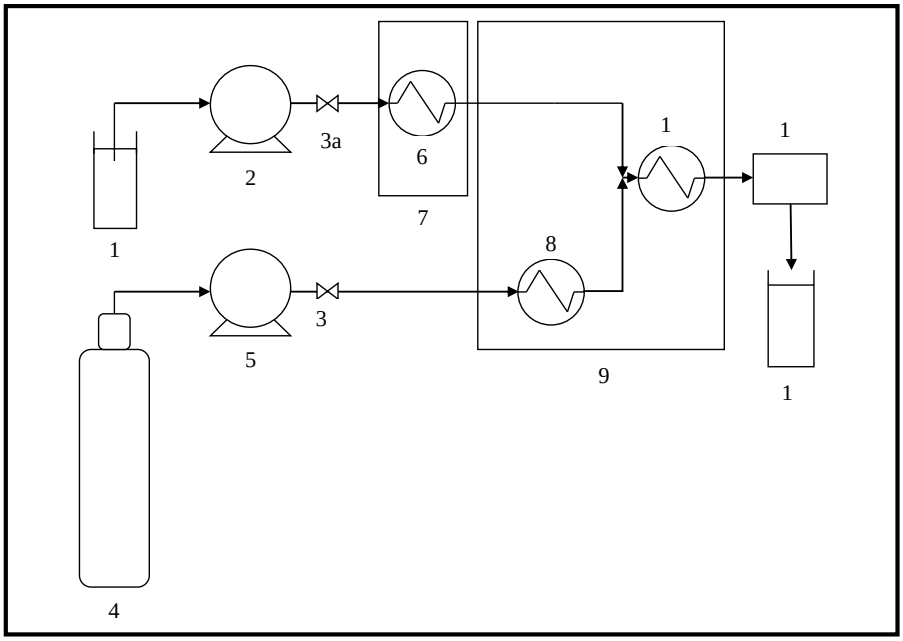

Figure 2.1. Schematic diagram of the continuous flow carbonic acid reactor with CO_2 pressure: 1, starch solution reservoir; 2, liquid pump; 3a&b, shutoff valves; 4, siphon CO_2 cylinder; 5, CO_2 pump; 6, starch solution preheating coil; 7, preheater oven; 8, CO_2 preheating coil; 9, oven; 10, reactor coil; 11, back pressure regulator; 12, collection vessel.

2.3 HPLC ANALYSIS

Hydrolysis product and decomposition byproduct contents were determined using the National Renewable Energy Laboratory biomass laboratory analytical procedure (NREL/TP-510-42623). HPLC analysis was carried out with a Hitachi (Schaumburg, IL, USA) LaChrom Elite HPLC system using a Bio-Rad (Hercules, CA, USA) Aminex

HPX-87H column (250×4.8 mm ID). A refractive index detector and UV-vis detector at a wavelength of 285 nm was used. The mobile phase was 0.001 N sulfuric acid at a flow rate of 0.6 ml/min and column temperature of 60 °C. Glucose, HMF and furfural were detected and quantified using authentic standards. All samples were filtered prior to injection using a 0.22 µm nylon membrane filter. The injection volume was 25 µL.

3. RESULTS AND DISCUSSION

Starch and cellulosic biomass are gaining global attention as low cost and renewable source of biofuels. Starch is one of the major components of the photosynthetic product and has wide applications from food to non-food industries. Hydrolysis of starch becomes an essential process to depolymerize into simple sugars for microbial fermentation to produce hydrogen or ethanol or methane for fuel applications.[10-11] Therefore, thermal hydrolysis of starch was investigated in continuous flow reactor using carbonic acid produced by pressurized CO_2. Orozco et al., (2012)[6] reported that the hydrothermal hydrolysis of starch at lower temperatures like 180°C resulted in low yields of fermentable sugars. Accordingly, new study was designed for the higher temperature range that will yield maximum amount of fermentable sugars with minimal formation of the degradation products.

Table 3.1. shows the yields of depolymerized products from starch under the influence of carbon dioxide at 200°C, 210°C and 220°C with varied range of reaction times. Graphs in Figure 3.1. indicates that the increase in reaction time has a large effect on the formation of hydrolysis products and degradation byproducts which can interfere with the subsequent biological fermentation to hydrogen or bioethanol. Glucose decomposition byproducts like HMF and furfural are well known be the inhibitors of microbial fermentation.[7-8]

The results show that at 210°C the maximum sugar yield of 77.7% was achieved with a 20 min residence time, producing 13.8% and 63.6% of maltose and glucose, respectively, with 7.8% of degradation product. Reaction time of 20 min at 210°C has

resulted in decreased yield of sugars. Though, the longer residence time of 40 min has produced increased yield (87.7%) of total hydrolysis products and subsequently the proportion of degradation products are also increased drastically by 53 % and 74 % compared to 20 min and 10 min reaction times respectively. These results indicate that the longer the reaction times greater the decomposition of sugars in to aldehydes (HMF and furfural). These results are in agreement with the observations made by Nagamori and Funazukuri (2004) and Orozco et al., (2012).[4,6]

Table 3.1. The percent yields of reducing sugars and degradation products from starch on weight basis for different reaction time and temperature conditions

		Sugars (%)				Degradation products (%)		
Temp.	Residence time (min)	Maltose	Glucose	Fructose	AG	GA	HMF	Furfural
230 °C	4	18.0 (±1.39)	53.0(±5.79)	0.9(±0.25)	2.1%(±0.49)	0.3%(±0.46)	2.4%(±1.68)	0.4%(±0.06)
	6	12.2(±0.81)	60.6(±1.82)	2.0(±0.06)	3.6%(±0.49)	1.0%(±0.17)	7.4%(±0.55)	0.7%(±0.02)
	8	10.3(±0.10)	56.7(±2.66)	2.4(±0.15)	4.2%(±0.87)	1.4%(±0.29)	9.7%(±0.32)	0.9%(±0.06)
220 °C	8	16.1(±0.83)	62.8(±2.51)	0.4(±0.61)	4.1%(±2.31)	nil/ n.d	4.3%(±0.15)	0.4%(±0.01)
	10	12.4(±0.53)	67.9(±2.11)	1.1(±0.90)	2.5%(±0.91)	0.3%(±0.52)	6.3%(±0.72)	0.5%(±0.01)
	20	9.5(±0.69)	60.1(±1.80)	2.8(±0.50)	3.5%(±1.83)	1.3%(±0.21)	11.5%(±0.81)	0.8%(±0.12)
210°C	10	19.8(±0.33)	54.1(±1.88)	nil/ n.d.	1.7%(±0.06)	nil/ n.d	2.0%(±0.21)	0.2%(±0.06)
	20	13.8(±1.25)	63.6(±2.76)	0.2(±0.35)	2.7%(±0.10)	nil/ n.d	5.1%(±.035)	0.4%(±0.01)
	40	10.5(±0.50)	60.6(±2.38)	2.1(±0.38)	3.3%(±0.44)	2.3%(±4.04)	9.0%(±1.12)	0.5%(±0.06)

In order to minimize the degradation products and to get the increased yields of fermentable sugars, starch was investigated to hydrolyze at relatively higher temperatures of 220°C and 230°C. Thermal hydrolysis of starch at 220°C has resulted with the total sugar yield of 81.3% at 10 min residence time, comprising 12.4%, 67.9% and 1.1% of maltose, glucose and fructose respectively, with 9.1% of degradation products (Table

3.1.). Further increase in reaction time to 20 min or decrease in reaction time to 8 min has resulted with decreased yields of total reducing sugars at 220°C.

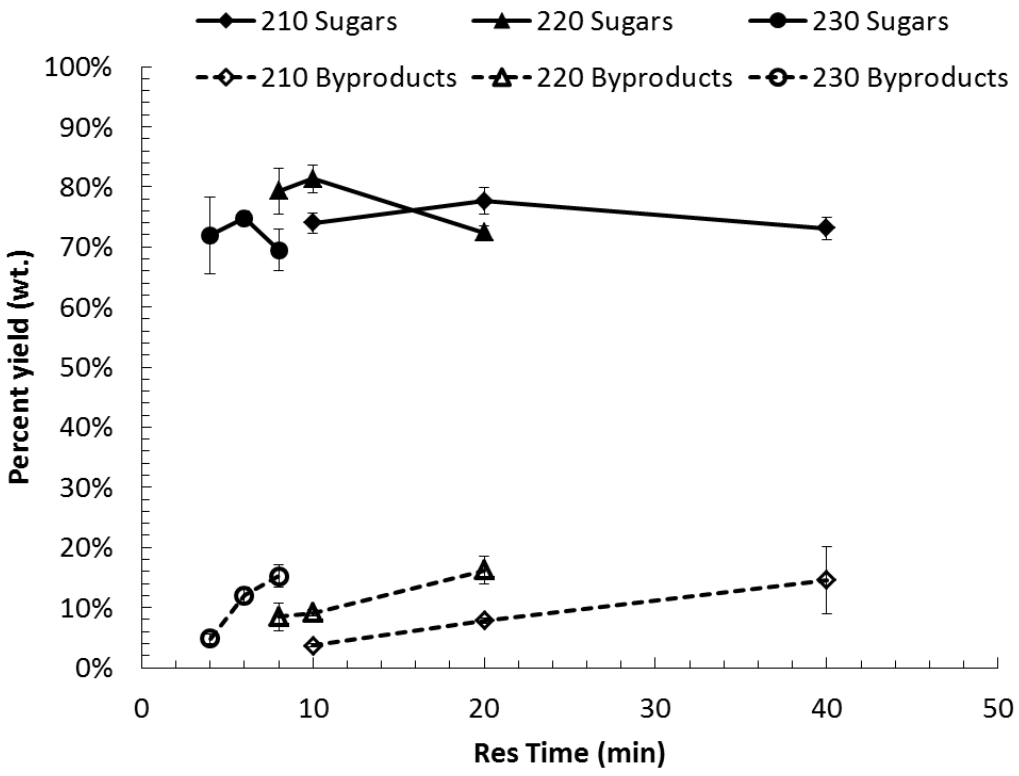

Figure 3.1. Sugar and degradation product yields from the hydrolysis of starch with carbonic acid at varying residence time and temperature

To see the influence of further increase in temperature up to 230°C with relatively reduced reaction or residence times on thermal hydrolysis of starch has been verified, but the total yields of both the reducing sugars as well as total hydrolysis products drastically reduced (Table 3.1 & Figure 3.1) at all the tested reaction times of 4, 6 and 8 min.

The maximum sugar yield of 74.8% was achieved with 6 min residence time at 230°C, producing 12.2%, 60.6% and 2.0% of maltose, glucose and fructose respectively,

with 12% of degradation products. Further decrease or increase in residence time than 6 min has resulted in decreased yields of hydrolysis products (Table 3.1.). It is also evident from this study that at all the tested temperature regimes of 210°C, 220°C and 230°C, the yields of oligosaccharides (maltose and fructose) are directly proportional with the increase in residence times and vice versa (Table 3.1.). Similarly, the yields of degradation products are also increased with the increase in residence times and vice versa (Table 3.1.). So, it appears that the yield of oligosaccharides is linked to the yields of glucose. Therefore, these results reveals that the moderate reaction conditions could yield better ratios of fermentable sugars than severe thermal condition that involve longer residence time or lesser residence time at high temperatures. These observations are comparable to the observations that are made by Nagamori and Funazukuri (2004)[4] and Orozco et al., 2012.[6] However, the glucose yields of 58.4% and 63% on weight basis were reported respectively by Oronoco et al., 2012 and Nagamori and Funazukuri, 2004, whereas the current study has recorded 67.9% of glucose on thermal hydrolysis of starch. Consequently, they have conducted thermal hydrolysis in batch reactor while the current study evaluated in contentious flow reactor.

To further evaluate the catalytic effect of carbon dioxide, control tests were carried out without CO_2 at the optimal reaction conditions of this study of 220°C for 10 min residence time, has conveyed 2.21 fold decreases in the yields of hydrolysis products with 67.9% of glucose on weight basis (Figure 3.2). So, it is evident from these observation that carbon dioxide attributed its intense effect on thermal hydrolysis of starch and these results are in agreement with several studies that used carbon dioxide to hydrolyze polysaccharides such as starch or cellulosic substances.[1-4,6]

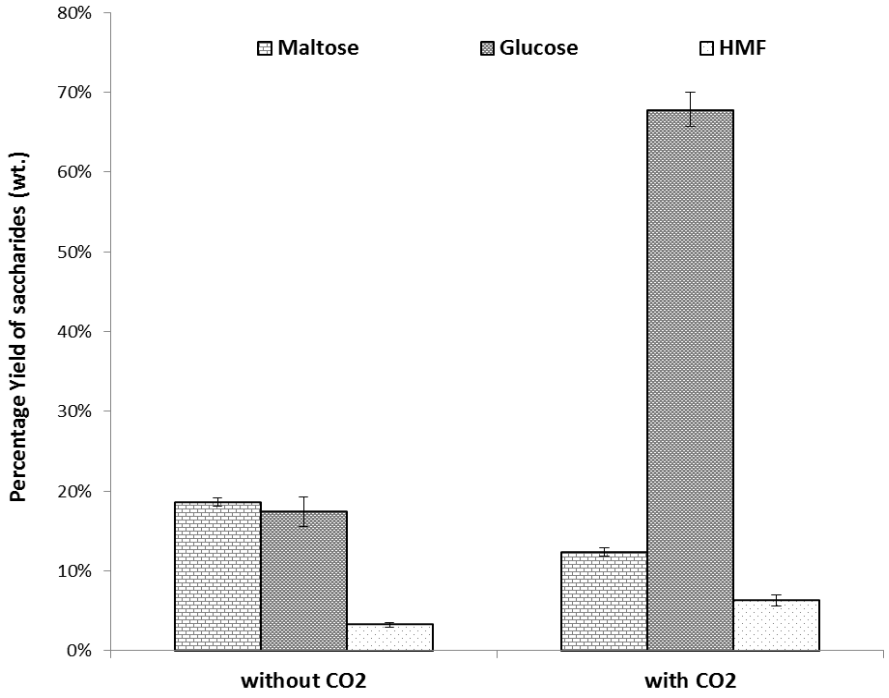

Figure 3.2. Yields of sugars from starch hydrolysis with and without carbon dioxide at 220°C for 10 minutes

4. CONCLUSIONS

Reaction time and temperature dictates the occurrence of depolymerization of starch into simple sugars and to degradation products. Carbon dioxide strongly contributes its catalytic effect on depolymerization of starch into simple sugars. Therefore the heating conditions should be moderate to achieve the targeted products, since high temperature and longer heating times will results in increased yields of aldehydes (HMF and furfural) and correspondingly decrease in the yields of glucose. In conclusion, ten minutes of residence time at 220°C with carbon dioxide produces significantly large amounts of fermentable sugars as well as relatively lesser yields of microbial fermentation inhibitors like HMF and furfural. However, further studies needs to be conducted to evaluate these results for its viability with the enzymatic hydrolysis of starch that is being currently preferred for the industrial use. Nevertheless, the scope of enzymatic hydrolysis over the thermal hydrolysis on various starch based biomass along with the clarification of degradation products like HMF and furfural from thermal hydrolysis as well as the cost of enzyme production for enzymatic hydrolysis remains as the major concerns while judging the competent methodologies. The scope of hydrothermal hydrolysis under the influence of carbon dioxide is very wide hence it could also be applied for various types of substances from carbohydrates to proteins and fats.

