CO-PRODUCTION OF FUMARIC ACID AND CHITIN USING *Rhizopus oryzae* FERMENTATION ON A NITROGEN-RICH AGRICULTURAL RESIDUE — DAIRY MANURE

By

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A dissertation submitted in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Department of Biological Systems Engineering

DECEMBER 2005

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of WEI LIAO find it satisfactory and recommend that it be accepted.

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ACKNOWLEDGMENT

Over the past four and half years, there are many people whose contributions and advices have made my graduate career one of the most valuable and enjoyable learning experiences of my life. First, I would like to take this opportunity to convey my sincere gratitude to my faculty advisor Dr. Shulin Chen who has taught me so much and given me tremendous support on the development and completion of this dissertation and other publications originating from this work. I would also like to express my appreciation to my committee members: Dr. Ron Kincaid, Dr. Brian He, and Dr. Armando McDonald for their helpful guidance, comments, and suggestions during the fulfillment of this dissertation.

Many thanks go to my fellow colleagues in our research group: Zhanyou Chi, Craig Frear, Dan Hardesty, Bo Hu, Chuanbin Liu, Simon Smith, and Zhiyou Wen for their valuable inputs, contributions, and friendship. Thanks are also extended to Naomi Calkins, Scott Economu, and Jonathan Lomber for their laboratory support.

My deepest gratitude goes to my parents as well. Their unconditional love and unending support gave me the strength to complete my Ph.D. program.

Finally, my most sincere thanks go to my lovely wife, Yan, and my adorable daughter, Clara. They helped me maintain a proper perspective about life by reminding me of things more important than the research.

CO-PRODUCTION OF FUMARIC ACID AND CHITIN USING *Rhizopus oryzae* FERMENTATION ON A NITROGEN-RICH AGRICULTURAL RESIDUE — DAIRY MANURE

Abstract

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December 2005

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Animal manure treatment remains a significant challenge to the United States livestock industries. As the traditional animal manure management practice of land application faces increasing environmental and regulatory scrutiny, the concept of using animal manure as a biomass for producing value-added products offers a potential alternative. Animal manure contains a variety of components including fiber, proteins/amino acids, and minerals. Such components are ideal feedstocks for producing value-added products by either biological or chemical conversion processes. The aim of this research was to develop a new process for the utilization of both nitrogen and carbohydrate sources in dairy manure to simultaneously produce two valuable chemicals: fumaric acid and chitin. The research included: 1) liquid/solid separation to obtain a nitrogen-rich manure liquid stream and carbohydrate-rich manure fiber stream, and their characterization; 2) investigation of effects of various hydrolysis methods including acid hydrolysis and enzymatic hydrolysis on manure fiber; 3) modeling enzymatic hydrolysis kinetics of differently treated manure fiber; 4) investigation of pellet formation for the

fungal strain – *Rhizopus oryzae*; and 5) co-production of fumaric acid and chitin from pre-treated dairy manure using a pelletized filamentous fungus — *Rhizopus oryzae*.

Several major conclusions have been obtained from the research: 1) nitrogen in dairy manure had a significant impact on the conversion of manure fiber into monosaccharides so that separation of the nitrogen-rich liquid stream must be carried out before the conversion process takes place; 2) enzymatic hydrolysis with dilute alkaline peroxide treatment was suggested to be the better pre-treatment method to complete the conversion of fiber to monosaccharides in terms of both technical and environmental concerns; 3) pelletized fungal biomass of the fumaric acid producing strain – *R. oryzae* 20344 was achieved using a simple and new culture method; 4) when compared to other nitrogen sources, manure liquid was shown to be a better nitrogen source for chitin accumulation during the fungal biomass cultivation step; and 5) a three-step fermentation process: pellet seed culture, biomass cultivation on liquid manure to produce the biomass and chitin, and fumaric acid production on the hydrolysate from manure fiber was found to be the optimal process to simultaneously produce the target chemicals of chitin and fumaric acid.

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Dedication

This dissertation is dedicated to my wife and my daughter

CHAPTER ONE

INTRODUCTION

1.1. Background

Finding an environmentally friendly method for disposal and utilization of animal manure is a significant challenge to the livestock industry. During the past decade, the United States livestock industry has undergone a substantial structural change featured with a rapid reduction in the number of animal operations with a corresponding increase in herd size on the remaining farms. These large, concentrated animal operations have created greater environmental concerns because of waste production at these facilities. An estimated 160 million tons of animal manure (dry basis) are produced annually in the United States (Council for Agricultural Science and Technology, 1995). Of this total approximately 55 million tons are collected for subsequent disposal. Nearly three-fourths of the manure comes from dairy and cattle feedlot. Currently, about 90% of collected manure is disposed through land application, small amount of manure are composted prior to disposal to reduce odors and stabilize organics. Because of excessive nutrient loading on the relatively limited amount of available land, direct land application can cause environmental problems such as stream and groundwater contamination from nitrate (Sutton et al., 1986), and air pollution from ammonia (Dewes et al., 1990) or nitrous oxide (Paul et al., 1993). Consequently, nutrient enrichment problems from excessive phosphorus and nitrogen in surface water have received extensive attention.

The increasing pollution control requirement for animal operations challenges the scientific community and the industry to develop new animal manure management

strategies. Recently, increasing attention has been given to the utilization of animal manure as a resource for producing bio-energy, as in the case of renewed interests in anaerobic digestion (AD) (Nielsen et al., 2004; Güngör-Demirci et al., 2004). With an energy content of about 13.4 MJ/kg (Klass, 1998), presently collected manures represent an annual renewable energy resource of approximately 7×10^{11} MJ. Meanwhile, animal manure contains large quantities of lignocellulosics, polysaccharides, proteins, and other biological materials. The possibilities to convert these materials into value-added products have been recognized as an attractive option. Innovative, environmentally friendly processes for converting manure to such renewable, bio-based high value chemicals are urgently needed. Such processes could potentially change manure from a disposal problem to an important biomass resource for chemical production. More effective utilization of manure could also assist the United States farmers in meeting environmental regulations and in developing a sustainable, economic livestock industry. The purpose of this research was to develop a process to utilize animal manure for simultaneously production of chitin and one of the most useful organic acids - fumaric acid using fungal fermentation.

1.2. Animal manure

There are three major types of manures in the United States livestock industries: poultry manure, swine manure, and manure from dairy and beef cattle. The characteristics of these three types of manure are presented in Table 1.1 (Wen et al., 2005). As data show, the fiber and crude protein contents varies among cattle, swine and poultry manures. It is apparent that the fiber (Neutral Detergent Fiber) accounts for a

large composition of cattle manure, whereas poultry manures have the highest protein content among the three types of manure. Such differences were attributed to the different diets fed to these animals and differences in their capability of digestion and utilization of the feed. These different compositions suggest that different treatments have to be applied to each type of manure in order to better utilize their nutrients.

This study focused on dairy manure, the largest portion among various animal wastes produced in the United States livestock industry. As Table 1.2 shows, dairy manure is rich in fiber and protein, accounting for 48% and 16% of the dry matter, respectively. The manure fiber is mainly composed by cellulose (22%), hemicellulose (12%) and lignin (14%). Cellulose and hemicellulose can be converted into monosaccharides such as glucose, xylose, arabinose, and galactose using various hydrolysis methods. Proteins in dairy manure include some undigested proteins, urea and a variety of amino acids. The detailed amino acids in dairy manure are presented in Table 1.3. Total amino acids account for about 8-10% of total protein in dairy manure. This means that dairy manure is not only a good fiber source but also a good nitrogen source. Thus, dairy manure provides a substantial resource to produce the useful chemicals of sugars and amino acids, which as intermediate substances, can be converted by microorganisms to more valuable chemicals such as organic acids and chitin.

1.3. Fumaric acid

Fumaric acid is a four-carbon unsaturated dicarboxylic acid. Its molecular formula is HOOCCHCHCOOH, and molecular weight is 116 g/mol. Fumaric acid is widely used as a acidulant and beverage ingredient in food industry. Due to its double

bond and two carboxylic groups, fumaric acid also has many other potential industrial applications, ranging from manufacture of synthetic resins and biodegradable polymers to production of intermediates for chemical syntheses (Tao et al., 1993). As an intermediate of the metabolic tricarboxylic acid (TCA) cycle, fumaric acid is often found as a metabolic product produced by microorganisms, which means that it could be produced by microorganism fermentation. However, currently most of the US production of fumaric acid is derived from petroleum-based materials. Only 15~20% of the used fumaric acid was produced by fermentation. It is apparent that there is a significant market opportunity for the development of bio-based fumaric acid production (Wise, 1983).

1.4. Chitin

Chitin is a polysaccharide, and is speculated to be the second most abundant biopolymer in the biosphere. The structure of chitin is a linear polysaccharide made up of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranosyl units where each individual residue is N-acetyl-D-glucosamine (Fig. 1.1) (Khor, 2001). The structural characters of chitin make it a useful biopolymer as coagulating agents in water treatment, plant seed coating agents in agriculture, biomaterials (e.g. absorbable sutures) in biomedical industry (Yusof et al., 2001). Chitin is widely distributed in the animal and plant kingdom, such as the shells of crustaceans and mollusks, algae, fungi (Muzzarelli, 1977). Traditionally, chitin is commercially produced from animal sources of shellfish and crab, which has some non-economic viabilities such as not from reusable sources, limitation on biomedical usage caused by residual proteins and trace minerals that have some side effects (Khor, 2001).

Producing chitin from fungi can avoid such problems. Moreover, the chitin content of fungi can be as high as 10% to 90% (Carlile, 2001). Therefore, chitin production from fungal sources using fermentation process has recently attracted increasingly attention in the related industry.

1.5. The strain of *Rhizopus oryzae* and its metabolism

Many species of mycelial fungi produce certain amount of citric, fumaric, malic, succinic, and other organic acids as metabolic by-products during the metabolism. Some mycelial fungi are highly productive in synthesizing fumaric acid (Romano et al., 1967; Kenealy et al., 1986). The genus *Rhizopus* within the order *Mucorales* is the most productive microorganisms for fumaric acid production (Foster et al., 1939; Rhodes et al., 1962; Osmani et al., 1985; Peleg et al, 1989; Werpy et al., 2004).

The metabolic pathway for fumaric acid synthesis is showed in Fig.1.2. Fumaric acid is an intermediate of the TCA cycle. The oxidative pathway of the TCA cycle will generate one mole of fumarate per mole of glucose consumed. During active fungal cell growth, however, this pathway cannot lead to a significant accumulation of fumarate. Fumarate generated in the TCA cycle is mainly utilized for the biosynthesis of cell constituents. Early research effort has shown that the production of fumaric acid is possible using the reductive branch of the TCA cycle within fumaric acid accumulation (Kenealy et al., 1986). The carbon dioxide fixing reductive branch is capable of producing two moles of fumarate per mole of glucose consumed as given in the follow reaction:

$$C_6H_{12}O_6+2CO_2 \Rightarrow 2HOOCCH=CHCOOH+2H_2O$$

The enzyme responsible for CO_2-fixation and fumarate accumulation in *Rhizopus* is pyruvate carboxylase, which is mainly localized in the cytoplasm (Osmani et al., 1985; Peleg et al., 1989). This enzyme catalyzes the ATP-dependent condensation of pyruvate and CO_2 to from oxaloacetic acid. Other enzymes involved in fumaric acid accumulation include fumarase and malate dehydrogenase. Both enzymes are also found in cytosol (Kenealy et al., 1986). Kenealy et al. (1986) studied the effect of inhibitors on fumarate accumulation and indicated that mitochondria are probably not involved in fumarate accumulation. The accumulation of fumarate from glucose by *Rhizopus* is believed to operate entirely through the cytosolic pathway. The cytosolic pathway is induced in the mycelial fungi under conditions of nitrogen limitation (Peleg et al., 1989; Kenealy et al., 1986). This means that by controlling the ratio of carbon to nitrogen, fungal cell growth can be directed to produce either fungal biomass or fumaric acid.

Meanwhile, as mentioned earlier, fungal biomass is rich in chitin content. *Rhizopus oryzae* (ATCC 20433) was used as the strain to simultaneously produce both fumaric acid and chitin.

1.6. Process strategy

Since dairy manure is rich in fiber and proteins and the growth of *Rhizopus oryzae* is limited by nitrogen, the strategy of the process is to first use nitrogen source in dairy manure to cultivate the fungal biomass in order to obtain one of the target products, chitin, furthermore, apply the sugar solution from fiber on the fungal biomass to produce fumaric acid. The process includes two main sections: pretreatment and fermentation (Fig.1.3).

1.6.1. Pretreatment

Prior to the fungal fermentation, pretreatments of dairy manure are needed in order to obtain both nitrogen and sugar streams. The nitrogen distribution data of dairy manure demonstrate that 50% of nitrogen in manure was soluble (Table 1.4). This means that the liquid-solid separation would be an effective method to separate the most of nitrogen from manure. The separation is also beneficial for minimizing negative effects of nitrogen on the conversion of manure fiber into monosaccharides such as some side reactions between proteins and carbohydrates (Zerbe et al., 1987; Nguyen, 1988).

As for pretreatment of manure fiber, aqueous phase catalytic processing techniques are widely used to convert carbohydrate components into intermediate monosaccharides that supply the main carbon source to the fungal fermentation. Various methods have been studied to hydrolyze lignocellulosic materials into individual sugars, including acid hydrolysis, enzymatic hydrolysis with proper pretreatments, and combinations of acid and enzymatic hydrolyses (Bhat et al., 1997; Bayat-makooi et al., 1985; Grohmann et al., 1985; Moore et al., 1972; Sun et al., 2002).

Acid hydrolysis is widely used to treat lignocellulosic materials in the pulp and paper industry. Acid hydrolysis cleaves the matrix structure of fiber into the components of cellulose, hemicellulose and lignin. Acid hydrolysis further reduces these polysaccharides to monosaccharides. The application commonly uses concentrated acid hydrolysis at low temperatures or dilute acid hydrolysis at high temperatures. Compared with concentrated acid hydrolysis, dilute acid hydrolysis with high temperature has the advantage of equivalent sugar yield while producing relatively small amount of by-

products (such as furfural and 5-hydroxylmethyl-2-furaldehyde or HMF). Dilute acid hydrolysis also eliminates the chemical hazards and potential environmental problems that are concerns commonly associated with the use of concentrated acid hydrolysis (Zerbe et al., 1987). Lee et al. (1997) reported that a temperature greater than 140°C, with dilute acid concentration varying from 0.5-2%, is best for hydrolysis of lignocellulosic materials.

Enzymatic hydrolysis has attracted increased attention as an alternative to acid hydrolysis for degrading lignocellulosic materials because it is highly specific, it has mild reaction conditions (pH around 5 and temperature less than 50°C) and it does not create corrosion problems (Sun et al., 2002). Cellulase is used to carry out this task. A typical cellulase consists of an endo-1,4-β-D-glucanase (E.C.3.2.1.4), an exo-1,4-β-D-glucanase (E.C. 3.2.1.91) and a β-glucosidase (E.C.3.2.1.21). The endo-glucanase cleaves cellulose to create free chain-ends, the exo-glucanase degrades the molecule by removing cellobiose from the free chain-end, and the β-glucosidase produces glucose by breaking down the cellobiose (Bhat et al., 1997). In lignocellulosic materials the matrix of hemicellulose and lignin around cellulose functions to prevent cellulase from attacking cellulose. Thus, various pretreatment must be used to fragment the matrix structure or remove hemicellulose and lignin in order to explore cellulose to the enzyme. There are a number of methods that are capable of changing the fiber structure (Sun et al., 2002). Sodium chlorite is one of the most effective reagents for the removal of lignin from lignocellulosic materials and is used by the pulp and paper industry to carry out deligninfication and bleaching (Ahlgren et al., 1971). Dilute acid treatment can completely remove hemicellulose with a limited loss of cellulose (Roberto et al., 2003).

Additionally, dilute alkaline peroxide treatment has been used as an effective and environmental friendly method that can break down the fiber structure and partially remove lignin to make cellulose accessible to enzymes (Sun et al., 2002; Curreli et al., 1997). Thus, chlorite treatment, dilute acid treatment, and dilute alkaline peroxide treatment were investigated in this study to treat manure fiber for enzymatic hydrolysis.

1.6.2. Fermentation

Since the fungal fermentation for fumaric acid production is limited by nitrogen, most of fumaric acid fermentation processes consist of three steps: 1) seed culture; 2) fungal biomass cultivation with nitrogen; 3) fumaric acid production with limited nitrogen (Kenealy et al., 1986; Zhou et al., 2002; Romano et al., 1967). According to the characteristics of dairy manure, the process of fumaric acid production is quite suitable to simultaneously produce fumaric acid and chitin using both nitrogen-rich manure liquid and sugar-rich hydrolysate from manure fiber. The manure liquid as a nitrogen source can be used to cultivate fungal biomass and further produce chitin. The hydrolysate can be used as the carbon source for fumaric acid production.

Meanwhile, fungal fermentation for organic acid production has some challenges such as the formation of cotton-like mycelia. The mycelia associated mass transfer and oxygen transfer difficulties cause reactor control concerns as well as the impossibility for fungal biomass reuse. These factors ultimately lead to a low efficiency and yield of organic acid in the fermentation process (Yin et al., 1998). Growing fungi in pellet form, however, can significantly improve the mass transfer condition and reactor performance,

and consequently benefit organic acid production. Thus, the pellet formation of the fungus *Rhizopus oryzae* ATCC 20344 was studied in this research as well.

1.7. Objectives of our research

The overall objective of this research was to develop a novel process for the utilization of both nitrogen stream and carbohydrate stream in a nitrogen-rich cellulosic material—dairy manure to produce value-added chemicals of fumaric acid and chitin. The process included solid-liquid separation, conversion of manure fiber to monosaccharides, and co-production of chitin and fumaric acid (Fig.1.3). Specific research objective included:

1). Studying effects of acid treatment on the components of dairy manure.

2). Optimizing acid hydrolysis of fiber from a nitrogen-rich cellulosic material dairy manure.

3). Studying the effects of hemicellulose, lignin on enzymatic hydrolysis of cellulose from dairy manure.

5). Modeling enzymatic hydrolysis kinetics of cellulose in differently pretreated fibers from dairy manure.

6). Developing a new approach of pellet formation of a filamentous fungus *Rhizopus oryzae.*

7). Co-producing fumaric acid and chitin from dairy manure using fungal fermentation.

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Table 1.1. Comparison of fiber and protein contents in different manures

Sample	Cattle			Swine		Poultry	
	Feedlot	Beef	Dairy	Nursery	Finisher	Chick starter	Pullet grower
Dry matter, %	26.6	23.6	13.4	25.9	27.3	24.5	24.0
Crude protein, % dry basis	17.0	12.1	18.1	25.1	22.0	39.8	48.4
Total fiber[a], % dry basis	41.7	51.5	52.6	39.2	39.1	31.7	36.4

a: total fiber = hemicellulose + cellulose + lignin

Table 1.2. Characteristics of dairy manure[a]

	Dairy manure
Dry Matter, %	15.50 ± 0.09
Crude Protein, % dry matter	16.44 ± 0.53
Neutral detergent fiber (NDF), % dry matter	48.27 ± 0.46
Acid detergent fiber (ADF), % dry matter	35.80 ± 0.14
Acid detergent lignin (ADL), % dry matter	13.91 ± 0.45
Cellulose (=ADF-ADL), % dry matter	21.89 ± 0.38
Hemicellulose(=NDF-ADF), % dry matter	12.47 ± 0.32
N, % dry matter	2.63 ± 0.09
C, % dry matter	45.49 ± 0.30

a: all data are the average of triplicates with standard deviations of the means (n=3)

Table 1.3. Amino acids in dairy manure

	Dairy manure
Taurine, % dry basis	0.06
Aspartic Acid, % dry basis	0.23
Serine, % dry basis	0.10
Proline, % dry basis	0.12
Glycine, % dry basis	0.13
Cysteine, % dry basis	0.07
Methionine, % dry basis	0.05
Leucine, % dry basis	0.21
Phenylalanine, % dry basis	0.13
Histidine, % dry basis	0.05
Lysine, % dry basis	0.12
Tryptophan, % dry basis	<0.04
Hydroxyproline, % dry basis	0.04
Threonine, % dry basis	0.11
Glutamic Acid, % dry basis	0.38
Lanthionine, % dry basis	0
Alanine, % dry basis	0.17
Valine, % dry basis	0.15
Isoleucine, % dry basis	0.21
Tyrosine, % dry basis	0.05
Hydroxylysine, % dry basis	0
Ornithine, % dry basis	0
Arginine, % dry basis	0.14
Total (g /100g dry manure)	2.43

Table 1.4. Percent distribution of nitrogen and carbon in dairy manure

	Nitrogen	Carbon
Liquid part, % total nitrogen or carbon	48.29	29.54
Solid part, % total nitrogen or carbon	51.71	70.46
Total(%)	100.00	100.00

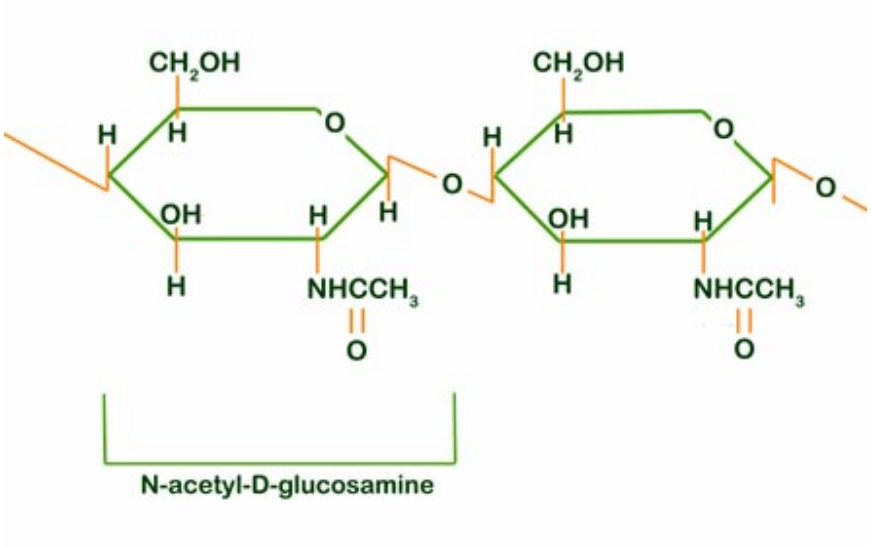

Fig 1.1. The structure of chitin

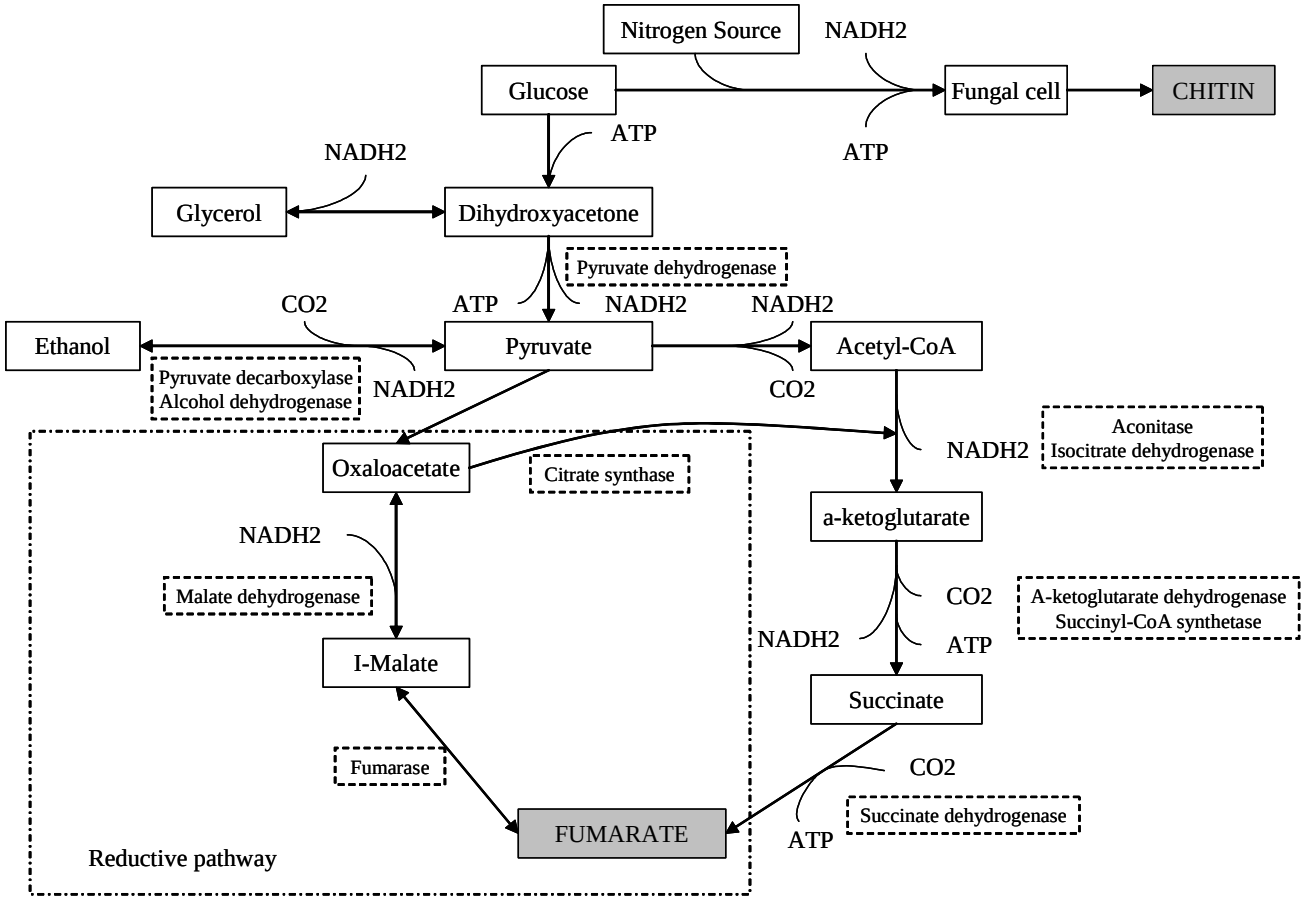

Fig.1.2. Metabolic pathways of fumaric acid and chitin production

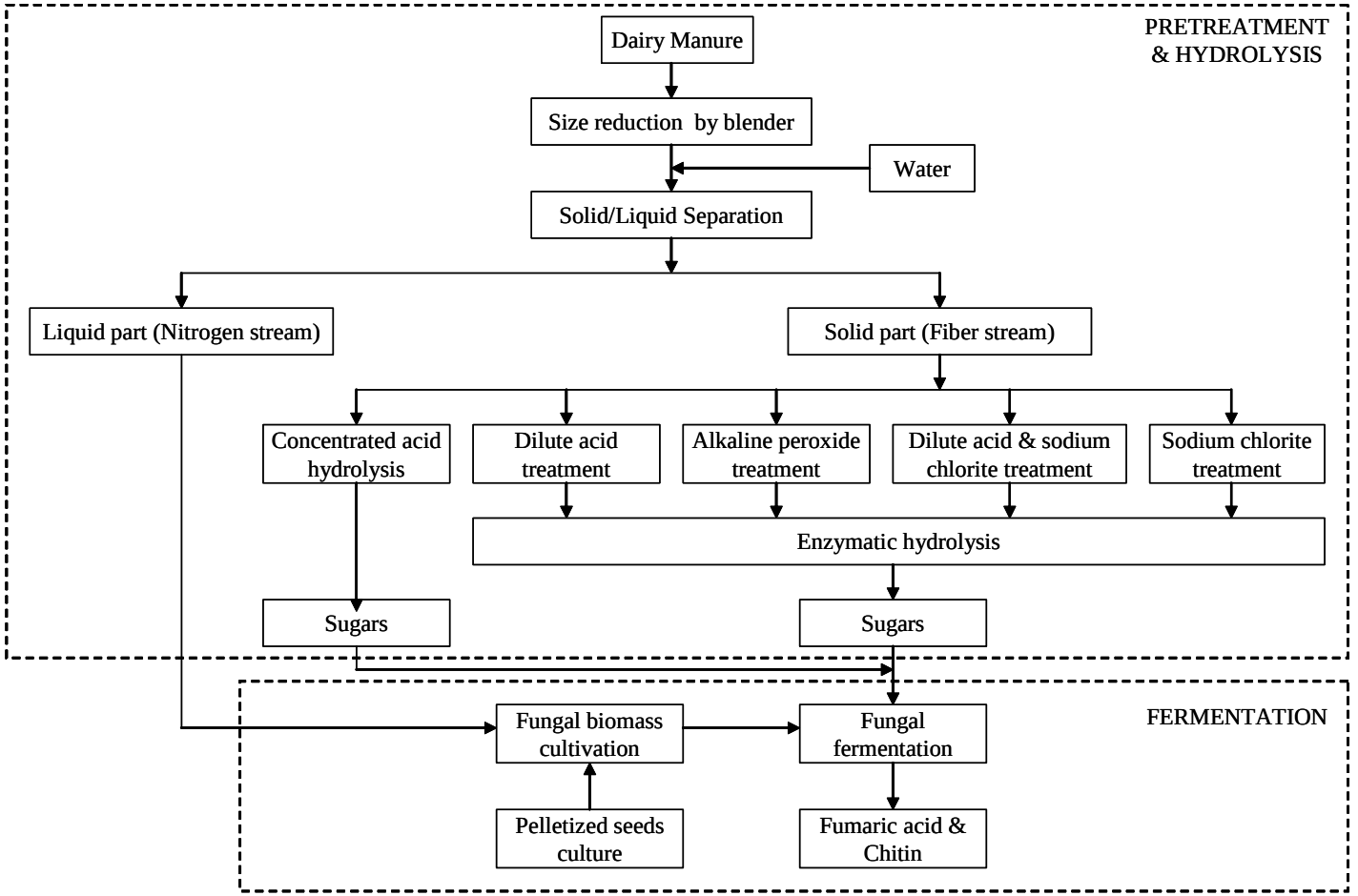

Fig. 1.3. Schematic flowchart of process of dairy manure utilization

CHAPTER TWO

EFFECTS OF DILUTE ACID TREATMENT ON COMPONENTS OF DAIRY MANURE

2.1. Abstract

Effects of dilute acid treatment on the main components of dairy manure were studied in order to better understand the behavior of each component during the treatment as well as further optimizing accumulation of cellulose, which could be later enzymatically hydrolyzed to produce glucose. A 2^3 full factorial design was adopted to investigate the effects of the reaction conditions including time, temperature and acid concentration on each individual component, further followed by a 3-factor central composite design that was used to obtain the optimal conditions for cellulose accumulation. The results indicated that acid concentration was the most important factor for the changes of all the components. The results also indicated that other two individual factors of reaction time and temperature, and the interactions among three factors also had significant influences on the changes. In addition, the optimal conditions for cellulose accumulation were 2.8 hours of reaction time, 140°C of reaction temperature and 1.0% of acid concentration. Under these conditions cellulose reached 32%, and hemicellulose, lignin and nitrogen were 3.15%, 20.8% and 2.35%, respectively.

Key words: cellulose, central composite design (CCD), dairy Manure, dilute acid treatment, full factorial design, hemicellulose, lignin, nitrogen

2.2. Introduction

Dairy manure contains 22% cellulose (w/w, all percentages used in this paper were based on weight), 12% hemicellulose, 13% lignin and 16% crude protein (Table 2.1). This represents a large potential source of carbohydrates and proteins that can be converted into useable intermediates of monosaccharides and amino acids that are widely used by the chemical industry to produce high-value products such as organic acids, sugar alcohol, etc. Dilute acid pretreatment followed by enzymatic hydrolysis is the typical process used to convert lignocellulosic material to carbohydrates and sugars (Iranmahboob et al., 2002; Torget et al., 1990; Torget et al., 1991a; Torget et al., 1991b; Torget et al., 1992). Previous study demonstrated that dilute acid treatment was able to completely hydrolyze manure hemicellulose (Liao et al., 2004) and provide cellulose that undergoes structure changes, which is more easily attacked by enzyme in the subsequent step of hydrolysis (Vlasenko et al., 1997; Liao et al., 2005). However, there was little information available on what changes and to what degree the changes occurred to other manure components such as cellulose, lignin and nitrogen during dilute acid pretreatment.