5. REFERENCES

(1) Modenbach, A. A.; Nokes, S. E. *Biotechnology and Bioengineering* **2012**, *109*, 1430.

(2) Hunter, S. E.; Savage, P. E. *Industrial & Engineering Chemistry Research* **2003**, *42*, 290.

(3) Alvira, P.; Tomás-Pejó, E.; Ballesteros, M.; Negro, M. J. *Bioresource Technology* **2010**, *101*, 4851.

(4) Nagamori, M.; Funazukuri, T. *Journal of Chemical Technology & Biotechnology* **2004**, *79*, 229.

(5) Miyazawa, T.; Funazukuri, T. *Biotechnology Progress* **2005**, *21*, 1782.

(6) Orozco, R. L.; Redwood, M. D.; Leeke, G. A.; Bahari, A.; Santos, R. C. D.; Macaskie, L. E. *International Journal of Hydrogen Energy* **2012**, *37*, 6545.

(7) Palmqvist, E.; Almeida, J. S.; Hahn-Hägerdal, B. *Biotechnology and Bioengineering* **1999**, *62*, 447.

(8) Taherzadeh, M. J.; Gustafsson, L.; Niklasson, C.; Lid´n, G. *Applied Microbiology & Biotechnology* **2000**, *53*, 701.

(9) Rachma Wikandari, R. M., Siti Syamsiyah, Ririn Muriana, and Yuliana Ayuningsih *Agricultural Journal* **2010**, *5*, 105.

(10) Goldemberg, J. *Science* **2007**, *315*, 808.

(11) Frigon, J.-C.; Guiot, S. R. *Biofuels, Bioproducts and Biorefining* **2010**, *4*, 447.

(12) Show, K. Y.; Lee, D. J.; Tay, J. H.; Lin, C. Y.; Chang, J. S. *International Journal of Hydrogen Energy* **2012**, *37*, 15616.

(13) Jones, C. S.; Mayfield, S. P. *Current Opinion in Biotechnology* **2012**, *23*, 346.

(14) Somrak Rodjaroen, N. J., Aparat Mahakhant, Kazuhisa Miyamoto *Nat. Sci*. **2007**, *41*, 57.

(15) John, R. P.; Anisha, G. S.; Nampoothiri, K. M.; Pandey, A. *Bioresource Technology* **2011**, *102*, 186.

III. HIGH PRESSURE CONTINUOUS FLOW REACTOR FOR CARBONIC ACID HYDROLYSIS OF MICROALGAL BIOMASS

ABSTRACT

Algal biomass is one of the most promising sources of biofuels, in particular bioethanol. Microalgae frequently store carbohydrates in the form of starch, which can be easily hydrolyzed and fermented into ethanol. Microalgae, *Chlorella pyrenoidosa* and *desmodesmus communis*, were hydrolyzed using carbonic acid in a high pressure continuous flow reactor. The hydrolysis was performed at a pressure of 7 MPa, and the reaction time and temperature were optimized for *desmodesmus communis* biomass. The reaction times ranged from 3 to 16 min, and temperatures of 200, 210, and 220°C were tested. The hydrolysates produced were analyzed using HPLC for the released sugars and degradation byproducts. The carbonic acid hydrolysis for algal biomass was optimized at 4.6min reaction time at 210°C and produced a glucose yield of 11.13% (wt. per wt. biomass). When compared to a dilute mineral acid hydrolysis, the carbonic acid hydrolysis produced a higher average glucose yield but also produced more degradation byproducts. The hydrolysates produced by carbonic acid hydrolysis where also fermented using yeast to produced ethanol, without any pH adjustment after the carbonic acid treatment.

1. INTRODUCTION

There is an increasing demand for alternative fuel sources because of the uncertainty of the oil reserves and the need to reduce CO_2 emissions. While, there are many renewable fuels which are derived from biomass, biomass produced from microalgae has a great potential for being a feed stock for a variety of renewable fuel, for example biodiesel or bioethanol. Microalgae have the possibility of year round production, and can have substantial growth rates, with most strain being able of double the biomass in 24hr.[1] While algae are cultured in aqueous media they require less water than land based crops, and can be cultured in waste water or brackish water.[2] Along with the production of biomass that can be utilized for biofuels algae also offer the potential of CO_2 fixation, bioremediation of heavy metals, and bio-treatment of waste waters.[3-5]

The production of bioethanol from microalgal biomass is of interest because they contain minimal amounts of lignin or hemi cellulose and can be processed into bioethanol easier than higher plant biomass.[6] In order for ethanol fermentation to occur algal biomass must first be treated to produce fermentable sugars. The most common methods used are either acid or enzyme based pretreatments. Acid pretreatments are conducted using concentrated or dilute acids, most commonly HCl or H_2SO_4. While concentrated acids can produce high monosaccharide yields of up to 90% for carbohydrates such as cellulosic biomass, they are hazardous, toxic, and corrosive.[7] Dilute acid treatments use acid concentration of 1-5% at temperatures between 100-120 °C. Dilute acid treatments do not require as much acid recovery and are not as hazardous when compared to concentrated acid treatments but only yield around 58% monosaccharides from cellulosic

biomass.[8] Enzymatic pretreatments can achieve high yields at milder conditions and from less by-products, when compared to acid treatments, however more cost and time effective methods are need.

Carbonic acid catalyst has been explored as an alternative to mineral acid hydrolysis for many reasons. Carbonic acid is a weak acid and therefore less corrosive and less toxic than strong mineral acids. The need to neutralize the hydrolysate or recover the acid material after processing is eliminated. Supercritical CO_2 pretreatments of lignocelluloses materials such as Aspen and southern yellow pine show that with a moisture content of 73% (w/w) sugar yields were increased as high as 84%.[9] Carbonic acid treatments of xylan showed that the hydrolysis of xylan is promoted by CO_2 at temperature of 170-230 °C.[10] Carbonic acid treatments have been shown to increase the production of xylose from corn stover[11], while no effect could be seen for aspen wood.[12] The high content of endogenous acid producing groups within the aspen wood biomass was used to explain why no effect was seen for the carbonic acid treatments. Carbonic acid pretreatment coupled to enzymatic saccharification of various biomass substrates show only a 6-10% decrease in sugar yields when compared the a more traditional sulfuric acid pretreatment, with the added benefit of not requiring a pH adjustment prior to fermentation.[13]

While promising reports of carbonic acid treatments preformed on lignocellulosic biomasses have been recently published, there have not been any study to date preformed on algal biomass. The aim of this study is to develop a continuous flow reactor that can utilized carbonic acid to hydrolyze algal biomass.

2. METHODS

2.1 MATERIALS

Ultrapure deionized (DI) water was obtained from a Milli-Q Gradient system (18.2M cm; Millipore, Bellerica, MA, USA). Pure glucose, maltose, galactose, xylose, fructose, cellubiose, 5-hydroymethyl-2-furaldehyde (HMF), and furfural standards were obtained from Sigma (St. Louis, MO, USA). Siphon tube CO_2 was supplied by Oz Arc Gas (Cape Girardeau, MO, USA). Sulfuric acid was obtained from Fisher (Waltham, MA, USA).

2.2 ALGAE CULTIVATION AND HARVESTING

Green algae, *Chlorella pyrenoidosa* and *desmodesmus communis*, cultures were provided by Lincoln University, Jefferson City MO. Algae were grown using F/2 media in two 30 L glass tanks with culture volumes of approximately 15 L each. The cultures were aerated continuously to aid in mixing. The growth of the algae cells was monitored by measuring optical density (OD) using a Spectronic 20D+ at a wavelength of 600nm. The cultures were harvested when they reached the stationary growth phase. Algal cells were harvested directly from their working cultures by centrifugation at 5,000 g for 5 min. The supernatant was removed and replaced with more culture and centrifuged again. This process was repeated until enough biomass was obtained. The cell pellet was then made into a slurry by suspending the pellet in distilled water. The biomass loading

(wt. biomass per L) was measured by filtering 10ml of the slurry through a glass microfiber filter, drying biomass over night at 80°C, and weighing the dried biomass.

2.3 SAMPLE PREPARATION

Prior to carbonic acid hydrolysis, sulfuric acid was added to the algal biomass slurries to make the acid concentration 0.010% to 0.100% by volume before being put through the reactor. Potato biomass was ground using a mortar and pestle and suspended in DI water to achieve a 1 g/L biomass loading. Potato biomass did not require the addition of any acid prior to carbonic acid hydrolysis.

2.4 CARBONIC ACID HYDROLYSIS REACTOR

A schematic of the reactor is shown in Figure 2.1. A Dionex series 600 SFC CO_2 pump (Sunnyvale, CA) was used for pressurizing the reactor coils and capture vessel with CO_2 before samples were pushed through. A Jasco PU-1580-CO_2 pump (Easton, MD) was used to pressurize the biomass holding/delivery vessel and push the biomass slurry through the reactor coils during operation. The biomass slurry flow rate through the reactor was controlled by the flow rate of this CO_2 pump. A Jasco CO-1580 intelligent column thermostat set at 80°C was used as a biomass slurry preheater. CO_2 was preheated in a Dionex series 600 SFC/GC oven which also housed the reactor coil and was set to temperatures of 200, 210, and 220 °C. The reactor coil was a 455 cm long stainless steel tubing with an internal diameter of 0.212 cm. The pressure in the reactor

coil was controlled by a Jasco BP-1580-81 back pressure regulator which was set to a pressure of 7 MPa and a temperature of 80 °C.

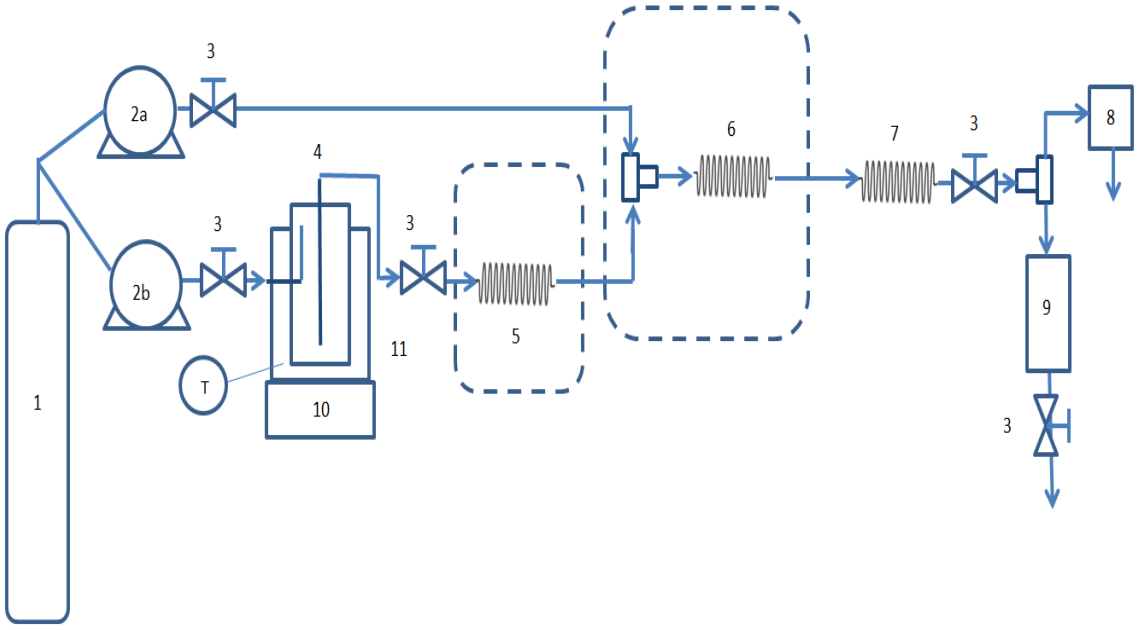

Figure 2.1. Carbonic acid hydrolysis reactor; 1. CO_2 tank, 2a,b. Pumps, 3. Shutoff valves, 4. Biomass holding/delivery vessel, 5. Preheater oven and coil, 6. Main oven and reactor coil, 7. Cooling coil, 8. Back pressure regulator/ gas outlet, 9. Capture vessel, 10. Heating/stir plate, 11. Heating block

During operation biomass slurry was placed into the holding vessel and stirred by a magnetic stir bar to prevent the slurry from settling. The reactor and holding vessel were then pressurized with CO_2 and the reactor was heated to the experimental temperatures. The biomass slurry was then allowed to pass through the reactor at desired flow rates to achieve the necessary residence times of 3 to 16 min. The pretreated samples were cooled and collected in a capture vessel. Solid residue in the collected was removed by centrifugation at 5,000 xg for 5 min. The supernatant was then filtered

through a 0.22 µm membrane filter and immediately analyzed with HPLC. All test parameters were measured in triplicate.