Each individual component has a different reaction to acid that may affect its physical and chemical properties. Cellulose is a homogeneous polymer of glucose connected by a beta-1,4-glycosidic bond. Cellulose polymers are associated via H-bonds to form a crystal structure. The structure makes cellulose strong and resistant to attack. Thus, severe conditions are required to break down H-bonds and glycosidic linkages and further depolymerize cellulose, such as concentrated acid, or long reaction time and high temperature (Sjostrom, 1993). Hemicellulose is a branched heterogeneous polymer

consisting of xylan, araban, galactan and mannan, which has a random, amorphous structure with little strength (Gatenholm et al., 2004). Therefore, it is relatively easily hydrolyzed by acid. It has been reported that 120°C of reaction temperature and 2% of sulfuric acid are able to completely remove hemicelluloses (Liao et al., 2004). Lignin is a 3-dimensional polymer comprising of phenylpropanoid units. Lignins are very sensitive to mineral acids such as sulfuric acid and hydrochloric acid, even under quite mild treatments (Sjostrom, 1993). Two reactions occur simultaneously during acid treatment: accumulation (condensation) and degradation (acidolysis) (Pearl, 1967). When reactions approach more severe conditions such as high temperature, long reaction time and concentrated acid, lignin-acid reaction will lean more toward degradation than accumulation (Sarkanen et al., 1971). Proteins in dairy manure are mainly in the form of indigestible forage proteins and proteins from the metabolism of rumen bacteria (Committee on Animal Nutrition Board on Agriculture National Research Council, 1983). Degree of protein degradation is increased following the increase of severity of reaction conditions. For example, under conditions of 6 M HCl and 24 hours, proteins can be completely hydrolyzed to soluble peptides and amino acids. It is apparent that each individual component in dairy manure has a different sensitivity to acid and consequently behaves differently during acid treatment. Therefore, a better understanding of their behaviors could guide the optimization of dilute acid treatment of dairy manure in order to accumulate or degrade target components.

A full factorial design as one of the most popular statistical experimental designs can be used to study the influences of factors on manure components of interest. The design investigates all of the possible factor combinations in order to elucidate the effects

of them and further identify the most important ones among them. The Response Surface Method (RSM) is another important experimental design that is mainly fulfilled to find improved or optimal process settings. It has the advantages of rotatability and is less complex in terms of optimization compared to full factorial designs (Haaland, 1989; Hogg, 1987). Thus, in this particular case, a 2^3 full factorial design was first adopted to study the effects of reaction factors on each individual component of dairy manure, a 3-factor central composite circumscribed design was then used to obtain the optimal conditions for cellulose accumulation.

The specific objectives of this study were statistically: (1) investigating the effects of reaction temperature, reaction time and acid concentration on cellulose, hemicellulose, lignin and nitrogen in manure and (2) determining the optimal conditions of accumulation of cellulose that could be utilized by enzymatic hydrolysis to produce sugars.

2.3. Materials and methods

2.3.1. Material and preparation

Fresh dairy manure was obtained from the Dairy and Beef Centers of Washington State University. The manure was composed of 15.3% dry matter (DM) with a total carbon content of 46.9g/100g DM and total nitrogen content of 2.6g/100g DM. The raw manure sample was made by mixing 10 kg of fresh dairy manure with 5 kg of water and then blending for 1 minute to achieve size reduction. The sample was used to study the effects of dilute acid on components of dairy manure. The data for the raw manure is shown in Table 2.1.

2.3.2. Experimental design

Each sample used for treatment contained 2.50 grams of dry raw manure mixed with 47.50 grams of acid solution. After hydrolysis, each sample was filtered through Whatman No. 5 filter paper to separate the liquid from the solid. The fiber analysis was conducted on the solid part.

2.3.2.1. Effects of temperature, reaction time, and acid concentration

The study on effects of temperature, reaction time and acid concentration were first carried out by a 2^3 full factorial design with six center points, which led to fourteen experiment runs. Two acid concentrations (1% and 3%) involving two durations (1 hour and 3 hours) were studied at two different temperatures (110°C and 130°C) on raw manure. The design matrix with both codes and real values of factors is presented in Table 2.2. The response was the content of each component in dry raw manure.

2.3.2.2. Optimization of cellulose accumulation

A 3^2 central composite circumscribed design with five coded levels was used to develop a mathematical model that was able to predict the cellulose accumulation as a function of acid concentration, reaction time and temperature. The design matrix with both codes and real values of factors is presented in Table 2.4 (Haaland, 1989). The response was the cellulose content in dry treated sample. Optimal conditions of accumulating cellulose were obtained from the model. Then, a verification run was conducted at the optimal conditions to ensure the accuracy of the predicted result.

The model for predicting cellulose accumulation was expressed as Eq. (1):

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (1)$$

Where Y represents cellulose content in dry sample, a_0 is the interception coefficient, a_1, a_2 and a_3 are coefficients for the linear terms, a_{12}, a_{13}, a_{23}, a_{11}, a_{22} and a_{33} are coefficients for the quadratic terms, and X_1, X_2 and X_3 represent the variables of reaction time, temperature, and acid concentration, respectively.

2.3.3. Statistical analysis

The effect of factors on each individual component of manure were compared using the special property of factorial designs whose effects can be simply estimated by the differences in average response values between the high and low codes of each factor. A ranked list that presented the relative importance among factors was formed by the comparison. The list is generally given by the left-to-right order of the spikes in the Pareto chart (Haaland, 1989). In addition, an ANOVA table was created to draw a vertical line on the chart so that factors on the left side of the line, which are statistically named as important factors, have significant influences ($P<0.05$) on the response.

Cellulose accumulation from central composite design was analyzed by multiple regression analysis using SAS 6.1 (SAS Institute Inc. NC). ANOVA table and R-square of the models were obtained from analyses, which were used to evaluate the model. Finally, the optimal conditions for cellulose accumulation were concluded from the model equation using Matlab 6.1 (Mathworks, Inc. MA).

2.3.4. Analytical methods

The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) of samples were analyzed using Van Soest Fiber Analysis System (Goering et al., 1970). NDF was used to estimate total cellulosic materials (cellulose, hemicellulose, lignin, and insoluble ash) while ADF was used to estimate the concentration of lignin and cellulose. Hemicellulose can be determined by the difference (%NDF-%ADF). Carbon and nitrogen contents in solid samples were measured by automated combustion techniques. A LECO CNS-2000 (LECO, MI) was used to measure the total carbon and total nitrogen of manure samples.

2.4. Results and discussion

2.4.1. Characteristics of dairy manure

As Table 2.1 denoted, the main components of dairy manure are cellulose, lignin, hemicellulose and protein. Cellulose was the largest component, containing 23% of dry matter. Lignin, hemicellulose and protein contents were 12%, 11% and 18%, respectively. Cellulose, hemicellulose and lignin in the manure and other lignocellulosic materials were connected to each other by chemical bonds, such as ether/ester bond and glycosidic bond, forming a very stable matrix structure (Sjostrom, 1993). The matrix structure is the major obstacle to further degrading them to produce monosaccharides. In order to convert cellulose and hemicellulose into monosaccharides, the structure needs to be broken down. Thus, dilute sulfuric acid as one of the most effective reagents is widely

applied by industry to accomplish the pretreatment (Torget et al., 1990; Torget et al., 1991a; Torget et al., 1991b; Torget et al., 1992).

2.4.2. Effects on manure components

2.4.2.1. Effects on cellulose

The change of cellulose under different reaction conditions is presented in Table 2.3. Cellulose contents were decreased from low to high level of acid concentration, reaction time, and temperature. ANOVA analysis elucidated that most of factors and their interactions, except temperature*acid interaction and time*acid interaction, were significant ($P<0.05$) on cellulose changes (Table 2.5). Meanwhile, Fig. 2.1a demonstrates that acid concentration had the greatest influence (about 50% of total effect) on cellulose, followed by temperature, time, time*temperature interaction, and time*temperature*acid interaction. Since acid concentration and temperature together have more than 70% of total effect, the major change of cellulose content could be obtained by simply adjusting these two factors.

2.4.2.2. Effects on hemicellulose

The change of hemicellulose during the treatment was similar to that of cellulose. Hemicellulose degraded once each factor changed from low to high level (Table 2.3). The results also indicated that hemicellulose was completely degraded by acid once reaction conditions became sufficiently severe, which is consistent with previous reports that hemicellulose is the only part in plant fiber that is completely hydrolyzed by dilute acid (Nguyen et al., 1998; Roberto et al., 2003). Statistical analysis demonstrates that all

of the factors and their interactions had significant influences ($P<0.05$) on hemicellulose degradation (Table 2.5), and acid concentration had the largest influence (Fig. 2.1b). In addition, time*temperature*acid interaction and temperature*acid interaction showed relatively larger effects than two individual factors of temperature and time (Fig. 2.1b). This means that time and temperature had less impact than some of their combinations with acid concentration (time*temperature*acid and temperature*acid) on hemicellulose degradation. Thus, in terms of maximizing the effects of treatment on hemicellulose, acid concentration, reaction time and temperature have to be changed simultaneously.

2.4.2.3. Effects on lignin

Unlike the changes of hemicellulose and cellulose, lignin content increased as conditions were changed from low to high level (Table 2.3). This means that lignin accumulation was greater than lignin degradation during dilute acid treatment. In other words, lignin condensation reaction with other components in manure was the main lignin-acid reaction since the reaction conditions used in this particular case were relatively moderate (Sarkanen et al., 1971). Meanwhile, based on statistical analysis, effects of acid, reaction temperature and time were significant ($P<0.05$) on lignin accumulation, and there were no significant interactions ($P>0.05$) among factors (Table 2.5). In addition, the Pareto chart of effects demonstrated the impact of the different factors on the reaction. Acid concentration was the most important one, temperature was second, and reaction time had the least impact on the reaction (Fig. 2.1c).

2.4.2.4. Effects on nitrogen

Nitrogen content kept decreasing with an increase of acid, reaction temperature and time (Table 2.3). Statistical analysis indicated that each individual factor of acid, reaction temperature and time showed significant (P>0.05) effect on the changes of nitrogen during the treatment, and there were also no significant interactions (P>0.05) among factors (Table 2.5). Acid concentration, once again, was the main factor affecting nitrogen degradation, and temperature showed more influence than reaction time (Fig. 2.1d).

2.4.3. Optimization of cellulose production

2.4.3.1. Mathematical model

Experimental data for cellulose accumulation are presented in Table 2.4. Multiple regression analysis was performed to fit the response function with the data. ANOVA table was used to evaluate the statistical significance of the model (Table 2.6). The R-square of the model was 0.98, which indicated that observations fell very well on the fitted regression surface.

Eq. (2) represented the content of cellulose as a function of acid concentration, reaction time and reaction temperature.

$$Y_{cellulose} = 2.62 - 1.05X_1 + 0.35X_2 + 8.90X_3 + 3.21\times10^{-2}X_1X_2 + 0.20X_1X_3 - 4.64\times10^{-2}X_2X_3 - 0.66{X_1}^2 - 1.30\times10^{-2}{X_2}^2 - 1.50{X_3}^2 \quad (2)$$

Where Y represents cellulose per dry sample, X_1 is reaction time, X_2 is reaction temperature, X_3 is acid concentration.

The response surface is shown in Fig. 2.2 that demonstrates the behaviors of cellulose accumulation during the reaction. Cellulose gradually increased following the

increase of acid concentration, and then quickly leveled off as acid concentration further increased (Fig. 2.2a). Raising temperature at low acid concentration and lowering temperature at high acid concentration were beneficial for increasing cellulose content (Fig. 2.2b). In addition, cellulose slightly increased as the temperature increased and the reaction progressed at each individual level of acid concentration (Fig. 2.2c). These behaviors can be chemically explained since components of hemicellulose and crude protein are relatively easier to be degraded than cellulose and lignin at low acid concentration. The degradation of hemicellulose and crude proteins at low acid concentration led to cellulose accumulation at the beginning of the treatment. Once acid approached higher levels, the rate of cellulose degradation increased to greater than that of cellulose accumulation since the breakdown of β-1-4-D-glycosidic bonds in cellulose is easier the greater the acid concentration is (Bayat-makooi et al., 1985), which causes the rapid decrease of cellulose content in treatments with high acid concentration.

2.4.3.2. Optimization

One of the most important tasks of the model was to find optimal conditions of cellulose accumulation in order to produce glucose in the next step of enzymatic hydrolysis. The optimization was conducted using the optimization toolbox of Matlab 6.1 based on the model equation (Eq. 2). The optimal values of the three factors are listed in Table 7. Under the optimal conditions, the model predicted cellulose content of up to 31.9% dry matter (Table 2.8). Compared to the composition of raw manure, around 30.0% cellulose was increased during dilute acid treatment.

A verification experiment was carried out to confirm the optimal conditions. Triplicate runs were conducted under the optimal conditions. The experimental data of cellulose was 30.9% (Table 2.8). The results presented that the model fitted ($P>0.05$) the experimental data well. Thus, the optimal conditions obtained from the central composite experimental design were valid. Meanwhile, the content of hemicellulose, lignin and nitrogen from the verification experiment were 3.3%, 21.3% and 2.3%, respectively (Table 2.8). Previous study elucidated that fiber with less hemicellulose from high temperature acid treatment can significantly increase glucose yield during following enzymatic hydrolysis (Liao et al., 2005) even though the fiber still contained a large amount of lignin which has been widely reported as an inhibitor to enzyme (Sewalt et al., 1997). This means that the optimal conditions concluded from the model were suitable to not only accumulate cellulose but also make it more hydrolysable by enzymes.

2.5. Conclusion

Each individual component of dairy manure behaved differently during dilute acid treatment. Cellulose, hemicellulose, and nitrogen contents were decreased following an increase of acid concentration, reaction temperature and time. Lignin continued to increase once reaction conditions became severe in the experiment. Among the three factors of dilute acid treatment, acid concentration was statistically indicated to be the most influential factor on all components; and the importance of other factors and their interactions were varied among different components. Meanwhile, optimal conditions for cellulose accumulation were reaction time of 2.80 hours, temperature of 140°C and acid concentration of 1.00%. Under these conditions, the corresponding contents of individual

components were 30.9% of cellulose, 3.3% of hemicellulose, 21.3% of lignin and 2.3% of nitrogen, respectively. The results demonstrate that dairy manure with proper dilute acid treatment could provide a substantial cellulose resource for the next step of enzymatic hydrolysis to produce glucose.

2.6. References

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Table 2.1. Characteristics of sample manure[a]

	Raw manure
Dry Matter, %	13.42 ± 0.10
Crude Protein, % dry matter	18.11 ± 0.27
Crude Fiber (NDF), % dry matter	45.89 ± 0.35
Cellulose (=ADF-ADL), % dry matter	23.13 ± 0.26
Hemicellulose(=NDF-ADF), % dry matter	10.64 ± 0.10
Lignin (ADL), % dry matter	12.11 ± 0.19
N, % dry matter	2.90 ± 0.045
C, % dry matter	45.35 ± 0.52

a: all data are the average of triplicates with standard deviations of the means (n=3)

Table 2.2. 2^3 full factorial design[a]

Run	Factors		
	Time(hrs)	Temperature(°C)	Acid Concentration (%)
1	1 (low)	110 (low)	1 (low)
2	1 (low)	110 (low)	3 (high)
3	1 (low)	130 (high)	1 (low)
4	1 (low)	130 (high)	3 (high)
5	3 (high)	110 (low)	1 (low)
6	3 (high)	110 (low)	3 (high)
7	3 (high)	130 (high)	1 (low)
8	3 (high)	130 (high)	3 (high)
9	2 (center)	120 (center)	2 (center)
10	2 (center)	120 (center)	2 (center)
11	2 (center)	120 (center)	2 (center)
12	2 (center)	120 (center)	2 (center)
13	2 (center)	120 (center)	2 (center)
14	2 (center)	120 (center)	2 (center)

a: Code values are in the parentheses, and actual values are out of the parentheses

Table 2.3. Experimental results from 2^3 full factorial design

Run	Cellulose (% raw manure, dry basis)	Hemicellulose (% raw manure, dry basis)	ADL (% raw manure, dry basis)	Nitrogen (% raw manure, dry basis)
1	23.64	10.31	16.01	2.22
2	17.96	2.47	17.43	1.87
3	22.63	8.04	17.45	2.05
4	17.25	-0.20	17.78	1.71
5	22.68	8.62	17.35	2.11
6	18.33	-0.53	17.33	1.74
7	21.40	3.50	17.63	1.81
8	15.57	2.80	17.96	1.51
9	21.99[a]	1.74[b]	18.13[c]	1.87[d]
10	21.60[a]	2.10[b]	17.97[c]	1.89[d]
11	21.03[a]	2.46[b]	18.45[c]	1.91[d]
12	21.23[a]	2.97[b]	17.87[c]	1.92[d]
13	22.09[a]	2.14[b]	18.36[c]	1.82[d]
14	21.22[a]	2.59[b]	17.88[c]	1.88[d]

a: The standard deviation of cellulose from run 9-14 is 0.44 with mean of 21.53.
b: The standard deviation of hemicellulose from run 9-14 is 0.43 with mean of 2.33.
c: The standard deviation of lignin from run 9-14 is 0.25 with mean of 18.11.
d: The standard deviation of nitrogen from run 9-14 is 0.04 with mean of 1.88.

Table 2.4. 3^2 central composite circumscribed design of factors with cellulose content as response[a]

Run	Factors			Cellulose content (% treated sample, dry basis)
	Time (hours)	Temperature (°C)	Acid Concentration (%)	
1	1 (-1)	110 (-1)	1 (-1)	29.76
2	1 (-1)	110 (-1)	3 (1)	25.33
3	1 (-1)	130 (1)	1 (-1)	29.97
4	1 (-1)	130 (1)	3 (1)	24.29
5	3 (1)	110 (-1)	1 (-1)	29.26
6	3 (1)	110 (-1)	3 (1)	26.23
7	3 (1)	130 (1)	1 (-1)	31.35
8	3(1)	130 (1)	3 (1)	25.86
9	0.27 (-1.682)	120 (0)	2 (0)	26.86
10	3.73 (1.682)	120 (0)	2 (0)	29.48
11	2 (0)	103 (-1.682)	2 (0)	29.73
12	2 (0)	137 (1.682)	2 (0)	29.81
13	2 (0)	120 (0)	0.27 (-1.682)	29.39
14	2 (0)	120 (0)	3.73 (1.682)	21.95
15	2 (0)	120 (0)	2 (0)	31.14
16	2 (0)	120 (0)	2 (0)	30.13
17	2 (0)	120 (0)	2 (0)	29.40
18	2 (0)	120 (0)	2 (0)	29.94
19	2 (0)	120 (0)	2 (0)	29.97
20	2 (0)	120 (0)	2 (0)	29.93

a: Code values are in the parentheses, and actual values are out of the parentheses.
b: The standard deviation of cellulose content from run 15-20 is 0.57 with mean of 30.08

Table 2.5. Analysis of variance (ANOVA) for factors and their interactions from 2^3 full factorial design[a]

	Degree of freedom	Mean square				F-value				P-value			
		Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4
Time	3	2.23	12.01	0.51	0.06	15.55	64.47	7.22	23.23	0.0057	0.0002	0.0289	0.0023
Temperature	3	5.95	5.86	0.61	0.06	41.40	31.46	8.70	21.95	0.0006	0.0011	0.0199	0.0026
Time×Temp.	1	1.42	1.24	0.23	0.007	9.88	6.66	3.20	2.48	0.0256	0.0494	0.1335	0.1762
Acid	3	18.05	58.19	2.69	0.15	125.58	312.49	38.19	52.02	<0.0001	<0.0001	0.0007	0.0003
Time×Acid	1	0.56	4.85	0.12	0.0006	2.87	26.05	1.71	0.23	0.1063	0.0038	0.2475	0.6497
Temp.×Acid	1	0.35	8.10	0.03	0.006	2.43	43.50	0.45	2.17	0.1801	0.0012	0.5340	0.2003
Time×Temp.×Acid	1	1.59	9.79	0.08	0.00007	11.08	52.57	1.14	0.03	0.0208	0.0008	0.3338	0.8764
Error	5	0.14	0.19	0.07	0.003								
Total	19												

a: Where Y1 represents cellulose, Y2 represents hemicellulose, Y3 represents lignin and Y4 represents nitrogen.

Table 2.6. Analysis of variance (ANOVA) for the model regressions from 3-factors central composite design [a]

	Sum of squares	Degree of freedom	F-value	P-value
Model	117.28	9	54.51	<0.0001
Error	2.39	10		
Total	119.67	19		
R-square	0.98			

a: Where the response is cellulose content in treated sample (% treated sample, dry basis)

Table 2.7. Optimal conditions for maximum of cellulose

	Optimal conditions
Time (hours)	2.80
Temperature (°C)	140
Acid conc. (%)	1.00

Table 2.8. Contents of each component at the optimal conditions

	Predicted content (%, dry basic)	Verified content (%, dry basic)	P value
Cellulose	31.90	30.94±0.59	0.17
Hemicellulose	-	3.15±0.25	-
lignin	-	20.80±0.74	-
Nitrogen	-	2.36±0.08	-

Verified data was the average of triplicates with standard deviations (n=3)

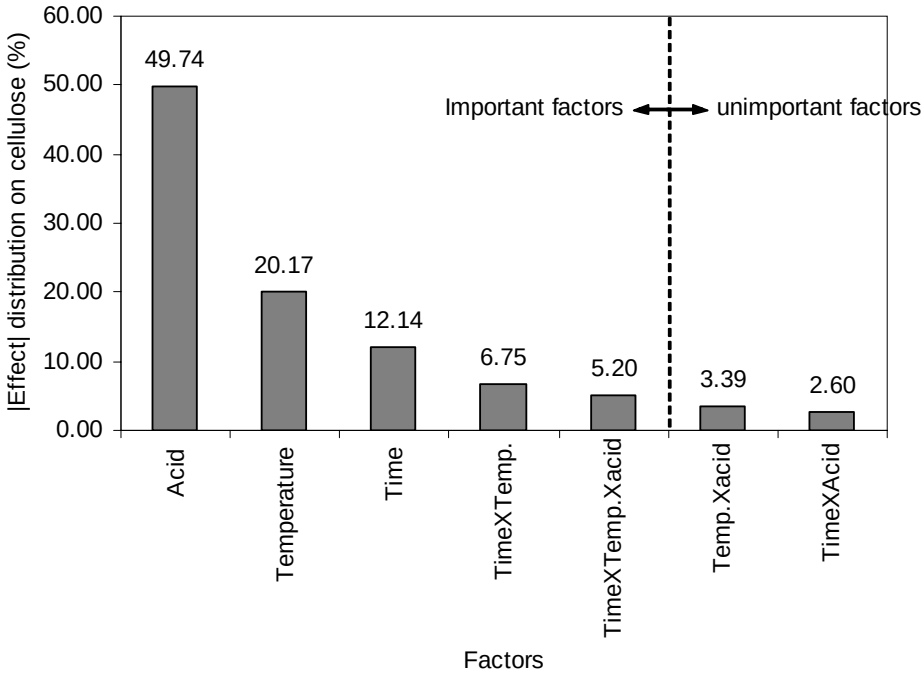

Fig. 2.1a. Effects on cellulose

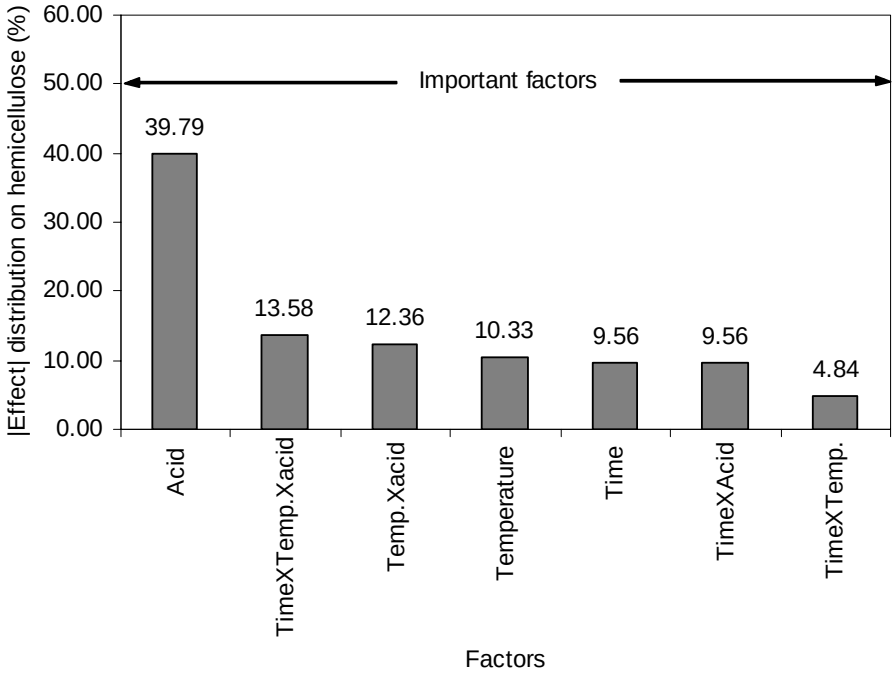

Fig. 2.1b. Effects on hemicellulose

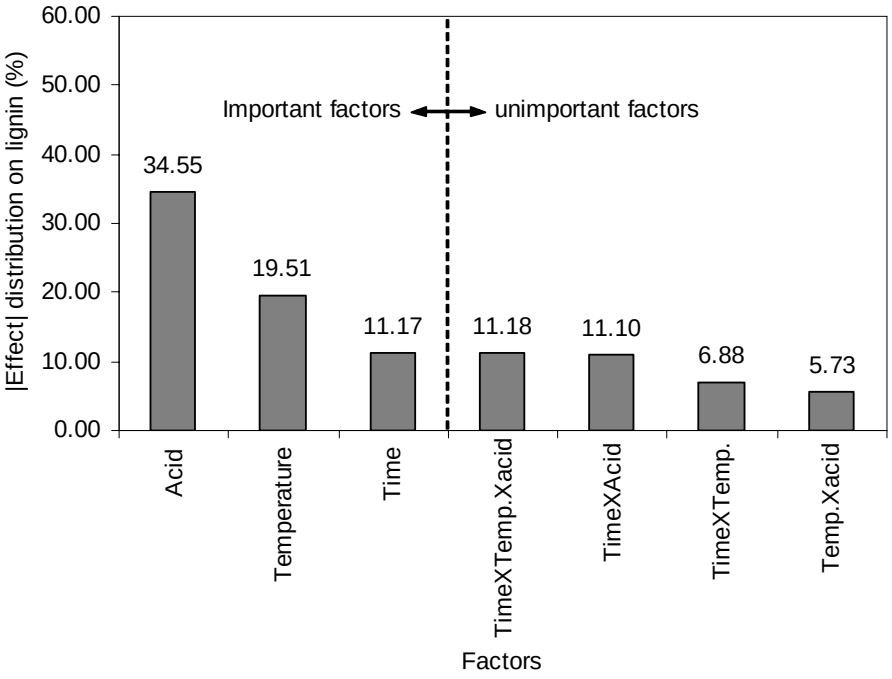

Fig.2.1c. Effects on lignin

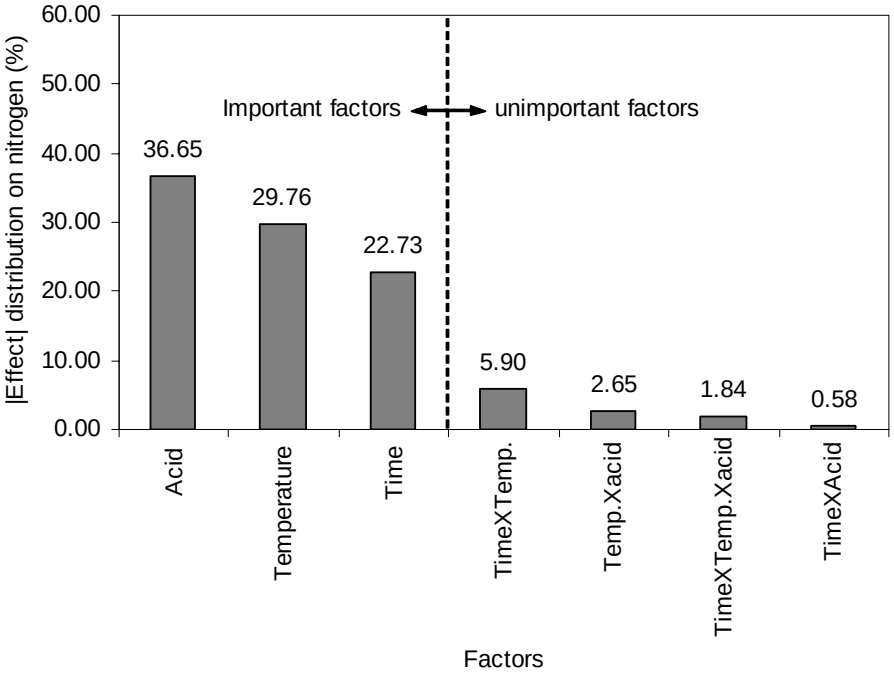

Fig. 2.1d. Effects on nitrogen

Fig. 2.1. Pareto charts of effects of factors and their interactions on each individual component in dairy manure

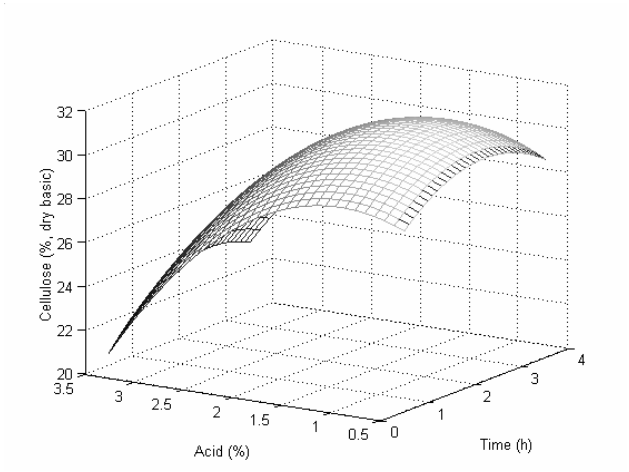

Fig.2.2a. at 120°C

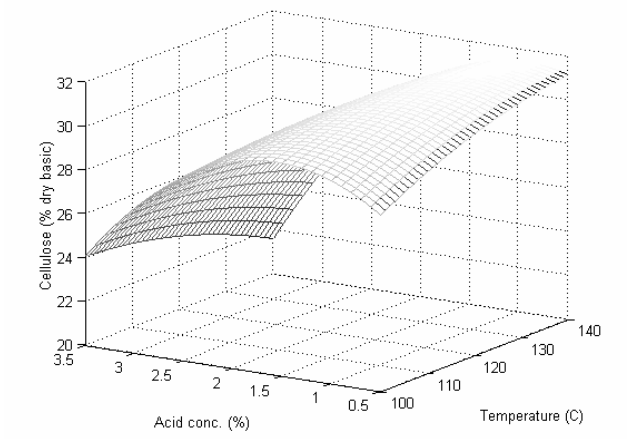

Fig.2.2b. at 2 hours

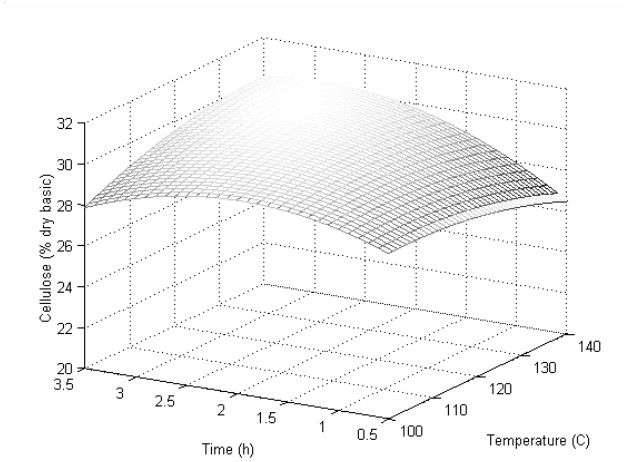

Fig.2.2c. at 2% acid concentration

Fig. 2.2. The effects of reaction conditions on cellulose accumulation

CHAPTER THREE

OPTIMIZING DILUTE ACID HYDROLYSIS OF HEMICELLULOSE IN A NITROGEN-RICH CELLULOSIC MATERIAL – DAIRY MANURE

3.1. Abstract

Effective dilute acid hydrolysis of dairy manure, which contains roughly 12% hemicellulose on a dry matter basis, produces a variety of monosaccharides such as arabinose, xylose and galactose. To enhance the effectiveness of this dilute acid hydrolysis, the effect of manure nitrogen content on the hydrolysis was studied because some reactions such as the browning reaction between amino acids and reducing sugars and acid-base reactions involving ammonia and acid interfere with the hydrolysis. Two dairy manure samples were used to study this nitrogen effect; the original manure and the pretreated manure derived from a solid/liquid separation pretreatment. The pretreated manure had a total nitrogen content of 1.3% dry matter while the original dairy manure had twice that amount with a total nitrogen content of 2.6% dry matter. Results found that the optimal conditions for hydrolysis of manure hemicellulose were 2 hours reaction time, 1% sulfuric acid concentration, 135°C, and 10% sample concentration using the pretreated dairy manure as raw material. Under these conditions the corresponding sugar yield from hemicellulose was 111% and sugar concentration in the solution reached 16.5 g/L. At the same time, the hydrolyzed solid had 43% dry matter of cellulose, which was much higher than both the original manure containing 22% and the pretreated manure with 32%.