2.4 DILUTE ACID HYDROLYSIS

Dilute acid hydrolysis was carried out by a modified method used by Nguyen et al.[8] An algae cell pellet was suspended in 5% sulfuric acid solution, to make a biomass loading of 3 to 4 g/L. Hydrolysis was carried out in 50ml glass tubes at 120 °C for 60 min. The samples were collected and the solid residue was removed by centrifugation at 5,000 xg for 5 min. The supernatant was then filtered through a 0.2-um membrane filter and immediately analyzed with HPLC. All test parameters were measured in triplicate.

2.5 ETHANOL FERMENTATION BY YEAST

Fermentation of hydrolyzed biomass was performed using store bought baker's yeast, Fleischmann's active dry yeast. Fermentation was done using the method described by Nguyen et al.[8] The yeast cell were pre-cultured aerobically in 250 ml flasks using 100 ml of YPD medium containing 10 g/L yeast extract, 50 g/L dextrose, and 20 g/L peptone. The YPD medium was autoclaved for 30 min at 121°C before being inoculated with yeast culture. The yeast culture was then placed in a shake incubator at 32°C for 24 hr. The yeast culture was then centrifuged at 5,000 xg for 5 min and the supernatant was removed. The yeast pellet was then suspended in distilled water. Then approximately 1ml of the washed yeast culture was then added to 40ml of the biomass

hydrolysate, and the shake incubator at 32°C for 24hr. The samples were then removed, filtered using a 0.20 um membrane filter, and analyzed using HPLC.

2.7 HPLC ANALYSIS

Hydrolysis products and byproducts as well as ethanol contents were determined using the National Renewable Energy Laboratory biomass laboratory analytical procedure (NREL/TP-510-42623).[14] Analysis was carried out with a Waters 2690 HPLC system (Waters Corporation, Milford, MA) using a Bio-Rad Aminex HPX-87H column (250mm×4.8mm) (Biorad Laboratories Inc., Hercules, California, USA), Waters 410 RI detector, and Waters 486 UV-vis detector at a wavelength of 285nm. The mobile phase was 0.001 N sulfuric acid at a flow rate of 0.6 ml/min and column temperature of 60°C. All samples used a 25 μL injection size.

3. RESULTS AND DISCUSSION

3.1 BIOMASS LOADING

One of the first issues that need to be addressed was the biomass loading that the reactor could operate at without any line clogging. To test the capacity of the reactor biomass slurries of *desmodesmus communis* were prepared ranging from 0.42 g/L to 5.74 g/L (dry biomass weight per volume). At biomass loading below 5.74 g/L the reactor operated normally and there was no sign of any line clogging. However, at a 5.74 g/L biomass loading inconsistences in the operating pressure were observed as well as line clogging during the cleaning and back flushing of the reactor. Due to the small internal diameters of some of the connecting pluming of the reactor the reactor has a biomass loading limit around 5.74 g/L when using algal biomass slurries. During these capacity testing the reactor was set to a temperature of 220 C and the residence time of 6.4 min was used. In Figure 3.1. the glucose yield shows a linear increase with the increase of biomass loading. The main limitation of the reactor being, in terms of biomass loading is a physical limitation as in at high biomass loading the lines clog. Figure 3.2. shows that maltose was detected in the 5.74 g/L biomass loading hydrolysate. Maltose is not detected in lower biomass loading sample. This presence of maltose in the 5.74 g/L biomass hydrolysate indicates that the hydrolysis of the biomass was not as effective as the lower biomass loading samples. At higher biomass loadings reactor temperatures and residence time might need to be further optimized, or that the higher biomass loadings require addition sulfuric acid to neutralize the biomass prior to the carbonic acid treatment. Unfortunately, because of the high risk of clogging while operating at higher

biomass loadings we were not able to test any of these. All other tests was performed on biomass loading of approximately of 4.0 g/L.

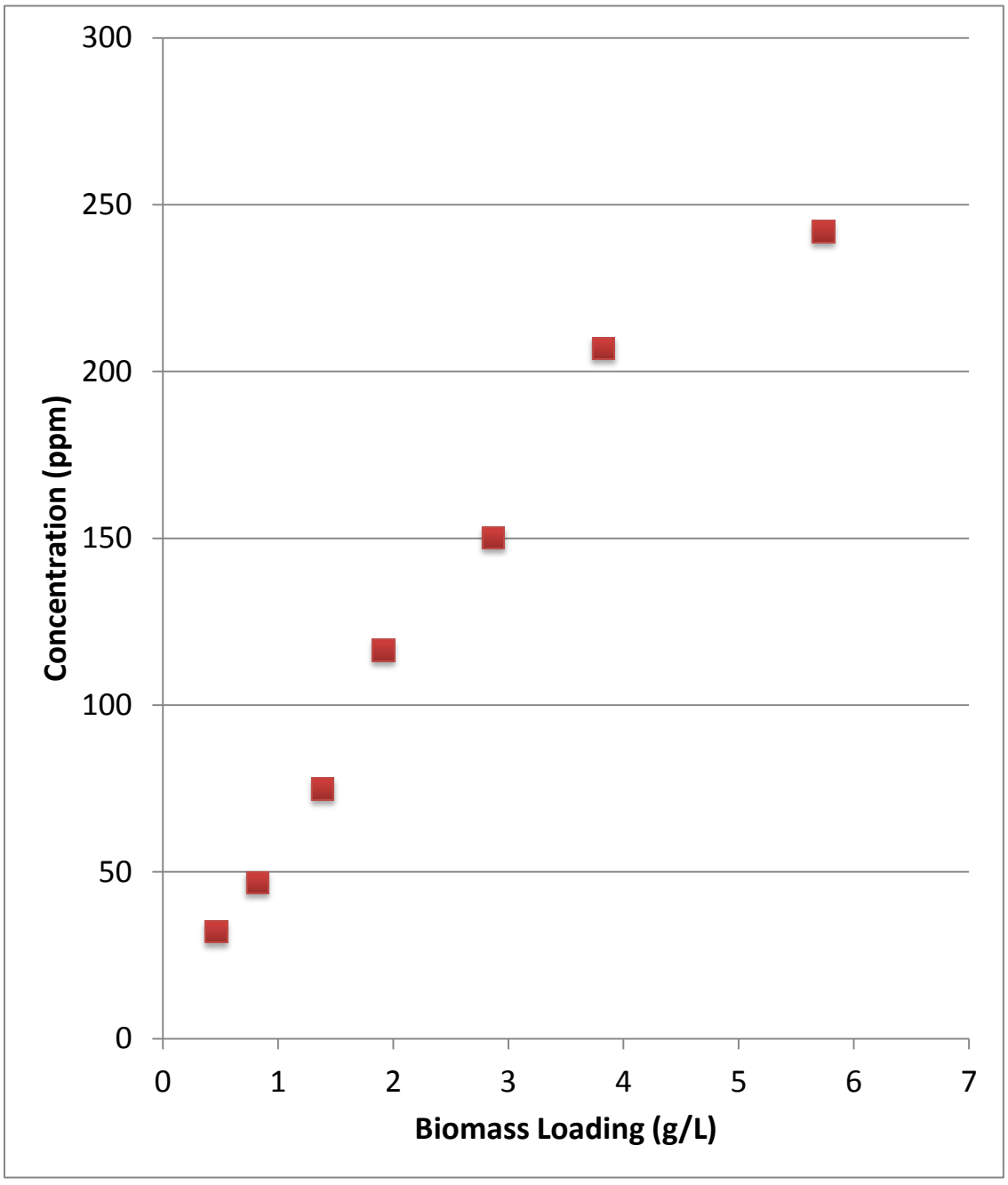

Figure 3.1. Effect of biomass loading on glucose yield for carbonic acid hydrolysis.

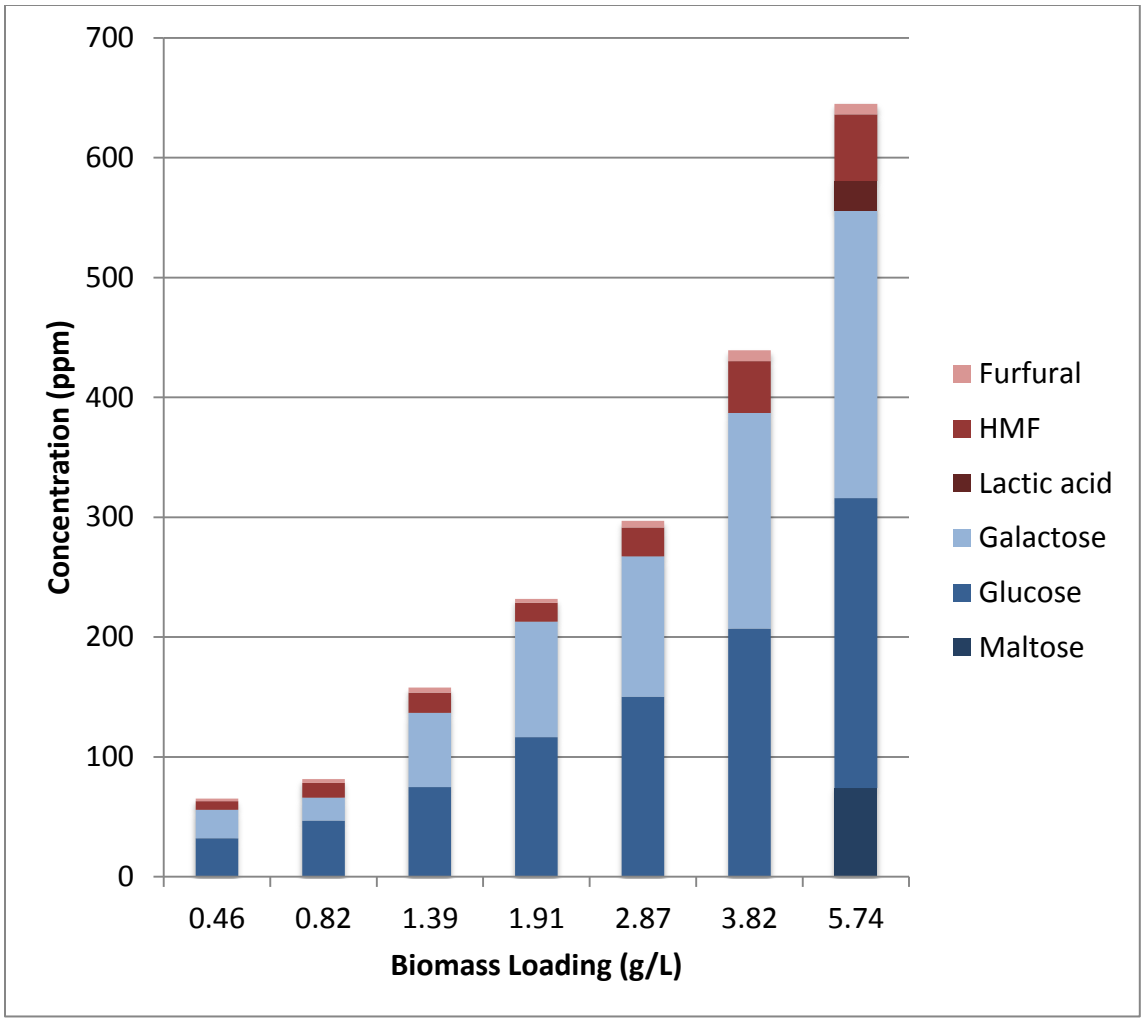

Figure 3.2. Effect of biomass loading on product yields for carbonic acid hydrolysis

3.2 ALGAE CELL STRUCTURAL CHANGE

The algae cells were observed under a microscope before and after carbonic acid hydrolysis. Figure 3.3.a depicts untreated *desmodesmus communis* cells, while Figure 3.3.b depicts cells after the carbonic acid treatment. The untreated cells are full and robust, with uniform size and morphology. After being treated in the high pressure carbonic acid, the cells are ruptured, collapsed, and have lost their original morphology.

3.3 OPTIMIZATION OF TEMPERATURE AND RESIDENCE TIME

Two main factors affecting the hydrolysis of *desmodesmus communis* biomass in your carbonic acid reactor investigated were the reactor temperature and residence time. Reactor temperatures of 200, 210, and 220 C were used during these optimization runs. Residence times ranged between 3.6 to 16 min depending on the reactor temperatures used, and the biomass slurries were pretreated with 0.05% sulfuric acid. For reactor temperatures of 200 C residence times of 4.57, 6.4, and 10.7 min were used. Figure 3.4. shows the yields from these tests. At 200 C we see little change in the glucose yields with increasing residence time and only a slight increase in HMF. Reactor temperature of 210 C was run at residence times of 3.6, 4.6, 5.3, 6.4, 8.0, 10.7, and 16.0 min. Figure 3.5 shows the yields from these tests. By increasing the reactor temperature from 200 to 210 C we see an increase in the glucose yield even at a smaller residence time of 3.6 min. The highest glucose yield of 11.13% was achieved at a residence time of 4.6 min and yields decreased at longer residence times. HMF yields increased with longer residence times. Further increasing the reactor temperatures to 220 C did not show any additional increase in the glucose yield but did result in larger deviations between runs and higher HMF yields. Figure 3.6 shows the yields from the 220 C reactor temperature tests performed at 4.6, 5.3, 6.4, and 16 min residence times. For these results the optimal condition for the carbonic acid hydrolysis of *desmodesmus communis* were found to be a reactor temperature of 210 C with a residence time of 4.6 min.