Key words: arabinose, cellulose, completely randomized design (CRD), dairy Manure, dilute acid hydrolysis, galactose, hemicellulose, nitrogen-rich cellulosic material, xylose, sugar yield

3.2. Introduction

Dairy manure is, by composition, 12% (w/w, all percentages used in this paper are based on weight) hemicellulose and 22% cellulose (Table 3.1), and represents a large potential source of carbohydrates capable of being converted to useable monosaccharides such as xylose, glucose etc. which can further produce value-added chemicals of xylitol and sorbitol using hydrogenation reaction (Fennema, 1996) and lactic acid using bacterial or fungal fermentation (Parajo et al., 1996; Woiciechowski et al., 1999). One hundred sixty million dry tons of animal manure is produced annually in the United States (Council for Agricultural Science and Technology, 1995). Nearly 55 million dry tons of this annual production is collected for subsequent disposal, with about 75% of the total coming from dairy and feedlot cattle. Finding effective bio-based uses for dairy and cattle manure such as carbohydrate production could effectively reduce the environmental liabilities related to manure management and disposal as well as provide an economic stimulus to the dairy farm.

Acid hydrolysis is the typical process used to treat and help convert lignocellulosic materials to sugars. The National Renewable Energy Laboratory (NREL) recently used a 190°C reaction temperature and 0.3% sulfuric acid to hydrolyze wood hemicellulose to yield 89% mannose and 82% galactose (Nguyen, 1998). Additionally, dilute acid hydrolysis of rice straw hemicellulose, under the optimal conditions of 1%

sulfuric acid concentration, 10% sample concentration, 27 minutes of reaction time, and 121°C of reaction temperature has resulted in a 77% xylose yield (Roberto et al., 2003).

Proper conditions for hydrolysis of manure hemicellulose, though, are potentially different from wood and straw fibers because of higher nitrogen levels and differences in composition and structure complexity. Dairy manure has a nitrogen content of around 2.6% which is considerably higher than the 1.0% of other fibrous materials such as wheat straw (Reinertsen et al., 1984). The nitrogen is in the form of indigestible forage proteins, proteins synthesized by rumen bacteria, and inorganic nitrogen such as urine and ammonia (Committee on Animal Nutrition Board on Agriculture National Research Council, 1983). During hydrolysis, ammonia and amino acids from hydrolyzed protein can react with monosaccharides in the solution under the high-temperature acidic conditions (Fennema, 1996; Collins et al., 1995), and ultimately can influence the final sugar yield. Additionally, the fiber in manure has already undergone some breakdown while passing through the animal's digestive tract. As a result, the conditions and yield for acid hydrolysis of dairy manure hemicellulose would most likely be affected.

Little research has been reported on hydrolysis of manure hemicellulose and the optimal conditions required. The specific objectives of this study were to add to this research by statistically: (1) studying the influence of nitrogen content on hydrolysis of dairy manure and (2) determining the optimal conditions of hydrolysis in regards to acid concentration, temperature, reaction time and sample concentration to produce monosaccharides such as arabinose, galactose, and xylose.

3.3. Materials and methods

3.3.1. Material and preparation

Fresh dairy manure was obtained from the Dairy and Beef Centers of Washington State University. The manure was composed of 15.3% dry matter (DM) with a total carbon content of 46.9g/100g DM and total nitrogen content of 2.6g/100g DM. The original manure and the pretreated manure were the two forms of manure used to study the effect of nitrogen on the hydrolysis of dairy manure. The original manure sample was made by mixing 10 kg of fresh dairy manure with 5 kg of water and then blending for 1 minute to achieve size reduction. The pretreated manure was obtained by washing 10 kg of fresh manure three separate times with 5 kg of water and then separating out the solid using a centrifuge at 3,000 rpm for 10 minutes. The three water washings were adequate to reduce the nitrogen content of the manure by half and release much of the soluble nitrogen to the liquid phase. The data for the original manure and pretreated manure are shown in Table 3.1.

3.3.2. Experimental design of acid hydrolysis

3.3.2.1. The effects of temperature, reaction time, acid concentration and nitrogen content

The effects of temperature, reaction time, acid concentration, and nitrogen content were first carried out by a Completely Randomized Design (CRD) with two replications of 120 treatment combinations. Five acid concentrations (1% to 5%) involving four durations (30 minutes to 3 hours) were studied at three different temperatures (105°C,

120°C, and 135°C) on each type of manure (original and pretreated manure). Each sample used for hydrolysis contained 2.50 grams of dry manure mixed with 47.50 grams of acid solution. After hydrolysis, each sample was filtered through Whatman No. 5 filter paper to separate the liquid from the solid. The General Lineal Model (GLM) for Analysis of Variance (ANOVA) of sugar yield from hemicellulose was tested using the Statistical Analysis System (SAS 8.0) program.

3.3.2.2. The effect of sample concentration

The effect of sample concentration was then studied based on the optimal conditions of temperature, reaction time, acid concentration, and nitrogen content. Four sample concentrations (2.5%, 5%, 7.5%, and 10%) with two replications were used by the same experimental design (CRD). The difference among the sugar yields of these samples was also analyzed using the Statistical Analysis System (SAS 8.0) program.

3.3.3. Sugar analysis

The concentration of each monosaccharide in the hydrolyzed solution was determined using a Dionex ion chromatograph (IC). Hydrolyzed solutions were neutralized to pH values of 5.0 to 6.0 using sodium hydroxide. Diluted hydrolyzed solutions (400x) were filtered with a 0.45 μm filter. Sugars were separated on CarboPac PA 10 guard (4x50 mm) and analytical (4x250 mm) columns at room temperature (approximately 25°C). Sugars were detected using an ED 40 electrochemical detector. An AS 40 auto-sampler was used for continuous running and Dionex PeakNet 5.1

chromatography software was used for data analysis. The eluent conditions for monosaccharide analysis are listed in Table 3.2.

3.3.4. Scanning Electron Microscopy

Structural changes in the manure during treatment were qualitatively studied using a Scanning Electron Microscopy (SEM). The dry samples were mounted on double-sided tape placed on aluminum stubs. A thin layer of gold (15 nm for 10 minute sputtering) was sputtered onto the mounted sample to reduce electron-altering effects using a Technics Hummer V Sputtering Device. Finally, the gold-coated samples were observed in a SEM (HITACHI S-570 Scanning electron microscope) with an accelerating voltage of 20 KW.

3.3.5. Other analytical methods

Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) of the manure were analyzed using a reflux apparatus (Goering et al., 1970). NDF estimated total cellulosic material content (cellulose, hemicellulose, lignin, and insoluble ash), ADF estimated the concentration of lignin-cellulose, and their difference (NDF-ADF) determined hemicellulose content. Nitrogen and crude protein were determined using the AOAC method (Association of Official Analytical Chemists, 1990). The total carbon of solid samples was measured by automated combustion techniques using a LECO CNS-2000.

3.4. Results and discussion

3.4.1. Characteristics of original manure and pretreated manure

The characteristics of the manure used in the study are listed in Table 3.1. The pretreated manure had 50% the nitrogen content of original manure. This means that more than half of the nitrogen source in dairy manure was from soluble sources such as urine, ammonia, and selected proteins. The nitrogen that remained was mainly from proteins bonded with the cell wall of the forage (Krishnamoorthy et al., 1982) and proteins associated with lignin (Sniffen et al., 1992), both of which were difficult to be removed via simple washing.

Fiber content was the largest component in both the original manure and pretreated manure. The pretreated manure had higher fiber content percentage than the original manure. It was this fiber that provided the source of cellulose, hemicelluose and lignin. Hemicellulose was the only part among them that was easily hydrolyzed by chemical or enzymatic methods to provide a variety of monosaccharides such as arabinose, galactose, and xylose.

3.4.2. Sugar analysis of hydrolyzed solution

Four major monosaccharides, arabinose, galactose, xylose, and glucose, were found in the hydrolyzed solutions (Fig. 3.1). Arabinose, galactose and xylose were from the hydrolysis of hemicellulose, while glucose was from the partial hydrolysis of cellulose. Although concentrations of each individual sugar varied because of the changing experimental conditions (data not shown), xylose and arabinose were the major monosaccharides from the hemicellulose hydrolysis (around 70% xylose and 10%

arabinose of total sugars in the solution, data not shown). This was consistent with the content of the precursors (xylan, araban) in the alfalfa forage (Hanson, 1972).

3.4.3. The effects of nitrogen content, temperature, reaction time and acid concentration on dilute acid hydrolysis of dairy manure

3.4.3.1. The effect of nitrogen content on dilute acid hydrolysis of dairy manure

Sugar yield (total amount of monosaccharides from hemicellulose to amount of hemicellulose in the sample) results from the hemicellulose hydrolysis experiments are outlined in Fig. 3.2-3.4. The sugar yields of pretreated manure with low nitrogen content were overall higher than those of the corresponding hydrolysis of original manure with high nitrogen content (Fig. 3.2-3.4). For instance, at the point of 2 hours, 3% acid, and 120°C, the hydrolysis of pretreated manure had a yield of 114%, while the corresponding original manure hydrolysis had only a yield of 102%. In addition, the hydrolyzed solution color of original manure was much darker than the pretreated manure (data not shown). These phenomena might have been caused by a much stronger browning reaction between reducing sugars and protein (Fennema, 1996) in the original manure. Meanwhile, Fig. 3.2-3.4 also demonstrate that hydrolysis of original manure needed more acid to reach the highest yield than did the pretreated manure. At the low acid concentration of 1%, sugar yields from hydrolysis of original manure for all three temperatures were much lower than those of the corresponding hydrolysis of pretreated manure, and sugar yields at the acid concentration of 1% increased a little following the increase of reaction time compared with yields at a much higher acid concentration. This means that a certain amount of acid is needed to start the hydrolysis reaction of original

manure. This is most likely because an acid-base reaction between acid and non-protein nitrogen like urine and ammonia occurred in original manure prior to hydrolysis of hemicellulose.

3.4.3.2. The effects of reaction time, reaction temperature, and acid concentration on hydrolysis of dairy manure

The sugar yield of both original manure and pretreated manure increased noticeably with increase of reaction time from 0.5 to 3 hours and acid concentration from 1% to 5% of hydrolysis at 105°C (Fig. 3.2). As temperature increased to 120°C (Fig. 3.3), the yields of both original manure and pretreated manure reached their highest values of 102% and 114%, respectively. Fig. 3.3 also presents that the sugar yields for both original manure and pretreated manure stopped increasing once reaction time and acid concentration were more than 2 hours and 2%, respectively. This means that side reactions such as dehydration and the browning reaction occurred very slowly at this temperature. At 135°C (Fig. 3.4), the sugar yields from hydrolysis of pretreated manure reached its highest value at an acid concentration of 1% which was lower than that achieved during the hydrolysis at 120°C. Also, the sugar yields from hydrolysis of original manure were lower than the corresponding hydrolyses at 100°C and 120°C. For both hydrolyses at this temperature, the increase in yield did level off as time progressed and acid concentration further increased. This is due to subsequent dehydration reactions of sugars under acid conditions and high temperature (Collins et al., 1995). For instance, under these circumstances xylose can be degraded to furfural (Zeitsch, 2000).

3.4.3.3. Statistical analysis

Statistical analysis of sugar yields from hemicellulose showed that not only were there significant differences ($P<0.01$) caused by the effects of temperature, reaction time, acid concentration and nitrogen content, but also significant two-way, three-way, and four-way interactions between the variables (Table 3.3). This means that the main effects could not be directly interpreted. Therefore, a simple pair-wise comparison, using least square means (lsmeans) was analyzed. The resulting analysis showed that there was no significant difference ($P>0.05$) among the nine hydrolyses of pretreated manure that had an average highest yield of 114% (Table 3.4). Meanwhile, the original manure had an average highest yield of 102% from two treatments (Table 3.5), which were not significantly different ($P>0.05$). The 11% difference in yield between the two nitrogen-differing manures, combined with the color change of hydrolyzed solution mentioned in section 3.4.3.1, indicates that nitrogen content had a noticeably negative effect on the hydrolysis of manure.

Since acid-sugar separation is usually the bottleneck for utilization of the entire acid hydrolysis system, the lower the acid concentration the hydrolysis used, the better the utilization of sugars. Thus, the best raw material for acid hydrolysis was the pretreated dairy manure with optimal conditions of reaction time of 2 hours, acid concentration of 1%, and reaction temperature of 135°C, with hydrolysis under these conditions having a yield of 114%. When compared to acid hydrolysis of softwood hemicellulose, which used 1% w/w acid at 185°C within a steam explosion reactor to reach a yield of 85% (Kim et al., 2001), the method of hydrolysis for hemicellulose of dairy manure was more moderate and had a higher sugar yield (114%).

3.4.4. The effect of sample concentration on acid hydrolysis of dairy manure

The effect of sample concentration on acid hydrolysis was investigated in terms of increasing the sugar concentration in solution. As stated in the discussion within the previous section, the optimal conditions obtained were based on a sample concentration of 5%. Although the reaction under this condition had a highest yield of 114%, the corresponding sugar concentration in solution was only 8.5 g/L, which was still low. Four sample concentrations (2.5%, 5%, 7.5% and 10%) of pretreated manure were used for this part of study. The reaction conditions were still the optimal conditions obtained from the previous section.

Fig. 3.5 demonstrates the effect of sample concentration on sugar yield from hemicellulose and sugar concentration in solution. Sugar concentration linearly increased with sample concentration. The concentration reached the highest value of 16.5 g/L with respect to 10% sample concentration. Meanwhile, the sugar yields were around 111%. Statistical analysis showed that there were no significant differences ($P>0.05$) among these four treatments in terms of sugar yield. Since more substrate was used, a higher sugar concentration was obtained and the 10% sample concentration of pretreated manure was adopted as the optimal sample concentration.

3.4.5. Structure changes of manure fiber during the hydrolysis

Manure samples before and after acid treatment were observed using Scanning Electronic Microscopy (SEM) in order to know what occurred on the structure of the manure fiber during the dilute acid hydrolysis. The difference in fiber structure between

original manure and pretreated manure are shown in Fig. 3.6a and 3.6b. The texture of original manure fiber was much rougher than that of fiber in pretreated manure. This means that there were more substances attached to the surface of the main structure of the fiber in original manure as compared to pretreated manure. Chemical composition comparisons of both samples showed that most of these substances were most-likely nitrogen-related materials such as proteins (Table 3.1).

Fig. 3.6d presents a very clear view of the main structure of manure fiber after dilute acid hydrolysis. Compared with Fig. 3.6b, the striations on the surface of the manure fiber are thinner. This means that more substances were washed from the manure fiber. In addition, fiber data for the hydrolyzed pretreated manure (Table 3.6) showed that there was almost no hemicellulose left in the hydrolyzed solid. Also, there were some holes that appeared in the main structure of the hydrolyzed fiber of pretreated manure (Fig. 3.6b). Sugar analysis shows that there was some glucose produced under the conditions (data not shown), which suggests that the phenomenon might be caused by partial degradation of cellulose. These results, combined with fiber data of the hydrolyzed pretreated manure (Table 3.6), indicate that the main structure of manure fiber was mainly composed of cellulose and lignin, with hemicellulose being only on the surface of the main structure. This might be a reason why hemicellulose was much easier to degrade than cellulose and lignin.

At the same time, the texture of the hydrolyzed fiber of original manure (Fig. 3.6c) was not as smooth as that of pretreated manure (Fig. 3.6d) and also there were more substances attached to the surface of the hydrolyzed fiber of original manure. This phenomenon combined with other results, such as the lower sugar yield from

hemicellulose (102%) for nitrogen-rich manure and the darker color of the hydrolyzed solution of original manure, indicates that the hydrolysis of original manure was not as complete as that of pretreated manure, which further elucidated that the effect of nitrogen on acid hydrolysis of manure hemicellulose.

3.5. Conclusion

In this study it was found that the nitrogen content in nitrogen-rich cellulosic material such as dairy manure was an important factor in the hydrolysis of hemicellulose to obtain monosaccharides. Effective removal of the nitrogen sources in this type of raw material contributed largely to an increase in the sugar yield from hemicellulose and made the reaction conditions more moderate. Optimal conditions for dilute acid hydrolysis of dairy manure hemicellulose involved the use of 10% pretreated manure under 2 hours reaction time, 1% acid concentration, and 135°C temperature. Under these conditions the corresponding sugar yield was 111% and sugar concentration in the solution reached 16.5 g/L. Meanwhile, the hydrolyzed solid had 43% dry matter of cellulose, which was much higher than original manure and pretreated manure (Table 3.6), and thus could supply a substantial cellulose resource for downstream glucose production.

3.6. References

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Table 3.1. Characteristics of sample manures[a]

	Original manure	Pretreated manure
Dry Matter, %	15.50 ± 0.09	13.26 ± 0.02
Crude Protein, % dry matter	16.44 ± 0.53	8.13 ± 0.09
NDF, % dry matter	48.27 ± 0.46	67.11 ± 0.68
ADF, % dry matter	35.80 ± 0.14	52.23 ± 1.54
ADL, % dry matter	13.91 ± 0.45	16.56 ± 0.65
Cellulose (=ADF-ADL), % dry matter	21.89 ± 0.38	35.67 ± 1.43
Hemicellulose(=NDF-ADF), % dry matter	12.47 ± 0.32	14.88 ± 0.94
N, % dry matter	2.63 ± 0.09	1.30 ± 0.01
C, % dry matter	45.49 ± 0.30	41.00 ± 0.88

a: all data are the average of triplicates with standard deviations of the means (n=3)

Table 3.2. Eluent conditions for monosaccharide analysis[a]

Time (min)	A(%)	B(%)	C(%)	Comments
Initial	20.00	80.00	0.00	TTL1
0.00	20.00	80.00	0.00	
0.10	20.00	80.00	0.00	Inject
27.00	20.00	80.00	0.00	
27.10	0.00	0.00	100.00	
37.00	0.00	0.00	100.00	
37.10	20.00	80.00	0.00	
57.10	20.00	80.00	0.00	

a: Eluent A: Water; Eluent B: 52 mM NaOH; C: 200 mM NaOH
Eluent flow rate: 1.0 mL/min; Pressure limit: 1500~3000psi.

Table 3.3. Analysis of Variance (ANOVA) table of the effect of nitrogen, temperature, reaction time and acid concentration on sugar yield from hemicellulose hydrolysis

Source term	Degree of freedom	Mean square	F-ratio	P-value*
Temperature	2	1.74	4353.10	<0.0001
Time	3	0.26	589.75	<0.0001
Acid concentration	4	1.40	3493.67	<0.0001
Nitrogen	1	2.61	6525.29	<0.0001
Temp.×Time	6	0.39	985.60	<0.0001
Temp.×Acid conc.	8	0.26	661.51	<0.0001
Temp.×Nitrogen	2	0.03	84.53	<0.0001
Time×Acid conc.	12	0.03	87.00	<0.0001
Time×Nitrogen	2	0.03	84.53	<0.0001
Acid conc.×Nitrogen	4	0.29	723.08	<0.0001
Temp.×Time×Acid conc.	24	0.02	56.47	<0.0001
Temp.×Time×Nitrogen	6	0.01	36.06	<0.0001
Time×Acid conc.×Nitrogen	12	0.01	18.99	<0.0001
Temp.×Acid conc.×Nitrogen	8	0.04	99.15	<0.0001
Time×Temp.×Acid conc.×Nitrogen	24	<0.01	5.96	<0.0001
Error	120	<0.01		
Total	239			

*: Term significant at alpha=0.05

Table 3.4. The highest sugar yield from hemicellulose in hydrolyzed solutions (pretreated manure) with different reaction conditions

Time (hour)	Acid conc. (%)	Temperature (°C)	Nitrogen content (%)	Mean of monosaccharide concentration from hemicellulose (g/100 dry nitrogen-lacking manure)	Mean of sugar yields from hemicellulose (%)
1	4	120	1.3	16.97	114
1	5	120	1.3	16.82	113
2	2	120	1.3	16.79	112
2	3	120	1.3	16.98	114
2	4	120	1.3	17.23	116
2	5	120	1.3	17.38	117
2	1	135	1.3	17.01	114
3	3	120	1.3	16.96	114
3	4	120	1.3	17.10	115

Table 3.5. The highest sugar yield from hemicellulose in hydrolyzed solutions (original manure) with different reaction conditions

Time (hour)	Acid conc. (%)	Temperature (°C)	Nitrogen content (%)	Mean of monosaccharide concentration from hemicellulose (g/100 g nitrogen-rich manure)	Mean of sugar yields from hemicellulose (%)
2	3	120	2.6	12.72	102
2	4	120	2.6	12.96	104

Table 3.6. Fiber characteristics of hydrolyzed pretreated manure[a, b]

	Hydrolyzed pretreated manure
Cellulose, % dry matter	42.67 ± 0.64
Hemicellulose, % dry matter	~ 0
Lignin, % dry matter	32.12 ± 0.97

a: all data are the average of triplicates with standard deviations of the means (n=3).
b: The hydrolyzed pretreated manure was from the treatments of temperature of 135°C, acid concentration of 1% and reaction time of 2 hours.

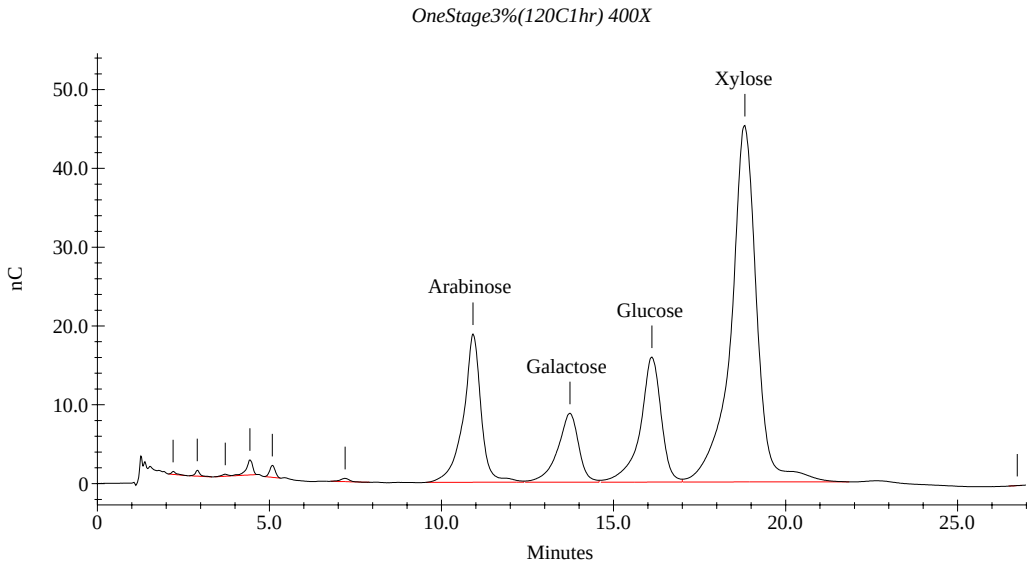


Fig. 3.1. Chromatogram at generated monosaccharides after hydrolysis [a]

a: from 120°C acid hydrolysis of nitrogen-rich manure under 3% acid and 1 hour. Sample is diluted by 400 times.

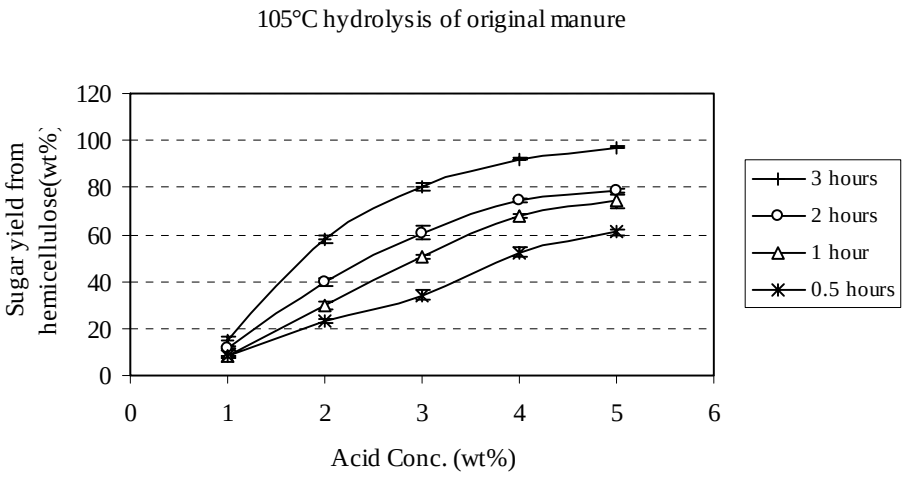


Fig. 3.2(a)

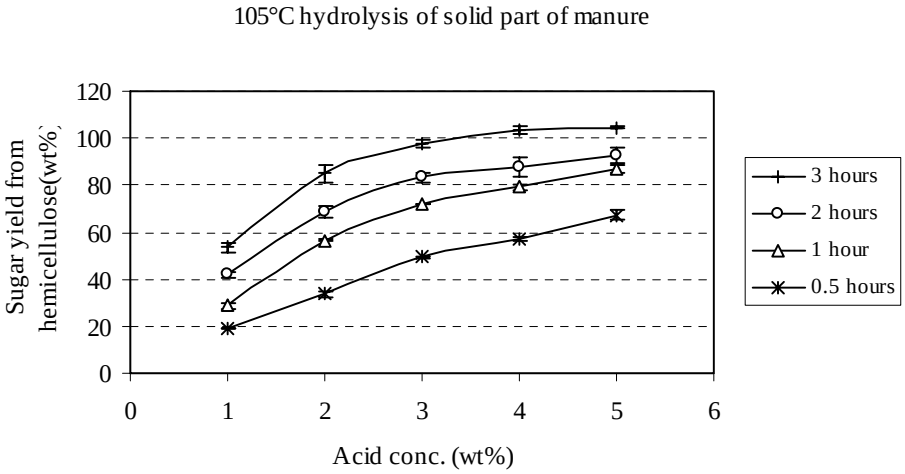


Fig. 3.2(b)

Fig. 3.2. Comparison of sugar yield from hemicellulose of acid hydrolysis of original manure and pretreated manure at 105°C[a,b]

a: Sugar yield from hemicellulose = grams of total monosaccharides (xylose + arabinose + galactose)/grams of hemicellulose in raw material
b: Data are presented as the mean of two replicates and the error bars show the standard deviation

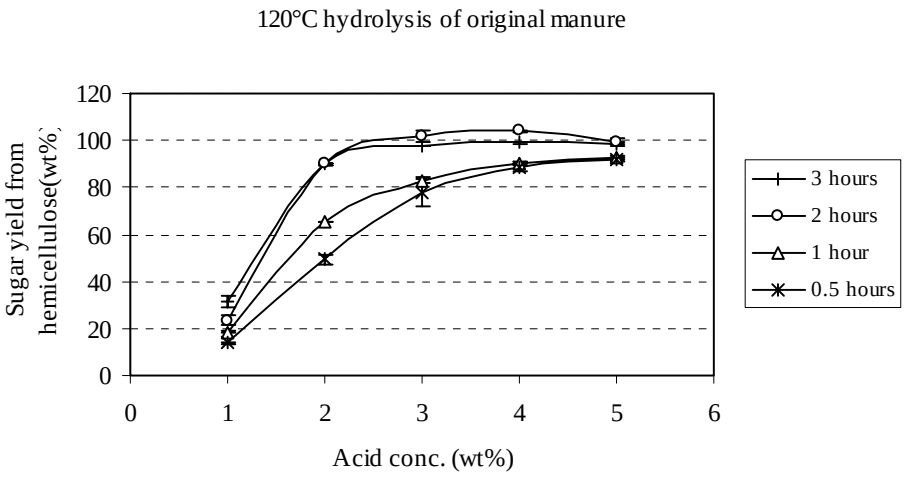


Fig. 3.3(a)

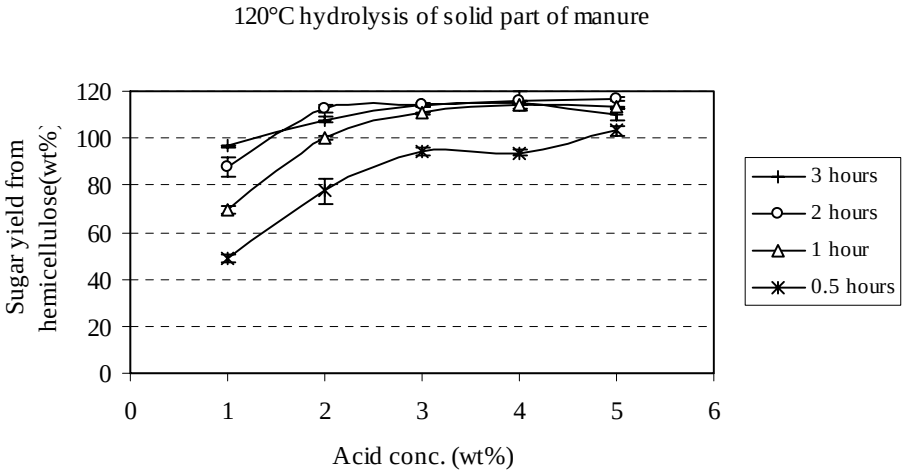


Fig. 3.3(b)

Fig. 3.3. Comparison of sugar yield from hemicellulose of acid hydrolysis of original manure and pretreated manure at 120 °C[a,b]

a: Sugar yield from hemicellulose = grams of total monosaccharides (xylose + arabinose + galactose)/grams of hemicellulose in raw material
b: Data are presented as the mean of two replicates and the error bars show the standard deviation