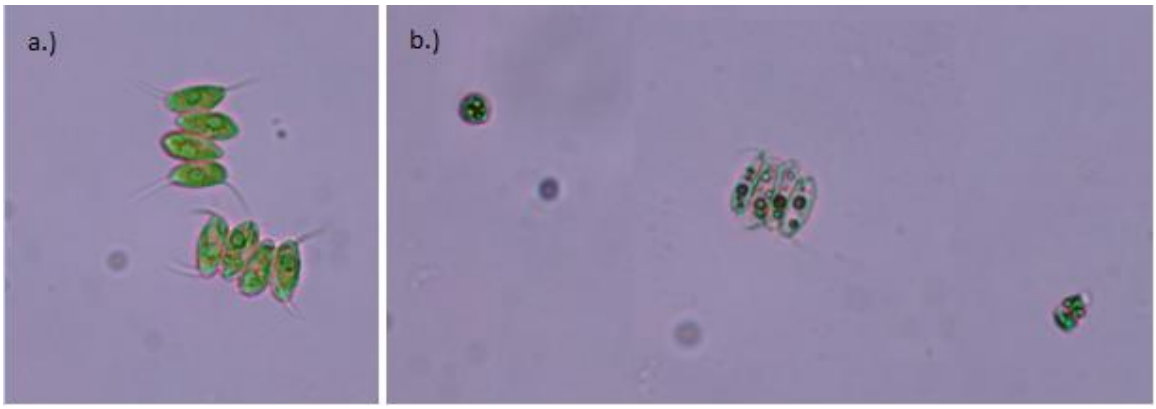

Figure 3.3. *Desmodesmus communis* cells at 400x magnification, a) untreated cells b) cells after carbonic acid treatment.

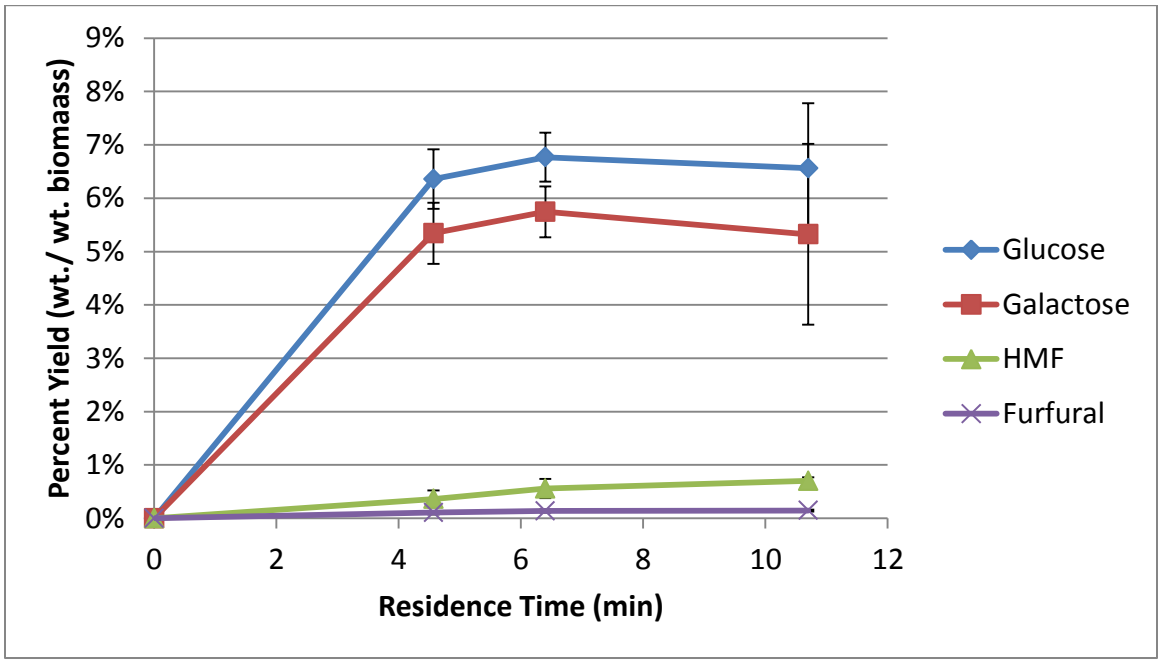

Figure 3.4. Yields of 200°C reactor temperature for carbonic acid hydrolysis.

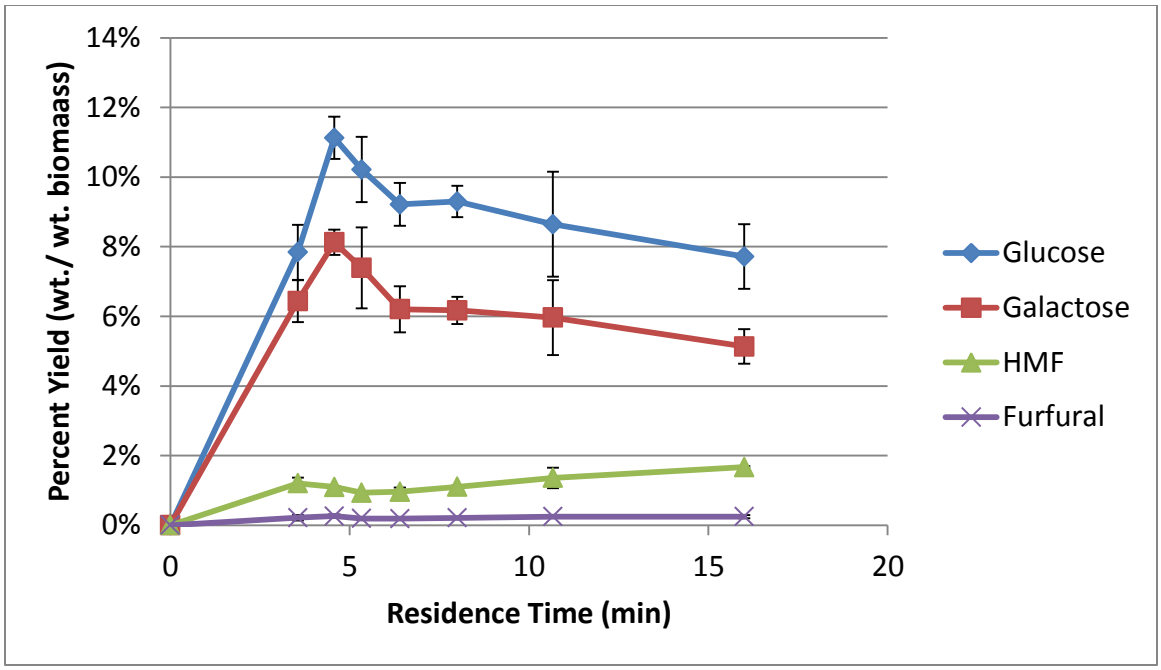

Figure 3.5. Yields of 210°C reactor temperature for carbonic acid hydrolysis.

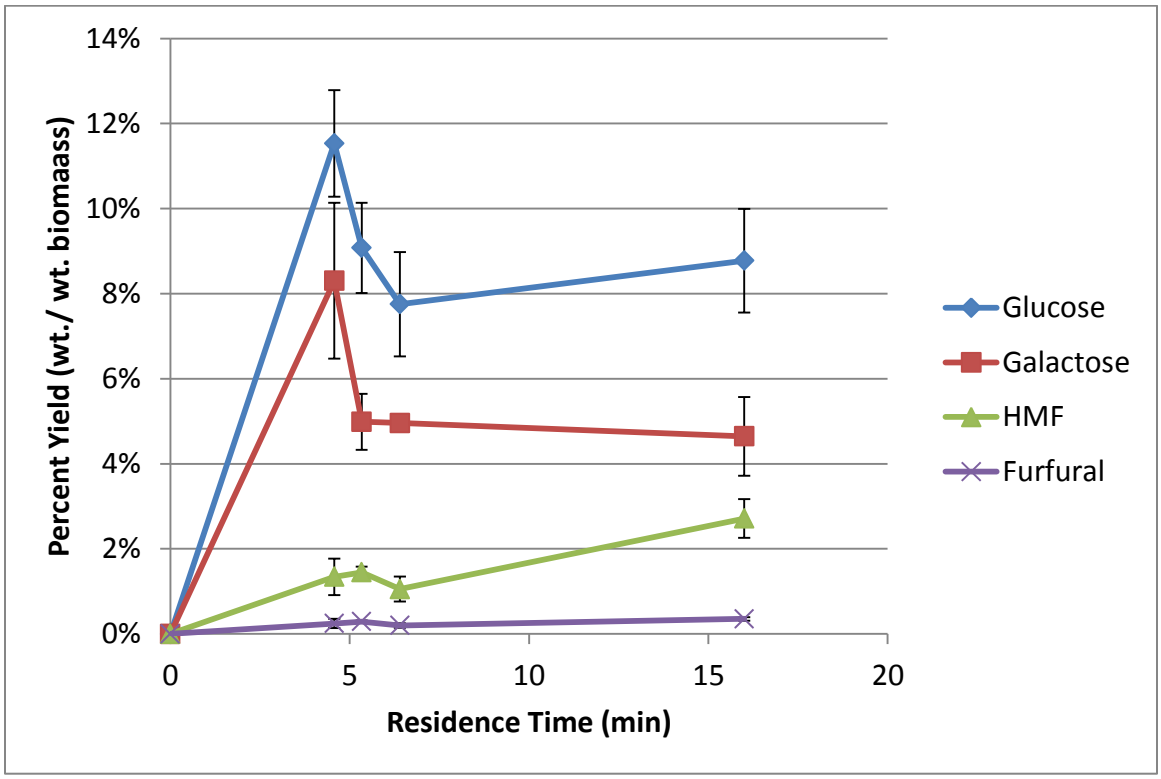

Figure 3.6. Yields of 220°C reactor temperature for carbonic acid hydrolysis

3.4 ACID PRETREATMENT CONCENTRATION

The main goal of this study was to replace conventional acids used in the hydrolysis process with carbonic acid, but because the algae cultures were growth to stationary phase the biomass was quit alkaline and required a small amount of acid to be used prior to being run through the reactor. There are a number of culturing techniques that could have been used to maintain a pH neutral algal culture but these would have led to increases in growth rates and the production of biomass quantities that could not have been processed. We wanted to investigate the effect of the acid pretreatment prior to the carbonic acid hydrolysis to insure that the smallest amount of acid was being used. For these tests *desmodesmus communis* biomass was used and the optimized conditions run (reactor temperature of 210 C with a residence time of 4.6 min). The sulfuric acid concentrations used for the pretreatment were 0.100, 0.050, 0.025, 0.010% by volume. The hydrolysis yields can be seen in Figure 3.7. At the lowest concentration of 0.010% sulfuric acid the glucose yield was greatly decreased and maltose was present in the hydrolysate. The presence of the maltose and low glucose yield shows that the carbonic acid hydrolysis was hindered by the alkalinity of the algal biomass substrate. Increasing the pretreatment acid concentration to 0.025% we no longer detect maltose in the hydrolysate and increasing to 0.050% we obtain the maximum yield of glucose. A further increase in the pretreatment acid concentration to 0.100% show a slight decrease in the average glucose yield and an increase in the HMF yields.

These results show that under our culturing conditions a pretreatment of 0.050% acid yields the best results. Increasing the acid concentration above 0.050% proved to be

counterproductive, the higher acid concentration producing a harsher reaction environment resulting in high degradation products.

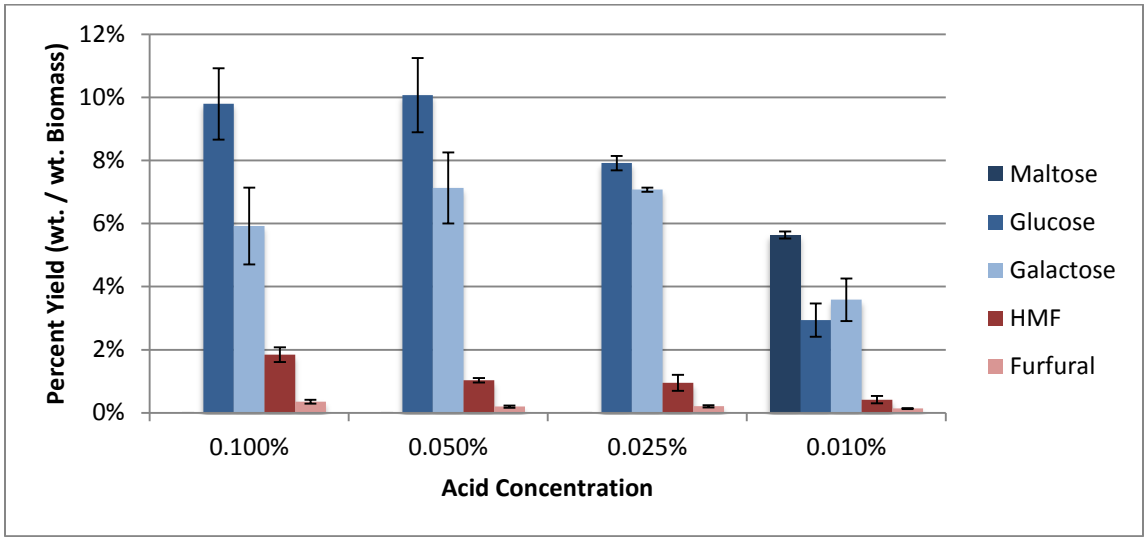

Figure 3.7. Effect of acid pretreatment on product yields for carbonic acid hydrolysis.

3.5 CARBONIC ACID AND DILUTE ACID HYDROLYSIS COMPARISON

In order to compare our carbonic acid hydrolysis to more common dilute acid hydrolysis, biomass of both *desmodesmus communis* and *chlorella pyrenoidosa* were hydrolysis using both methods and then compared. Optimized condition for the carbonic acid hydrolysis were used, reactor temperature of 210 C and a residence time of 4.6 min. The hydrolysis yields for both carbonic acid and dilute acid hydrolysis of *desmodesmus communis* are shown in Figure 3.8, while *chlorella pyrenoidosa* are shown in Figure 3.9.

The carbonic acid hydrolysis of *desmodesmus communis* resulted in a higher average yield of glucose 10.07% compared to the dilute acid hydrolysis which had an average yield of 8.31%. The carbonic acid hydrolysis also resulted in an average HMF

yield of 1.03%, one order of magnitude higher than the dilute acid hydrolysis. The carbonic acid hydrolysis of *chlorella pyrenoidosa* showed a lower average glucose yield of 9.31% compared to the dilute acid hydrolysis average yield of 10.63%, but maltose was also detected in the carbonic acid hydroslate showing some incomplete hydrolysis. The presence of maltose could mean that the reactor conditions that were optimized for the *desmodesmus communis* biomass may not be the same conditions needed for the *chlorella pyrenoidosa* biomass. Never the less these results show that the two hydrolysis methods are comparable.

3.6 FERMENTATION OF HYDROLYSATE

One of the advantages of use carbonic acid for the hydrolysis of biomass comes from the fact that neutralization of the hydrolysate is not required. Under the conditions of our carbonic acid reactor the biomass hydrolysates produced have pH ranging from 4 to 5 the pH at which yeast fermentation takes place. To insure that the hydrolysates produced could be fermented directly without pH adjustment carbonic acid hydrolyzed *desmodesmus communis*, *Chlorella pyrenoidosa*, and potato biomass were fermented to produce ethanol. The potato biomass was used to create a mash. The mash was then hydrolyzed in the carbonic acid reactor at 210 C with a residence time of 4.6 min. The hydrolysate showed an average glucose yield of 86.0%. The hydrolyzed mash was then fermented and analyzed showing no detectible glucose and an average yield of ethanol of 45.0%, shown in Figure 3.10. The fermentation yields of *desmodesmus communis* and *chlorella pyrenoidosa* are shown in Figure 3.11, with *desmodesmus communis* yielding

an average of 41.2mg ethanol per gram biomass and *chlorella pyrenoidosa* yielding an average of 68.3mg ethanol per gram biomass.

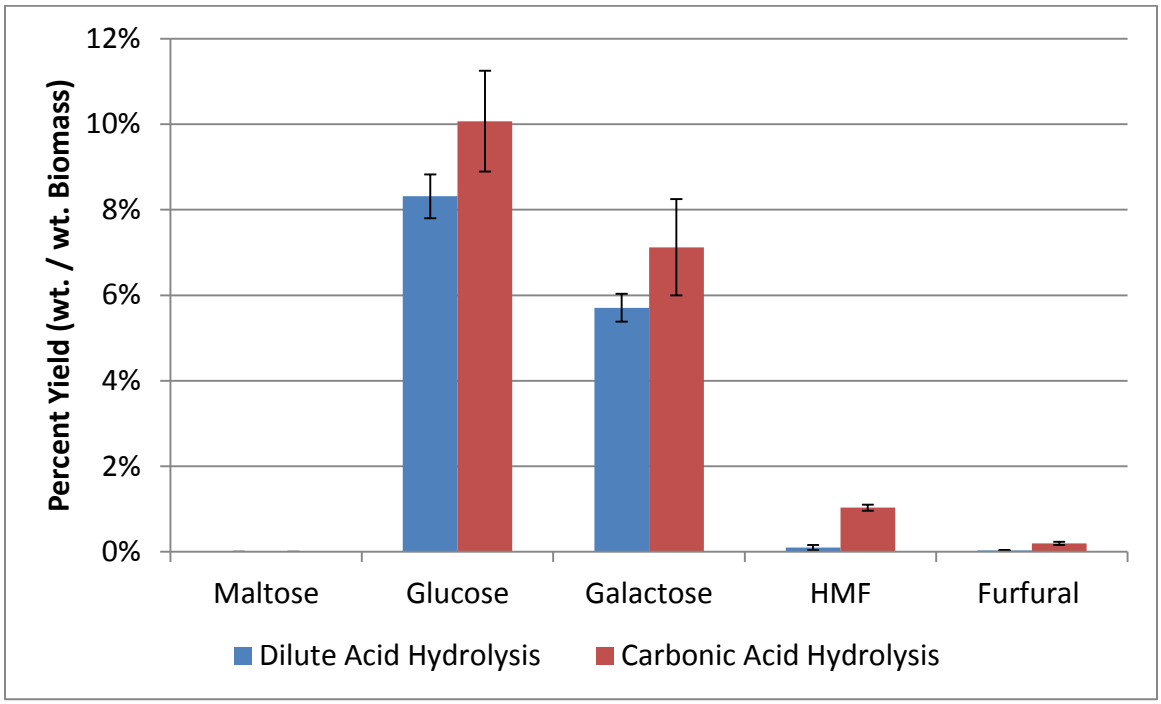

Figure 3.8. Comparision of dilute acid and carbonic acid hydrolysis of *Desmodesmus communis* biomass.

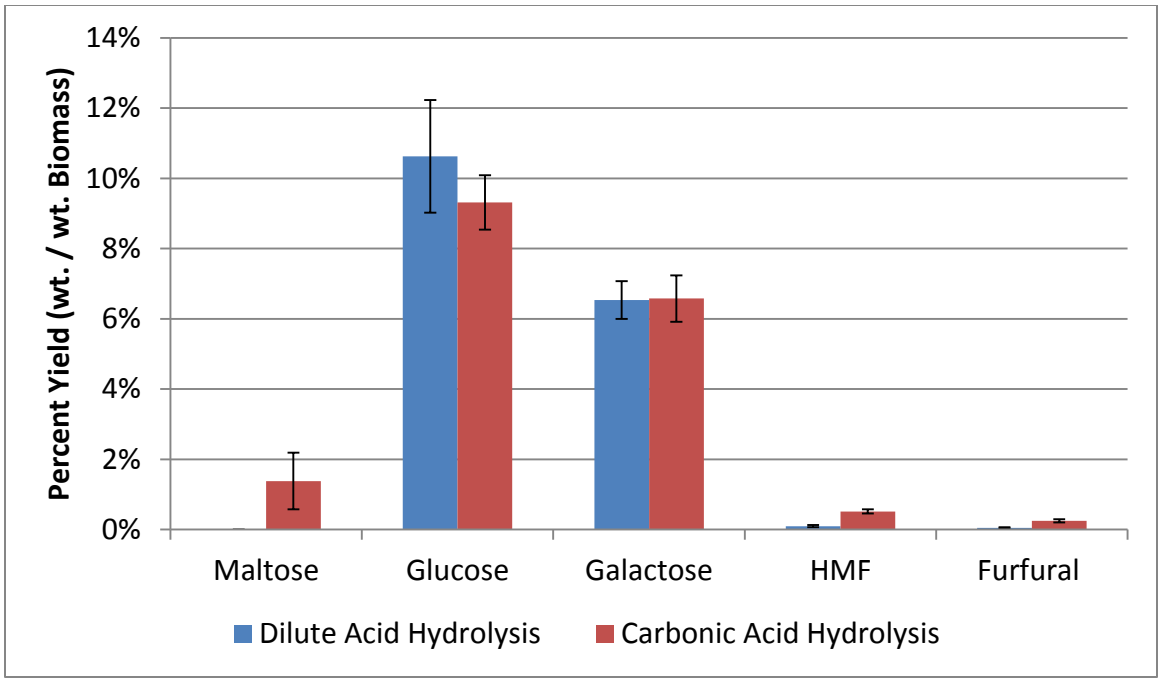

Figure 3.9. Comparision of diulte acid and carbonic acid hydrolysis of *Chlorella pyrenoidosa* biomass.

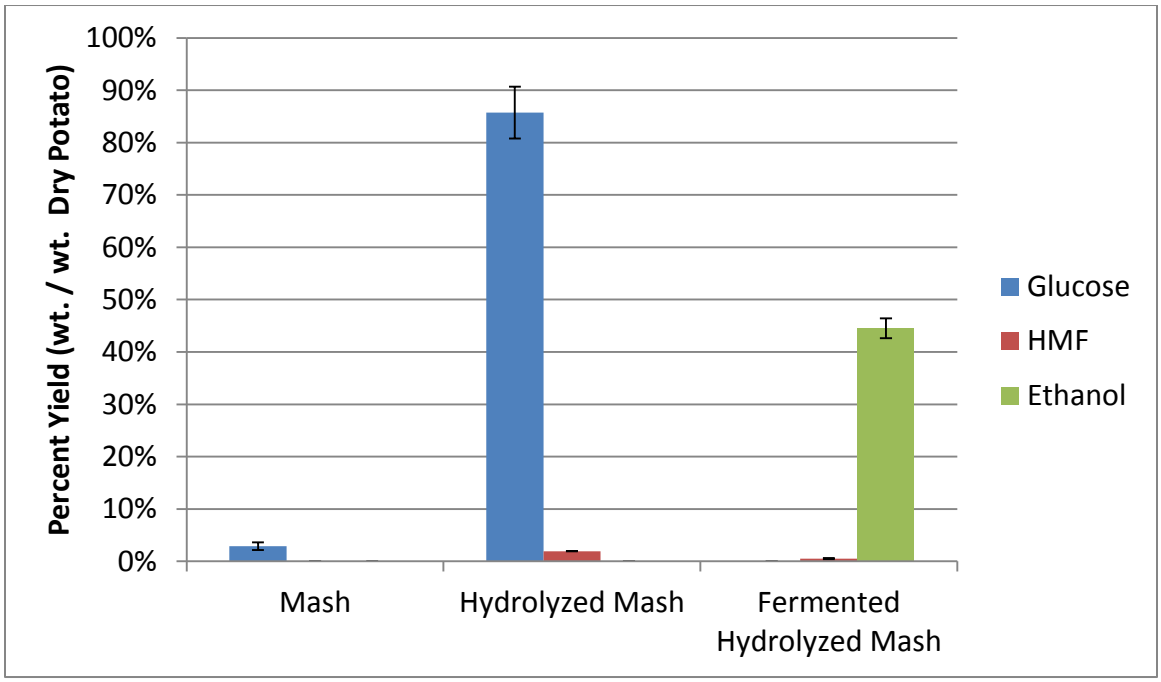

Figure 3.10. Carbonic acid hydrolysis and fermentation of potato mash

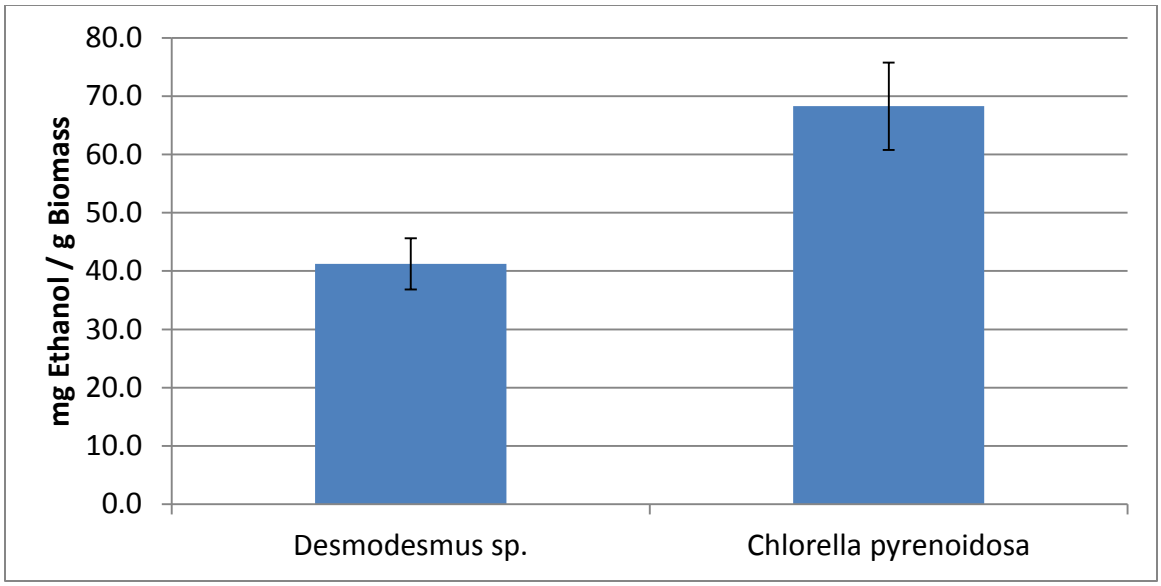

Figure 3.11. Ethanol yield from algal biomass after carbonic acid hydrolysis.