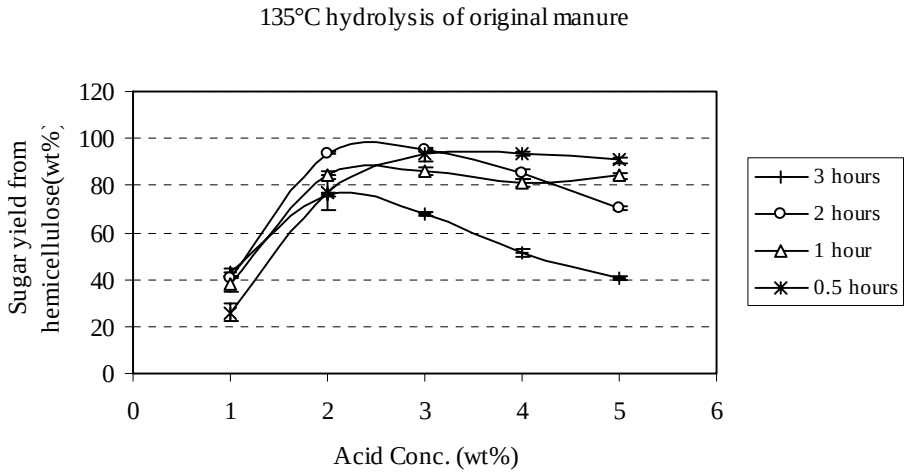


Fig. 3.4(a)

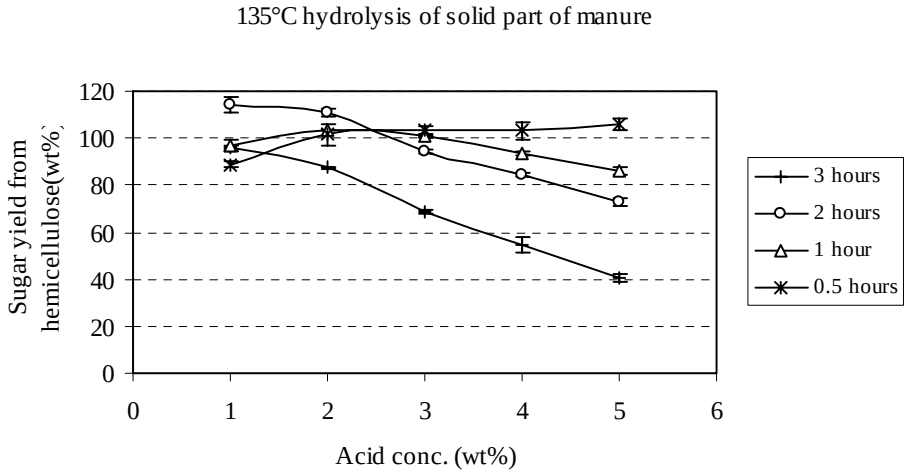


Fig. 3.4(b)

Fig. 3.4. Comparison of sugar yield from hemicellulose of acid hydrolysis of original manure and pretreated manure at 135°C[a,b]

a: Sugar yield from hemicellulose = grams of total monosaccharides (xylose + arabinose + galactose)/grams of hemicellulose in raw material
b: Data are presented as the mean of two replicates and the error bars show the standard deviation

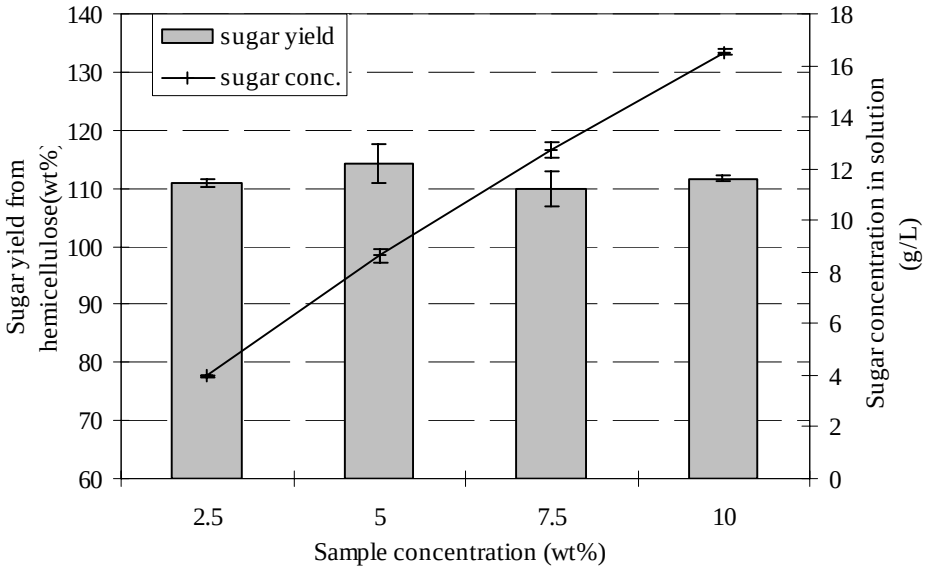
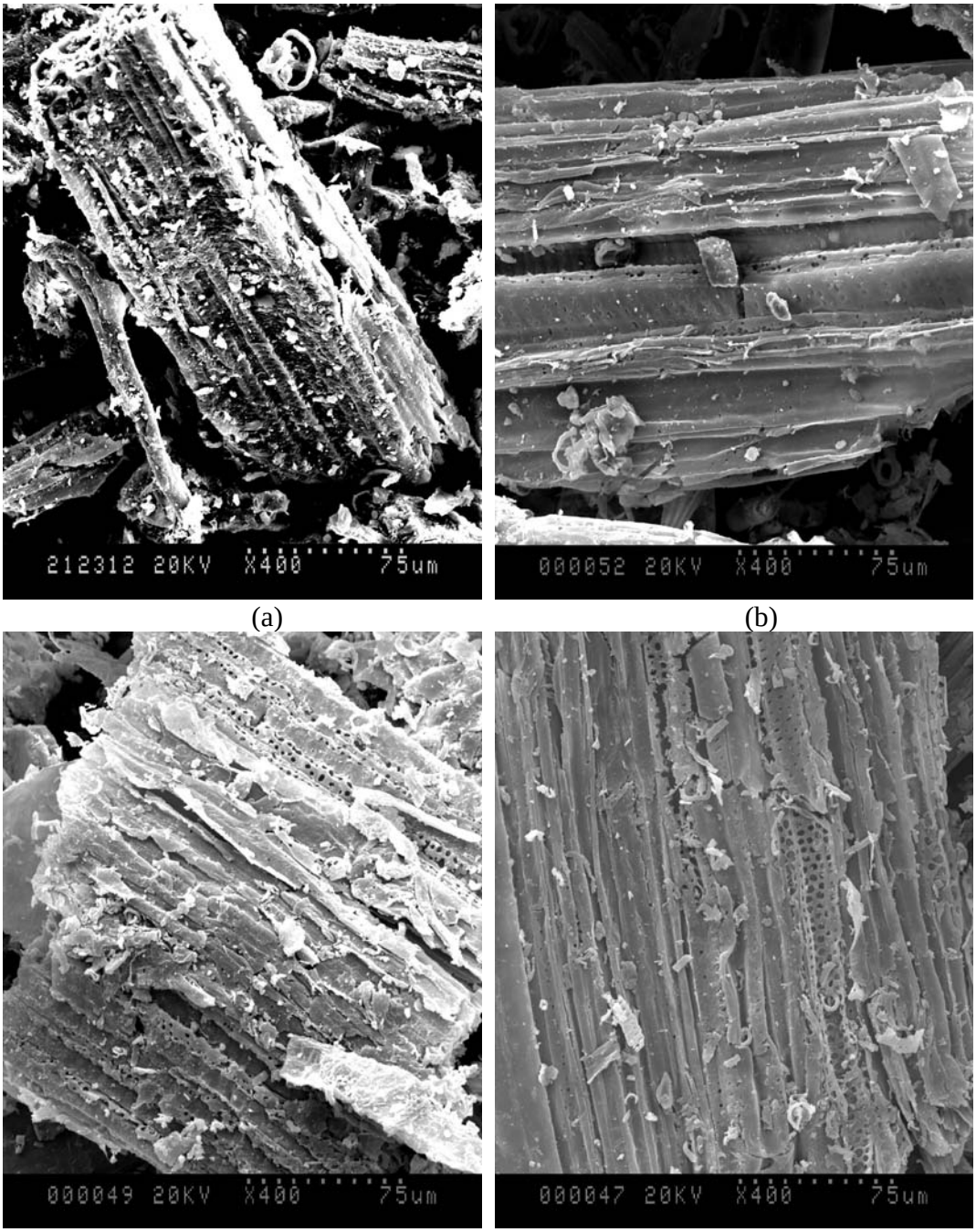


Fig. 3.5. The effect of sample concentration on sugar concentration in solution and sugar yield from hemicellulose[a, b]

a: Sugar yield from hemicellulose = grams of total monosaccharides (xylose + arabinose + galactose)/grams of hemicellulose in raw material
b: Data are presented as the mean of two replicates and the error bars show the standard deviation



(a) (b)

(c) (d)

Fig. 3.6. Scanning electron micrograph of dilute-acid-treated dairy manure (400x)

a: Original dairy manure
b: Pretreated dairy manure (after washing)
c: Solid of dilute acid hydrolysis of original manure (2 hours, 3% sulfuric acid, 120°C)
d: Solid of dilute acid hydrolysis of pretreated manure (2 hours, 1% sulfuric acid, 135°C)

CHAPTER FOUR

ACID HYDROLYSIS OF FIBER FROM A NITROGEN-RICH CELLULOSIC MATERIAL – DAIRY MANURE

4.1. Abstract

Concentrated acid hydrolysis of lignocellulosic materials is a conventional treatment process for the production of monosaccharides. However, this method is ineffective and undesirable for the treatment of dairy manure due to the high nitrogen content of dairy manure and the environmental issues caused by the use of highly concentrated acid solution. In an effort to overcome these barriers, a modified acid hydrolysis process with short reaction time was introduced that involved a nitrogen-removing pretreatment followed by decrystallization with concentrated acid and then hydrolysis using dilute acid. The effects of nitrogen, acid concentration, reaction time, and temperature were investigated. A pretreated manure with a low nitrogen content of 1.3% was used as the substrate. The results indicated that the optimal conditions for fiber decrystallization were 75% acid concentration, 3:5 sample to acid ratio (weight basis), and 30 minutes of reaction time; while the optimal conditions for acid hydrolysis were 12.5% acid and 10% dry sample at 135°C for 10 minutes. These conditions produced 26 g/L glucose at a yield of 84% and 11 g/L hemicellulose-sugars at a yield of 80%.

Keywords: acid decrystallization, cellulose, dairy manure, dilute acid hydrolysis, glucose, hemicellulose, sugar yield

4.2. Introduction

Animal manure as a feedstock has great potential for producing value-added chemicals such as monosaccharides from manure fiber. Presently, nearly 55 million dry tons of animal manure is collected every year for subsequent disposal, with about 75% (w/w, all percentages used in this paper are based on weight) of the total coming from dairy and feedlot cattle (Council for Agricultural Science and Technology, 1995). Increasingly stringent requirements as well as a lack of land availability are limiting the farmers' ability to use their land for direct manure disposal, requiring the development of new animal waste management strategies and alternatives, such as processes to turn the manure into saleable high value chemical products.

Acid hydrolysis, particularly sulfuric acid hydrolysis, is widely used to treat lignocellulosic materials to obtain monosaccharides (Choi et al., 1996; Kim et al., 2001; Nguyen, 1998; Patrick Lee et al., 1997; Zerbe et al., 1987). In the process, acid first breaks the matrix structure of the fiber into more accessible cellulose, hemicellulose, and lignin (Forest Products Laboratory, 1990), and then further reduces these polysaccharides to monosaccharides (Fengel et al., 1984). This type of application commonly utilizes either concentrated acid at a low temperature or dilute acid at a high temperature (Sun et al., 2002). In general, concentrated acid hydrolysis is much more effective than dilute acid hydrolysis (Harris, 1949; Grohmann et al., 1985). It involves two steps: a decrystallization step to break down the crystal structure of fiber using sulfuric acid of more than 60% concentration and a hydrolysis step with acid of around 20%-30% concentration to liberate sugars from the decrystallized fiber (Bayat-makooi et al., 1985). Glucose yields of 72-82 % can be achieved from mixed wood chips by using such a

concentrated acid hydrolysis process (Iranmahboob et al., 2002). However, concentrated acid hydrolysis has a major drawback because use of highly concentrated acid can cause serious environmental concerns (Sun et al., 2002). Therefore, a new process of concentrated acid decrystallization followed by relatively dilute acid hydrolysis should be studied for the purpose of combining the positive aspects of shorter reaction time and lower temperatures of concentrated processes with the positive aspects of complete degradation of lignocellulosic and diminished chemical toxicity in dilute processes.

Dairy manure is a special type of lignocellulosic material as its nitrogen content (about 3%) is high compared to that of wood and straw, which contain less than 1% nitrogen (Reinertsen et al., 1984). Most of the nitrogen in dairy manure is from indigestible forage proteins, urine and/or ammonia (Committee on Animal Nutrition Board on Agriculture National Research Council, 1983). This relatively high concentration of nitrogen can negatively influence the hydrolysis process and eventual sugar yield by introducing the potential for debilitating side reactions such as the Browning reaction (Fennema, 1996; Collins, 1995). Therefore, hydrolysis of dairy manure is much more complicated than of other lignocellulosic materials. The effect of nitrogen on hydrolysis of dairy manure has to be studied, as well as the other important factors such as reaction temperature, acid concentration, and reaction time.

The objective of this research was to study a two-step hydrolysis process of dairy manure involving a concentrated acid decrystallization step followed by a dilute (less that 15%) acid hydrolysis step. More specifically, this study was designed to investigate: (1) the effects of nitrogen, acid concentration, temperature and reaction time on concentrated acid decrystallization of dairy manure, (2) the effects of temperature and reaction time on

the ensuing dilute acid hydrolysis, and (3) the optimal conditions for producing highest glucose and hemicellulose-sugars yield.

4.3. Materials and methods

4.3.1. Material and preparation

Dairy manure contains 12 % hemicellulose and 22 % cellulose (Table 4.1), both of which are capable of being converted to useable monosaccharides, such as glucose, xylose, arabinose, and galactose, using acid hydrolysis. Fresh dairy manure was obtained from the Dairy Center of Washington State University. The fresh manure had 15.3% dry matter (DM) with a carbon content of 46.9 g/100g DM and nitrogen content of 2.6 g/100g DM. Ten kilograms of raw manure were mixed with 5 liters of deionized water and blended (using a Oster blender) for 1 minute to achieve manure particle size reduction, and then 10 kg of the mixture were washed three separate times with 5 liters of water each time. Solid-liquid separation using a centrifuge at 3,000 rpm for 10 minutes isolated most of the soluble nitrogen and impurities within the liquid fraction. The solid part was collected and dried as pretreated manure for hydrolysis. The data for the raw manure and the pretreated manure are shown in Table 4.1.

4.3.2. Concentrated acid decrystallization

4.3.2.1. Effects of main components in dairy manure on concentrated acid decrystallization

Urea (2.60 g per 100 g dry dairy manure) and a mineral mixture of 0.42 g sulfur, 7.55 g $CaSO_4$, 2.30 g $MgSO_4$, 0.65 g Na_2SO_4, 6.38 g K_2SO_4, and 1.60 g H_3PO_4 per 100 g

dry dairy manure were used as representatives of nitrogen source and minerals, respectively. Four samples of raw manure, pretreated manure with mineral mixture, pretreated manure with urea, and pretreated manure were decrystallized at a fixed sample-acid ratio of 1:5 (by weight), acid concentration of 70%, and reaction time of 60 minutes. Each sample used for acid decrystallization contained 2 g of dry manure sample mixed with 10 g of acid solution. The decrystallization was carried out in a 200 ml glass mortar using a pestle for continuous stirring. All samples obtained from the decrystallization step were first diluted to the same sample concentration (5%), and then extra acid was added into samples to increase acid concentration to 20%. A titration method using sodium hydroxide was used to measure the acid concentration of all samples. The dilute samples were hydrolyzed at 100°C for 1 hour in order to produce sugars used as the criterion for optimal conditioning.