4. CONCLUSIONS

This study has shown the potential of a carbonic acid hydrolysis on biomass for the production of fermentable sugars. The reactor temperature and residence time were optimized for *desmodesmus communis* biomass cultured by batch processing and grown to stationary phase. The results show that the carbonic acid hydrolysis is comparable to common dilute acid hydrolysis. Furthermore, the hydrolysates produced from the carbonic acid hydrolysis were able to be fermented without any pH adjustments.

Improvements to the reactor design need to be addressed. Changes to the reactor that could accommodate high biomass loadings could be made to study at what biomass loadings would carbonic acid no longer be effective, if at any. One of the major limitations of the reactor was the operating pressure. The back pressure regulator used only operated consistently at a pressure of 7 MPa, so the effect of pressure could not be investigated in this study. With increasing pressures, the solubility of CO_2 increases producing more carbonic acid which could allow for lower reactor.

There are many other parameters that can be further investigated for the upstream processing. The primary strain used in this study, *desmodesmus communis*, was chosen for its high growth rates and ease at which it could be cultured in the lab. Studies on carbonic acid hydrolysis could be performed on more suitable algal strain with higher carbohydrate compositions in the biomass. Alternative culturing techniques could also be used such as monitoring and controlling culture pH to eliminate the need to pretreat the algal biomass with acid prior to carbonic acid hydrolysis or culture strains in a way to induce carbohydrate accumulation.

5. REFERENCES

(1) Chisti, Y. *Biotechnology Advances* **2007**, *25*, 294.

(2) Brennan, L.; Owende, P. *Renewable and sustainable energy reviews* **2010**, *14*, 557.

(3) Cantrell, K. B.; Ducey, T.; Ro, K. S.; Hunt, P. G. *Bioresource Technology* **2008**, *99*, 7941.

(4) Saunders, R. J.; Paul, N. A.; Hu, Y.; Nys, R. d. *PLoS ONE* **2012**, *7*, 1.

(5) Yun, Y.-S.; Lee, S. B.; Park, J. M.; Lee, C.-I.; Yang, J.-W. *Journal of Chemical Technology & Biotechnology* **1997**, *69*, 451.

(6) John, R. P.; Anisha, G. S.; Nampoothiri, K. M.; Pandey, A. *Bioresource Technology* **2011**, *102*, 186.

(7) Zhou, N.; Zhang, Y.; Wu, X.; Gong, X.; Wang, Q. *Bioresource Technology* **2011**, *102*, 10158.

(8) Minh Thu Nguyen, S. P. C., Jinwon Lee, Jae Hwa Lee, Sang Jun Sim *Journal of Microbiology and Biotechnology* **2009**, *19*, 161.

(9) Kim, K. H.; Hong, J. *Bioresource Technology* **2001**, *77*, 139.

(10) van Walsu, G. P. *Appl Biochem Biotechnol* **2001**, *91-93*, 317.

(11) van Walsum, G.; Shi, H. *Bioresource Technology* **2004**, *93*, 217.

(12) McWilliams, R.; van Walsum, G. P. *Appl Biochem Biotechnol* **2002**, *98-100*, 109.

(13) King, J. W.; Srinivas, K.; Guevara, O.; Lu, Y.-W.; Zhang, D.; Wang, Y.-J. *The Journal of Supercritical Fluids* **2012**, *66*, 221.

(14) A. Sluiter, B. H., R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton *Technical Report NREL/TP-510-42623* **2008**.

SECTION

3. FINAL CONCLUSIONS

This study focused on the development of a high pressure continuous flow reactor for the hydrolysis of biomass using carbonic acid. The reactor temperatures and residence times were studied and optimized to give maximum yield of simple sugars suitable for fermentation. The reactor operation and capacity was also tested to achieve high and consistent output. The carbonic acid hydrolysis produced similar yields compared to a 5% sulfuric acid hydrolysis. It also showed that the hydrolysates produced from the carbonic acid hydrolysis were able to be fermented directly and without any pH adjustment.

The first paper covers the evaluation of carbonic acid for the hydrolysis of micro crystalline cellulose (MCC). The carbonic acid hydrolysis was performed in a batch reactor that was assembled in house. Reaction temperature of 150, 170, 190, and 210°C and reaction times of 60, 90, 120, and 180 min were investigated. The addition of carbonic acid showed a large increase in the production of glucose at 210°C with a 60 min reaction time giving a maximum yield 20% (w/w). However, these conditions did produce a large amount of degradation products. Never the less the potential of carbonic acid to hydrolyze MCC was demonstrated.

The second paper of the study covered the design of a more optimized reactor which could be operated with a continuous flow. Starch solutions were hydrolysis using carbonic acid and the newly designed reactor. The reactor temperatures used were 210,

220, and 230°C with reaction times ranging from 4 to 40 min. A maximum yield for simple sugars of 90.5% (w/w) was achieved at a temperature of 220°C at 10 min. The improvements to the reactor also resulted in far less degradation products being produced. The continuous flow reactor was also ideal for the processing of algal biomass. Algal biomass is far less complex when compared to higher order plant biomass, contains no lignocellulosic material, and microalgae can be easily pumped through the reactor without any milling or prior size adjustment.

The third paper describes the development and evaluation of a continuous flow reactor and the carbonic acid hydrolysis of mircoalgal biomass for the production of bioethanol. Microalgae strains *Chlorella pyrenoidosa* and *desmodesmus communis* were used and hydrolyzed with carbonic acid in a continuous flow reactor. Reaction temperatures of 200, 210, and 220°C, and reaction times of 3 to 16min were used. The algae biomass slurries required the addition of 0.05% sulfuric acid prior to carbonic acid hydrolysis to neutralized the biomass substrate. A glucose yield of 11.13% (wt. per wt. biomass) was obtained at the optimized conditions of 210°C with a reaction time of 4.6min. The glucose yields of the carbonic acid hydrolysis were comparable to conventional dilute acid hydrolysis. Additionally, the hydroslates produced from the carbonic acid hydrolysis were able to be fermented with no pH adjustment.

APPENDIX A.
HPLC STANDARD CURVES

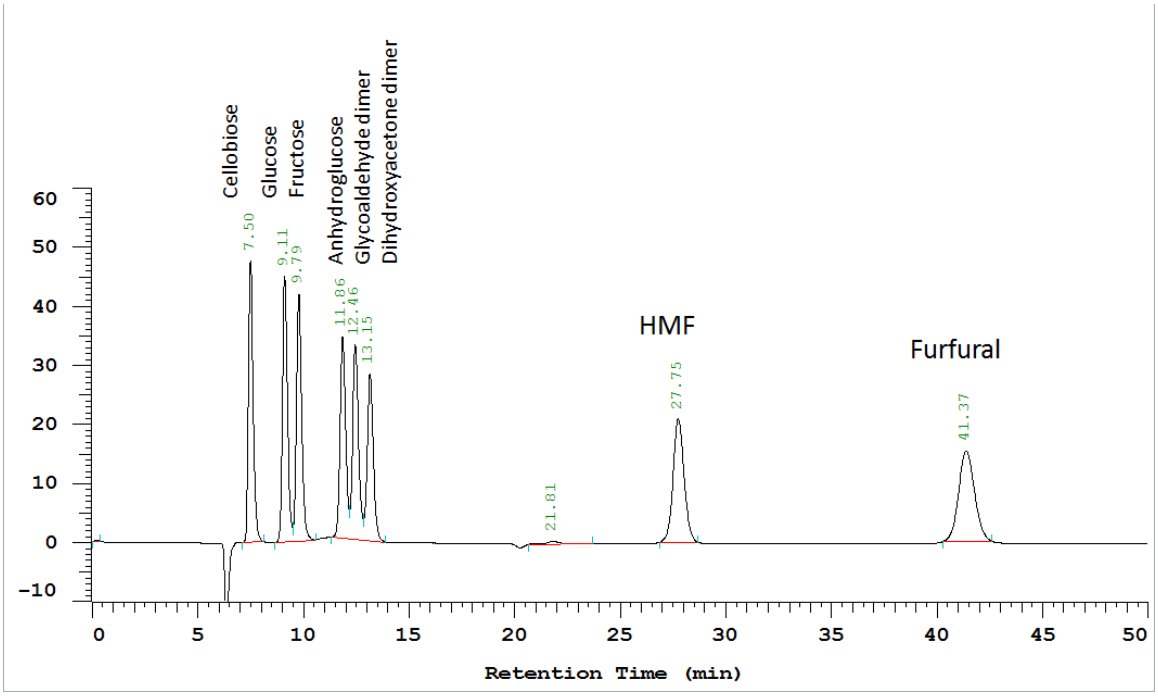

Figure 1. Example HPLC chromatogram of selected standards all at 1.0 mg/ml.

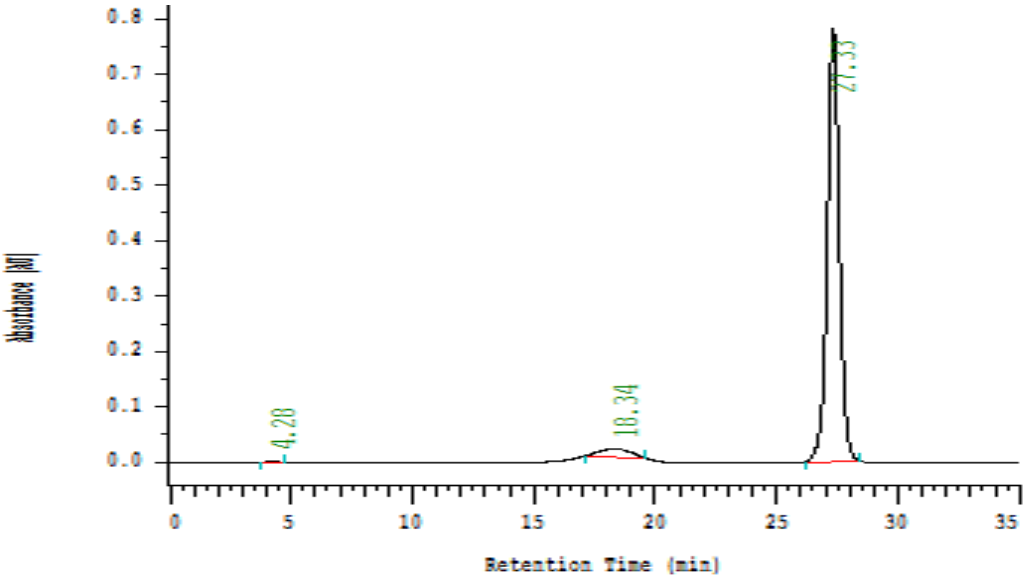

Figure 2. HMF standard chromatogram with UV-vis detector at concentration of 0.100 mg/ml.

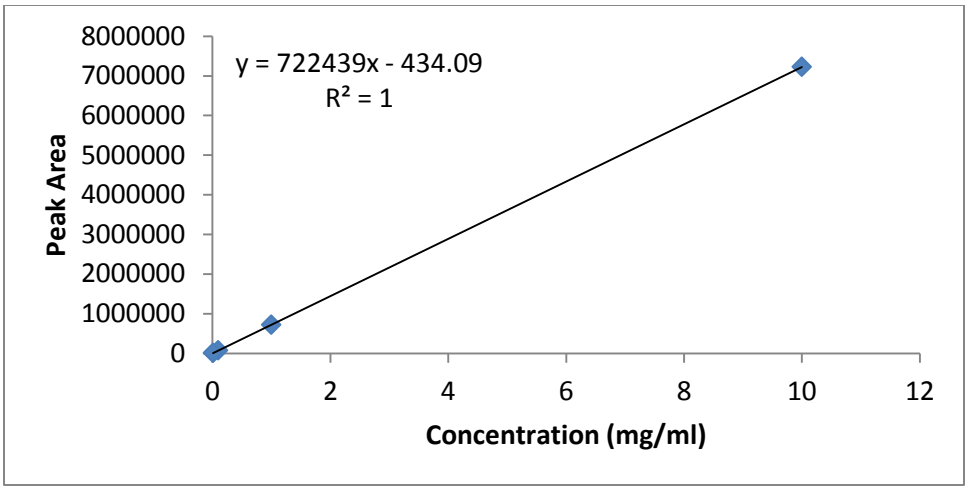

Figure 3. HPLC calibration curve generated for cellobiose using RI detector.

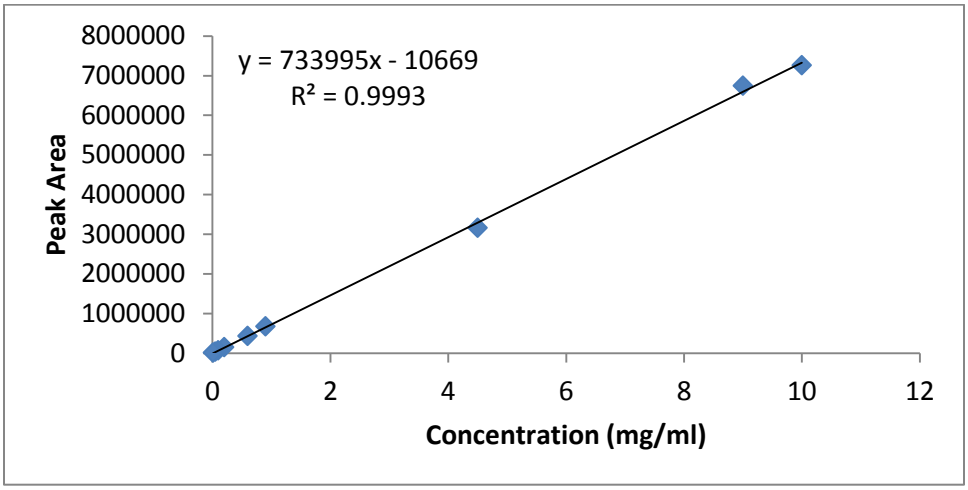

Figure 4. HPLC calibration curve generated for glucose using RI detector.

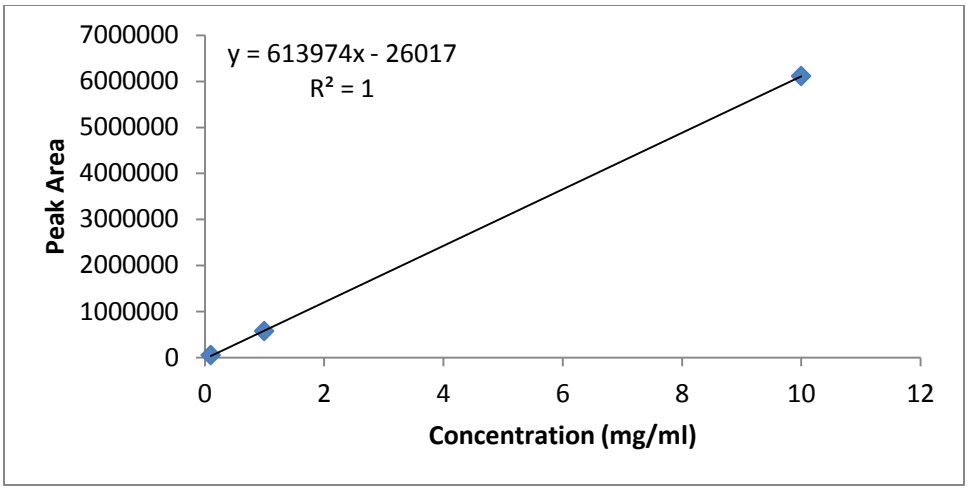

Figure 5. HPLC calibration curve generated for glycoaldehyde dimer using RI detector.

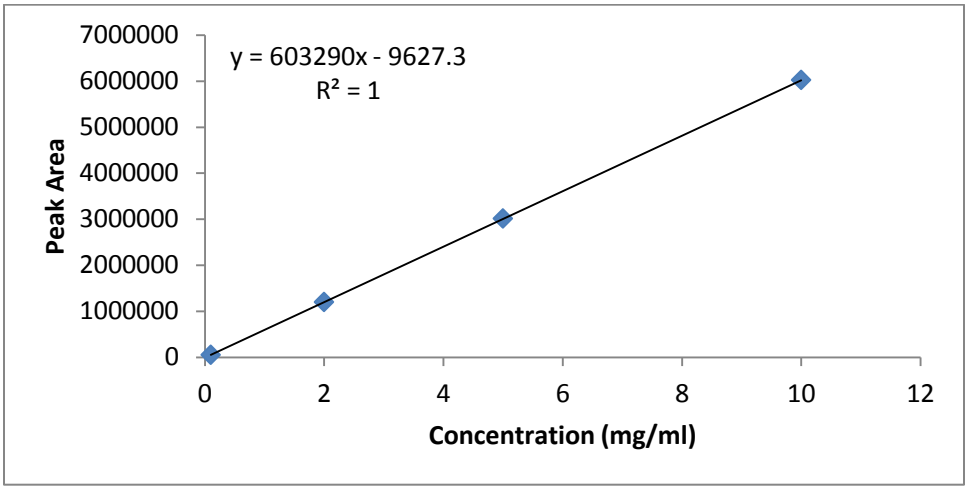

Figure 6. HPLC calibration curve generated for dihydroxyacetone dimer using RI detector.

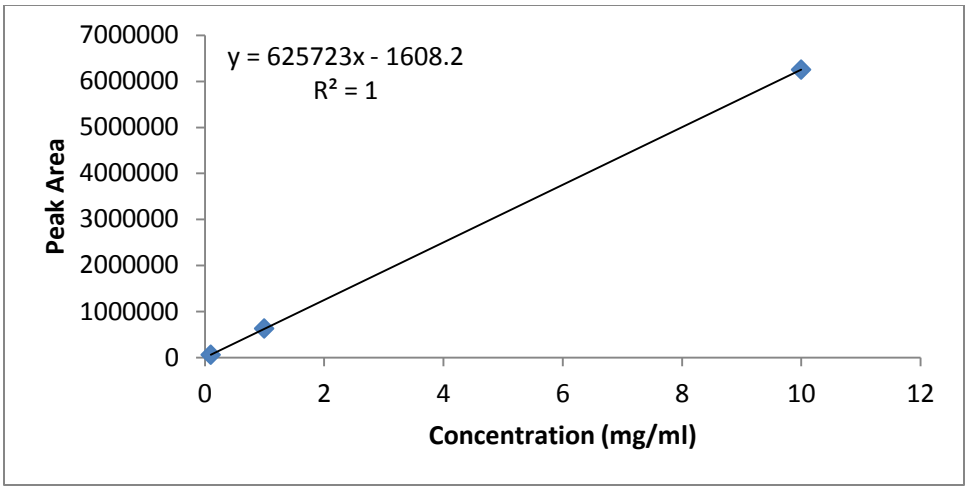

Figure 7. HPLC calibration curve generated for anhydroglucose using RI detector.

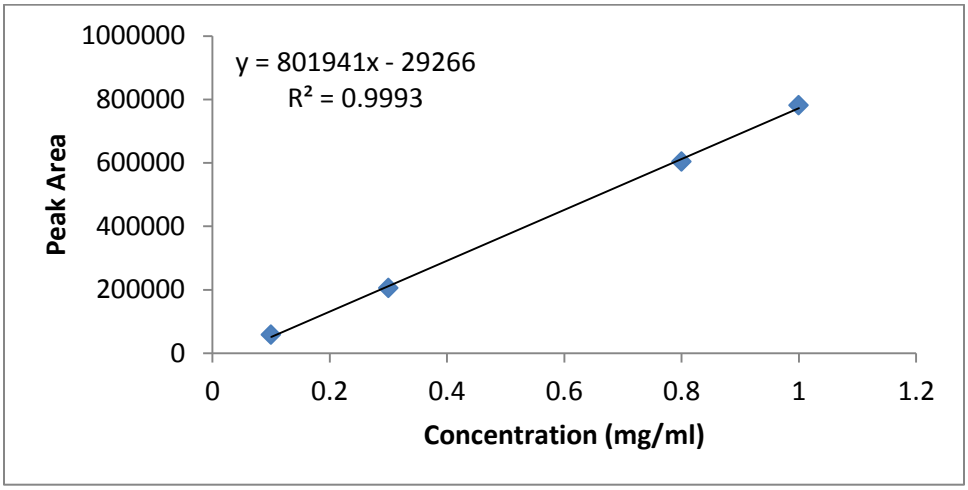

Figure 8. HPLC calibration curve generated for HMF using RI detector.

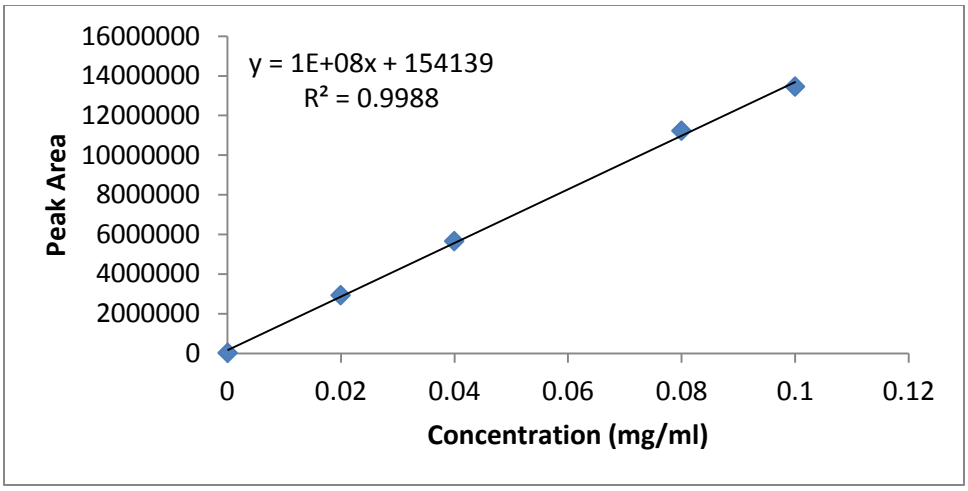

Figure 9. HPLC calibration curve generated for HMF using UV/Vis detector.

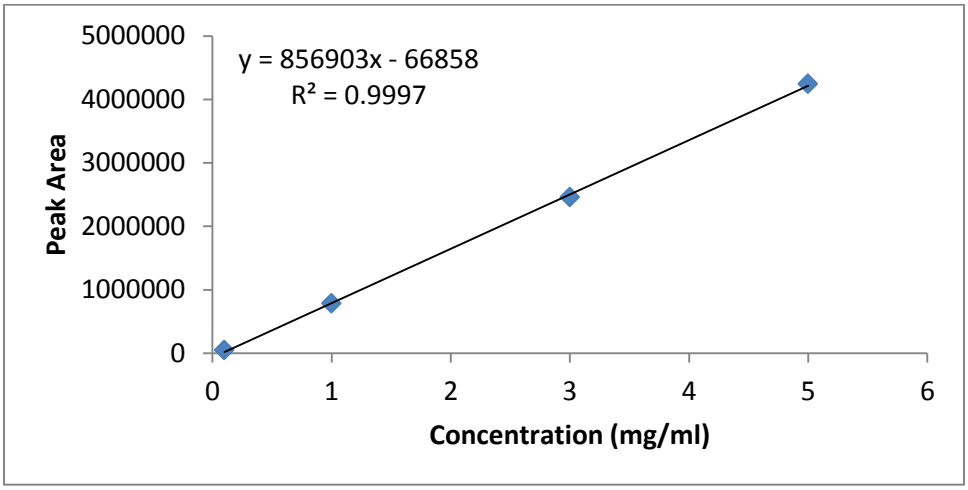

Figure 10. HPLC calibration curve generated for furfural using RI detector.

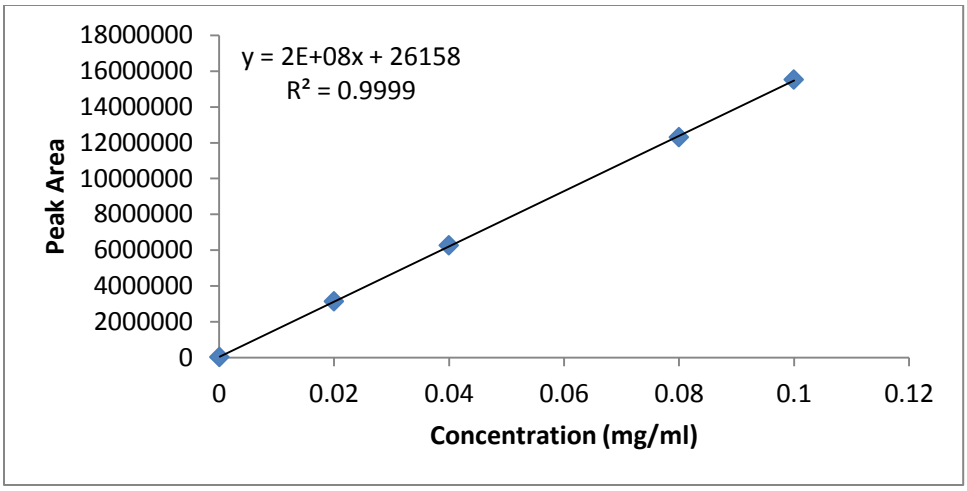

Figure 11. HPLC calibration curve generated for furfural using UV/Vis detector.

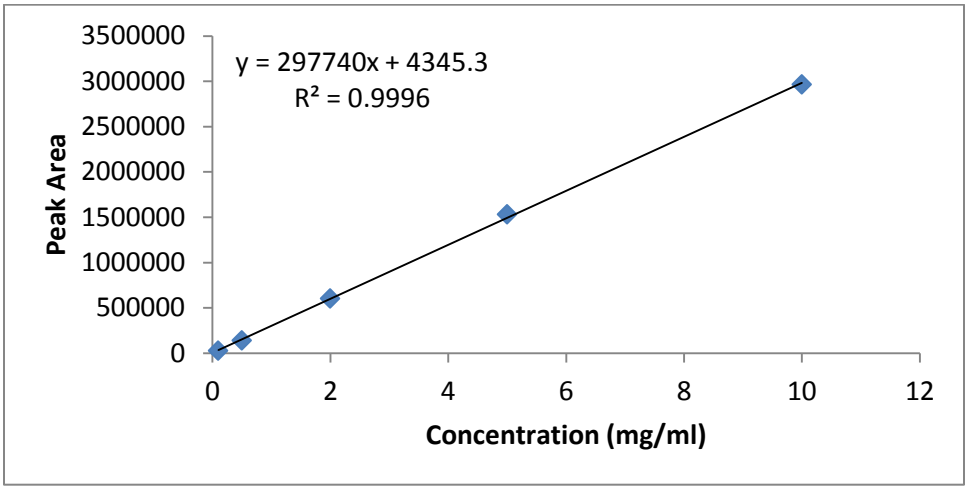

Figure 12. HPLC calibration curve generated for ethanol using RI detector.

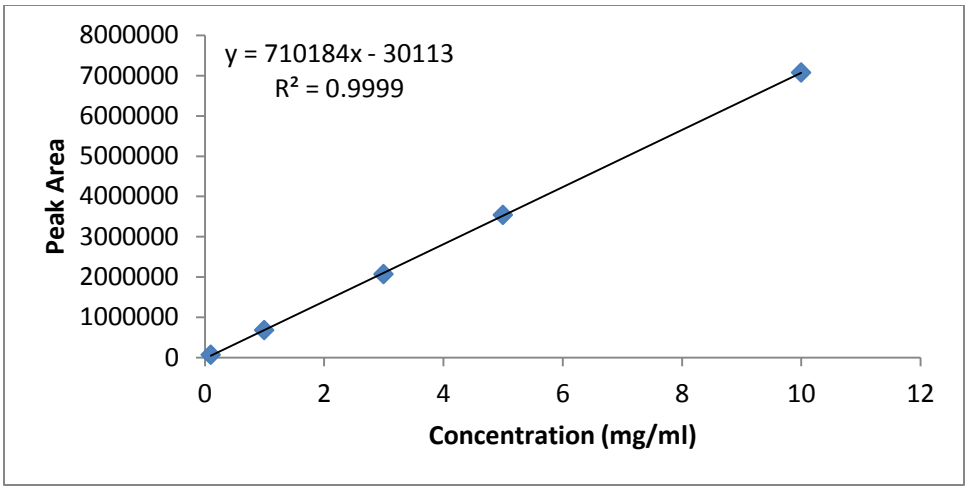

Figure 13. HPLC calibration curve generated for xylose using RI detector.

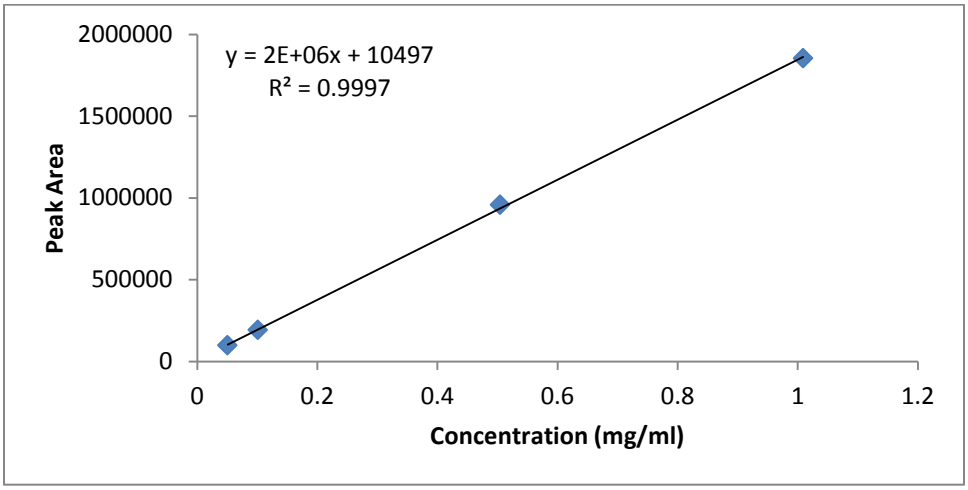

Figure 14. HPLC calibration curve generated for galactose using RI detector.

APPENDIX B.
CONTINUOUS FLOW REACTOR

Figure 1. Image of continuous flow reactor use for the carbonic acid hydrolysis of algal biomass

APENNDIX C.

PRODUCTION OF ALGAL BIOMASS UTILIZING FLUE GAS FROM COAL FIRED POWERPLANT

Algae used in this study were isolated from cultures grown in Chamois, MO. Cultures were grown in five large open ponds which were aerated and carbon dioxide was supplemented into the culture. The carbon dioxide was generated from the coal fired power plant on site, Central Electric Cooperative power plant. Figures 1-3 show the power plant, algae ponds, and harvested biomass.

Figure 1. Coal fired power plant, Central Electric Cooperative power plant Chamois, MO.

Figure 2. Algae ponds in Chamois MO.

Figure 3. Harvested algae biomass grown using flue gas.

REFERENCES

(1) Ghasemi, Y.; Rasoul-Amini, S.; Naseri, A. T.; Montazeri-Najafabady, N.; Mobasher, M. A.; Dabbagh, F. *Appl Biochem Microbiol* **2012**, *48*, 126.

(2) Nigam, P. S.; Singh, A. *Progress in Energy and Combustion Science* **2011**, *37*, 52.

(3) Ho, S.-H.; Chen, W.-M.; Chang, J.-S. *Bioresource Technology* **2010**, *101*, 8725.

(4) John, R. P.; Anisha, G. S.; Nampoothiri, K. M.; Pandey, A. *Bioresource Technology* **2011**, *102*, 186.

(5) Brennan, L.; Owende, P. *Renewable and sustainable energy reviews* **2010**, *14*, 557.

(6) Chiaramonti, D. In *Improvement of Crop Plants for Industrial End Uses*; Ranalli, P., Ed.; Springer Netherlands, 2007.

(7) Subhadra, B.; Edwards, M. *Energy Policy* **2010**, *38*, 4897.

(8) Donner, S. D.; Kucharik, C. J. *Proceedings of the National Academy of Sciences* **2008**, *105*, 4513.

(9) Fargione, J.; Hill, J.; Tilman, D.; Polasky, S.; Hawthorne, P. *Science* **2008**, *319*, 1235.

(10) Hill, J.; Polasky, S.; Nelson, E.; Tilman, D.; Huo, H.; Ludwig, L.; Neumann, J.; Zheng, H.; Bonta, D. *Proceedings of the National Academy of Sciences* **2009**.

(11) Landis, D. A.; Gardiner, M. M.; van der Werf, W.; Swinton, S. M. *Proceedings of the National Academy of Sciences* **2008**, *105*, 20552.

(12) Searchinger, T.; Heimlich, R.; Houghton, R. A.; Dong, F.; Elobeid, A.; Fabiosa, J.; Tokgoz, S.; Hayes, D.; Yu, T.-H. *Science* **2008**, *319*, 1238.

(13) Tilman, D.; Hill, J.; Lehman, C. *Science* **2006**, *314*, 1598.

(14) Kim, S.; Dale, B. E. *Biomass and Bioenergy* **2004**, *26*, 361.

(15) Mtui, G.; Nakamura, Y. *Biodegradation* **2005**, *16*, 493.

(16) Kádár, Z.; Szengyel, Z.; Réczey, K. *Industrial Crops and Products* **2004**, *20*, 103.

(17) Fan, Z.; South, C.; Lyford, K.; Munsie, J.; van Walsum, P.; Lynd, L. *Bioprocess Biosyst Eng* **2003**, *26*, 93.

(18) Lissens, G.; Klinke, H.; Verstraete, W.; Ahring, B.; Thomsen, A. B. *Journal of Chemical Technology & Biotechnology* **2004**, *79*, 889.

(19) Wen, Z.; Liao, W.; Chen, S. *Bioresource Technology* **2004**, *91*, 31.

(20) Harun, R.; Danquah, M. K.; Forde, G. M. *Journal of Chemical Technology & Biotechnology* **2010**, *85*, 199.

(21) Chen, C.-Y.; Yeh, K.-L.; Aisyah, R.; Lee, D.-J.; Chang, J.-S. *Bioresource Technology* **2011**, *102*, 71.

(22) Becker, E. W. *Biotechnology Advances* **2007**, *25*, 207.

(23) Chen, P.; Min, M.; Chen, Y.; Wang, L.; Li, Y.; Chen, Q.; Wang, C.; Wan, Y.; Wang, X.; Cheng, Y.; Deng, S.; Hennessy, K.; Lin, X.; Liu, Y.; Wang, Y.; Martinez, B.; Ruan, R. *International Journal of Agricultural and Biological Engineering* **2009**, *2*, 1.

(24) Waltz, E. *Nat Biotech* **2009**, *27*, 15.

(25) Packer, M. *Energy Policy* **2009**, *37*, 3428.

(26) Adams, J.; Gallagher, J.; Donnison, I. *J Appl Phycol* **2009**, *21*, 569.

(27) Matsumoto, M.; Yokouchi, H.; Suzuki, N.; Ohata, H.; Matsunaga, T. *Appl Biochem Biotechnol* **2003**, *105*, 247.

(28) Dismukes, G. C.; Carrieri, D.; Bennette, N.; Ananyev, G. M.; Posewitz, M. C. *Current Opinion in Biotechnology* **2008**, *19*, 235.

(29) Shilton, A. N.; Mara, D. D.; Craggs, R.; Powell, N. *Water Science & Technology* **2008**, *58*, 253.

(30) Sheehan, J. *Nat Biotech* **2009**, *27*, 1128.

(31) Brown, L. M. *Energy Conversion and Management* **1996**, *37*, 1363.

(32) Mata, T. M.; Martins, A. A.; Caetano, N. S. *Renewable and Sustainable Energy Reviews* **2010**, *14*, 217.

(33) Borowitzka, M. A. *Journal of Biotechnology* **2008**, *136*.

(34) Lee, Y.-K. *J Appl Phycol* **2001**, *13*, 307.

(35) Chisti, Y. *Biotechnology Advances* **2007**, *25*, 294.

(36) Ugwu, C. U.; Aoyagi, H.; Uchiyama, H. *Bioresource Technology* **2008**, *99*, 4021.

(37) Ho, S.-H.; Chen, C.-Y.; Chang, J.-S. *Bioresource Technology* **2012**, *113*, 244.

(38) Dragone, G.; Fernandes, B. D.; Abreu, A. P.; Vicente, A. A.; Teixeira, J. A. *Applied Energy* **2011**, *88*, 3331.

(39) Yeh, K.-L.; Chang, J.-S. *Biotechnology Journal* **2011**, *6*, 1358.

(40) Ho, S.-H.; Huang, S.-W.; Chen, C.-Y.; Hasunuma, T.; Kondo, A.; Chang, J.-S. *Bioresource Technology* **2013**, *135*, 191.

(41) Wargacki, A. J.; Leonard, E.; Win, M. N.; Regitsky, D. D.; Santos, C. N. S.; Kim, P. B.; Cooper, S. R.; Raisner, R. M.; Herman, A.; Sivitz, A. B.; Lakshmanaswamy, A.; Kashiyama, Y.; Baker, D.; Yoshikuni, Y. *Science* **2012**, *335*, 308.

(42) Rubin, E. M. *Nature* **2008**, *454*, 841.

(43) Demirbaş, A. *Energy Conversion and Management* **2001**, *42*, 1357.

(44) Bush, R. A.; Hall, K. M.; US6508768 B1, 2006.

(45) Lynd, L. R.; Weimer, P. J.; van Zyl, W. H.; Pretorius, I. S. *Microbiol Mol Biol Rev* **2002**, *66*, 506.

(46) Gírio, F. M.; Fonseca, C.; Carvalheiro, F.; Duarte, L. C.; Marques, S.; Bogel-Łukasik, R. *Bioresource Technology* **2010**, *101*, 4775.

(47) Moxley, G.; Zhang, Y. H. P. *Energy & Fuels* **2007**, *21*, 3684.

(48) Hunter, S. E.; Savage, P. E. *Industrial & Engineering Chemistry Research* **2003**, *42*, 290.

(49) van Walsu, G. P. *Appl Biochem Biotechnol* **2001**, *91-93*, 317.

(50) McWilliams, R.; van Walsum, G. P. *Appl Biochem Biotechnol* **2002**, *98-100*, 109.

(51) Peter van Walsum, G.; Shi, H. *Bioresource Technology* **2004**, *93*, 217.

(52) Peter Van Walsum, G.; Garcia-Gil, M.; Chen, S.-F.; Chambliss, K. *Appl Biochem Biotechnol* **2007**, *137-140*, 301.

(53) Rogalinski, T.; Liu, K.; Albrecht, T.; Brunner, G. *The Journal of Supercritical Fluids* **2008**, *46*, 335.

(54) Rogalinski, T.; Ingram, T.; Brunner, G. *The Journal of Supercritical Fluids* **2008**, *47*, 54.

(55) Schacht, C.; Zetzl, C.; Brunner, G. *The Journal of Supercritical Fluids* **2008**, *46*, 299.

(56) Morais, A. R. C.; da Costa Lopes, A. M.; Bogel-Łukasik, R. *Chemical Reviews* **2015**, *115*, 3.

(57) Xiang, Q.; Lee, Y. Y.; Pettersson, P. O.; Torget, R. W. In *Biotechnology for Fuels and Chemicals: The Twenty-Fourth Symposium*; Davison, B. H., Lee, J. W., Finkelstein, M., McMillan, J. D., Eds.; Humana Press: Totowa, NJ, 2003.

(58) van Putten, R.-J.; van der Waal, J. C.; de Jong, E.; Rasrendra, C. B.; Heeres, H. J.; de Vries, J. G. *Chemical Reviews* **2013**, *113*, 1499.

(59) Teng, H.; Yamasaki, A. *Journal of Chemical & Engineering Data* **1998**, *43*, 2.

(60) Wiebe, R.; Gaddy, V. L. *Journal of the American Chemical Society* **1940**, *62*, 815.

(61) Stewart, P. B.; Munjal, P. K. *Journal of Chemical & Engineering Data* **1970**, *15*, 67.

(62) Sabirzyanov, A. N.; Il'in, A. P.; Akhunov, A. R.; Gumerov, F. M. *High Temperature* **2002**, *40*, 203.

(63) Hunter, S. E.; Savage, P. E. *AIChE Journal* **2008**, *54*, 516.

VITA

Nicholas Dudenhoeffer was born in Jefferson City, Missouri on June 3, 1987. He received his Bachelor's degree in Biology with a minor in Chemistry from Lincoln University, Jefferson City, MO in 2010. Upon completion, Nicholas joined Missouri University of Science and Technology to purse his PhD degree in Chemistry in September 2010 and begun his doctoral studies under the direction of Dr. Paul Nam. He received his PhD in Chemistry from Missouri University of Science and Technology in May 2016.