4.3.2.2. Effects of acid concentration and nitrogen content at different reaction duration

In order to obtain the optimal conditions for decrystallization, three acid concentrations (65%, 70%, and 75%), four treatment durations (30, 60, 90, and 120 minutes) and two nitrogen content levels (1.3% and 2.6%) were studied at a fixed sample-acid ratio of 1:5 (by weight) using a completely randomized design (CRD) with two replications of 24 treatment combinations. Again, each sample used for acid decrystallization contained 2 g of dry manure mixed with 10 g of acid solution. The samples were decrystallized, and further treated to produce sugars under the same conditions as in 4.3.2.1.

4.3.2.3. Effects of sample-acid ratio and reaction time

Using the same experimental design (CRD), the tests were then repeated for three levels of sample-acid ratio (1:5, 3:5, 5:5) and four levels of reaction time (30, 60, 90, and 120 minutes) at the optimal acid concentration and nitrogen content, which were obtained from the previous optimization experiment to conduct the optimal sample-acid ratio and reaction time for decrystallization. All samples after reaction were once again diluted to the same sample concentration (5%) and the same acid concentration (20%). The dilute samples were further hydrolyzed to produce sugars under the same conditions as in 4.3.2.1.

4.3.3. Dilute acid hydrolysis

4.3.3.1. Effects of acid concentration and temperature on acid hydrolysis of decrystallized manure

Two levels of acid concentrations (12.5% and 20%) were tested under a fixed sample concentration of 10% at two different temperatures (100°C and 120°C) to find the relationship between acid concentration and reaction temperature for the acid hydrolysis of decrystallized manure.

4.3.3.2. Effects of reaction time and temperature on dilute acid hydrolysis of decrystallized manure

The ensuing dilute acid hydrolysis was studied by changing temperature and reaction time under fixed sample and acid concentrations of 10% and 12.5%,

respectively. The samples were first decrystallized using the optimal decrystallization conditions determined previously. Then, three levels of temperature (100°C, 120°C and 135°C) and six reaction times (10, 20, 30, 60, 90, 120, 150, 180 minutes) were tested in order to obtain the optimal conditions for dilute acid hydrolysis of decrystallized manure. The hydrolyzed samples were then analyzed for monosaccharides.

4.3.4. Statistical analysis

An Analysis of Variance (ANOVA) of sugar yields was performed for each individual experiment mentioned above using the Statistical Analysis System (SAS 8.0) program.

4.3.5. Analytical methods

Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and NDF ash of the manure were analyzed using Van Soest Fiber Analysis System (Goering et al., 1970). NDF was used to estimate total cellulosic materials (cellulose, hemicellulose, lignin, and insoluble ash) while ADF was used to estimate the concentration of lignin and cellulose. Hemicellulose was determined by a difference (%NDF-%ADF). The content of each monosaccharide in the hydrolyzed solution was determined by an ion chromatography (Dionex, CA) method, which was described previously (Liao et al., 2004). Metal elements, including casium, magnesium, sodium and potassium were analyzed using atomic absorption spectrophotometry (Perkin-Elmer, 1982). Phosphorus and nitrogen and crude protein were determined using AOAC method (Association of Official Analytical Chemists, 1990). Carbon, sulfur and nitrogen contents

in solid samples were measured by automated combustion techniques. A LECO CNS-2000 (LECO, MI) was used to measure the total carbon, the total sulfur and total nitrogen of manure samples. The structure changes during the acid hydrolysis were qualitatively studied using a scanning electron microscope (HITACHI, Japan) (Liao et al., 2004).

4.4. Results and discussion

4.4.1. Concentrated acid decrystallization

4.4.1.1. Effects of nitrogen, acid concentration, and reaction time on concentrated acid decrystallization

4.4.1.1.1. Effects of main components in dairy manure on concentrated acid decrystallization

The comprehensive data of samples shows that, besides the components of lignocellulosics, both of raw manure and pretreated manure contain nitrogen and some minerals (Table 4.1). The pretreated dairy manure contained 50% of the total nitrogen content of the raw manure. This means that more than half of the nitrogen in dairy manure is in soluble forms such as urine, ammonia, and selected proteins. Meanwhile, raw manure has a higher potassium content, and the pretreated manure has a relatively higher calcium content. The effects of nitrogen and mineral mixture on acid decrystallization demonstrated that nitrogen had a significant ($P<0.05$) influence on both glucose yield and hemicellulose-sugar yield during the reaction (Fig. 4.1). Thus, the effects of nitrogen combined with acid concentration and reaction time on decrystallization have to be studied.

4.4.1.1.2. Effects of nitrogen content, acid concentration at different reaction duration

The data demonstrate the effects of nitrogen on decrystallization at different duration (Figs. 4.2 & 4.3). The pretreated manure with low nitrogen content showed higher glucose yields than raw manure at longer reaction times (90 and 120 minutes), although there was no significant difference ($P>0.05$) in glucose yields between the manure samples at shorter reaction time (i.e., 30 and 60 minutes at 65% acid concentration, 30 minutes at 70% and 75% acid concentration) (Fig. 4.2). This can be explained as that the longer the decrystallization time, the more the glucose from the raw manure was consumed by browning reaction because of the presence of both concentrated acid and a relatively high concentration of nitrogen in the raw manure (Fennema, 1996). Hemicellulose-sugar (i.e., xylose + arabinose + galactose) yields of raw manure at all reaction times were lower than those of pretreated manure (Fig. 4.3). Compared to glucose yield from acid decrystallization of manure cellulose, nitrogen content had a much more pronounced impact on hemicellulose-sugar yield during decrystallization of manure fiber.

Meanwhile, sugar yields were also changed following the increase of acid concentration and reaction time. At the low acid concentration of 65%, glucose yields of both raw manure and pretreated manure showed an increase during the period from 30 to 120 minutes, and peaked at roughly 32% and 46% at the reaction time of 120 minutes, respectively (Fig. 4.2). The glucose yields for both samples continued increasing following the increase of acid concentration from 65% to 75%. At the high acid concentration of 75% however, the glucose yields of both samples reached the maximum

of around 90% at 30 minutes. The glucose yields thereafter continuously decreased as reaction time progressed. Also, the glucose yields from raw manure decreased at a much faster rate than that of pretreated manure. These phenomena might have been caused by a much stronger browning reaction in raw manure at high acid concentration (Fennema, 1996). The results from both manure samples indicated that a higher acid concentration and a shorter reaction time were beneficial to glucose production from decrystallization of manure cellulose.

In the same reactions, changes in hemicellulose-sugar yields showed some difference from the glucose yields reported above (Fig. 4.3). Hemicellulose-sugar yields of both manures decreased following the increase in acid concentration. The highest hemicellulose-sugar yield from the hydrolyses of raw manure and pretreated manure were 80% and 100%, respectively, which were obtained at 65% acid concentration and 30-minutes reaction time. On the other hand, hemicellulose-sugar yields of both samples at different levels of acid concentrations barely changed following an increase of reaction time. This result, combined with higher sugar yield at relatively low acid concentration (65%), suggested that hemicellulose was more easily degraded than cellulose.

Statistical analysis of sugar yields further indicated that there were significant differences ($P<0.05$) caused by the effects of each individual factor (nitrogen content, reaction time, and acid concentration) on the production of both glucose and hemicellulose-sugar (Table 4.2). The analysis also presents that there were significant two-way interactions ($P<0.05$) between some variables (i.e., nitrogen/time, time/acid) on glucose production, and no significant two-way, three-way interactions ($P>0.05$) on hemicellulose-sugar production (Table 4.2). This means that only the effects on

hemicellulose-sugar production be directly interpreted by comparison of mean values of each factor. Furthermore, a pair-wise comparison using least squares means (lsmeans) has to be used for interpreting glucose production. The resulting analysis indicates that nitrogen influenced hemicellulose-sugar yield at all reaction conditions and glucose yield at reaction times longer than 60 minutes during decrystallization. At the reaction time of 30 minutes, there were no significant differences ($P>0.05$) in glucose yield between raw manure and pretreated manure at each individual acid level. The analysis also shows that there was no significant difference ($P>0.05$) among three treatments that had an average highest yield of 89% (Table 4.3); and hemicellulose-sugar yield reached an average highest value of 97% from two treatments (Table 4.4).

In summary, the results indicate that nitrogen content had a negative effect ($P<0.05$) on acid decrystallization of dairy manure. It was also concluded from the results that a higher acid concentration and shorter time of decrystallization were beneficial for obtaining the highest glucose yields from cellulose in both raw manure and pretreated manure, while a lower acid concentration and shorter time of decrystallization were good for obtaining the highest hemicellulose-sugar yield from pretreated manure. Since dairy manure fiber is mainly composed of cellulose (Table 4.1), glucose yield from cellulose was the main concern once the conditions of decrystallization were optimized. The pretreated manure had a higher cellulose and hemicellulose content than the raw manure, thus it consequently yielded higher sugar concentrations (6 g hemicellulose-sugar/L, 16 g glucose/L) in the hydrolyzed solution than the raw manure (4 g hemicellulose-sugar/L, 11 g glucose/L)(Fig. 4.4). Therefore, considering all the facts, the optimal conditions for decrystallization of manure samples under the specific sample-acid ratio of 1:5 were (1)

30-minutes reaction time, and (2) 75% acid concentration using pretreated manure with low nitrogen content.

4.4.1.2. Effects of sample-acid ratio and reaction time on concentrated acid decrystallization of pretreated dairy manure

Statistical analysis of sugar yields showed that the sample-acid ratio was a major factor that has significant influence ($P<0.05$) on the production of glucose and hemicellulose-sugar (Table 4.5). Since there was two-way interaction between sample-acid ratio and reation time on sugar yields, a pair-wise comparison was analyzed again to obtain the optimal ratios.

The result indicated that glucose yields were greater for ratios of 1:5 and 3:5 than for ratios of 5:5 (Fig. 4.5a) and there were no significant differences ($P>0.05$) between samples from the two ratios at each individual reaction time. This means that a small amount of acid (large sample-acid ratio) was insufficient to break down the structure of cellulose in manure fiber to release glucose. Thus, increasing the total amount of acid (lowering the sample-acid ratio) in the reaction system effectively improved the performance of acid decrystallization. Also, once the amount of acid reached a certain level, for instance of a 3:5 sample-acid ratio, the acid was able to almost completely break down the structure of cellulose, while a further increase in the amount of acid did not benefit the final sugar production. Therefore, the 3:5 of sample to acid ratio was the optimal one for the acid decrystallization of cellulose from pretreated manure.

Meanwhile, the same analysis on hemicellulose-sugar production shows that the ratio of 3:5 was the best ratio for decrystallization of hemicellulose as well (Fig. 4.5b).

Hemicellulose-sugar yield reached 96% at the ratio of 3:5 in 30 minutes of decrystallization time, which was much higher than the 78% yield from the ratio of 1:5. Also, compared to 100% hemicellulose-sugar yield from the combination of 1:5 ratio and 65% acid from the previous experiment described in Section 4.4.1.1, there was no significant difference ($P>0.05$) between yields. This finding suggests that the negative effect of increasing decrystallization acid concentration on hemicellulose hydrolysis can be overcome by increasing the sample-acid ratio. An increase of sample-acid ratio to 75% acid concentration can increase both sugar yields to their highest yields (i.e., 96% for hemicellulose-sugar, 90% for glucose).

In addition, Fig. 4.5 also shows that, at sample-acid ratios of 1:5 and 3:5, sugar yields from both cellulose and hemicellulose at 30 minutes of reaction time were significantly higher ($P<0.05$) than yields from other reaction time. The result further indicated that shorter time of decrystallization at relatively low sample-acid ratios was beneficial for acid decrystallization of pretreated dairy manure.

Thus, the optimal sample-acid ratio for the concentrated acid decrystallization of pretreated dairy manure was 3:5, and the optimal reaction time was 30 minutes.

4.4.2. Dilute acid hydrolysis

4.4.2.1. Determination of acid concentration

Both the glucose and hemicellulose-sugar yields decreased as reaction temperature increased when the higher acid concentration (20%) was used (Tables 4.6 & 4.7). At a lower acid concentration of 12.5%, although both sugar yields were relatively lower, an increase in glucose yield (+12%) was significant ($P<0.05$). The results

suggested that an increase in temperature for dilute acid hydrolysis at an acid concentration of 12.5% will allow for the production of more sugars from decrystallized pretreated manure. For this reason and the added environmental benefit of using a lower acid concentration, a 12.5% decrystallization acid concentration was used for the remaining studies involving dilute acid hydrolysis.

4.4.2.2. Effects of reaction time and reaction temperature

Sugar yields varied with different reaction temperatures and time (Fig. 4.6). Glucose and hemicellulose-sugar yields both showed that low temperature under dilute acid condition did not convert much cellulose and hemicellulose to sugars, although the sugar yields did increase with an increase in reaction time. It is also shown that the higher the temperature, the more rapid the sugars were consumed by the side reactions such as dehydration reaction and browning reaction. Thus, shorter time and higher temperature were beneficial for increasing sugar yields, especially glucose yield. A pair-wise comparison using least squares means (lsmeans) was used again to statistically obtain the optimal conditions of sugar production. The treatment of 135°C for 10 minute was the only treatment that gave the highest glucose yield of 84% (Fig. 4.6a), achieving a glucose concentration of 26 g/L. Meanwhile, hemicellulose-sugar yield and concentration reached the highest values of 91% and 13 g/L, respectively, under the condition of 120°C and 10 minutes (Fig. 4.6b).

Considering the fact that the cellulose content of pretreated dairy manure (about 32%) was much higher than hemicellulose (about 14%) (Table 4.1), the priority of dilute acid hydrolysis should be to degrade manure cellulose for glucose. Thus, 10% of sample

concentration, 12.5% of acid concentration, 135°C, and 10 minutes of reaction time were determined as the optimal conditions for dilute acid hydrolysis of decrystallized pretreated dairy manure. Under the optimal conditions, dilute acid hydrolysis of hemicellulose produced an 83% sugar yield and 11.5g/L of hemicellulose-sugar. Meanwhile, compared to other lignocellulosic materials such as woods, the glucose yield of acid hydrolysis of dairy manure was as high as that from the hydrolysis of sweetchip (glucose yield of 85%) (Bayat-makooi et al., 1985). However, the acid concentration of 12.5% used in the second step of hydrolysis was much lower than the acid concentration in the hydrolysis of sweetchip, which was 68%.

4.4.3. Structural changes of manure fiber during the hydrolysis

The difference in fiber structure between raw manure and pretreated manure was discussed in the previous study (Liao et al., 2004). Figure 4.7, generated using a SEM, demonstrated that, after acid decrystallization, the crystal structure of pretreated manure fiber was completely destroyed and turned into an amorphous powder. Meanwhile, fiber data of decrystallized pretreated manure showed that the remaining amorphous powder were mainly lignin (data not shown), and there was very little glucose and hemicellulose-sugar produced from the decrystallization step (data not shown). These results indicated that most of the hemicellulose and cellulose were degraded to poly- or oligo-saccharides by concentrated acid and then dissolved in the solution during the dilute process. As a result, the poly- or oligo-saccharides in the solution were rather easy to convert to corresponding monosaccharides using dilute acid hydrolysis.

4.5. Conclusion

The nitrogen content in nitrogen-rich cellulosic material such as dairy manure has a considerable impact on acid hydrolysis of fiber for producing sugar, which is consistent with our previous study of dilute acid hydrolysis of hemicellulose in manure fiber (Liao et al., 2004). The removal of nitrogen from dairy manure made acid decrystallization of manure fiber more effective. During the ensuing acid hydrolysis, acid concentration can be effectively decreased by increasing the reaction temperature. The optimal conditions for acid hydrolysis of pretreated dairy manure were 75% acid concentration, 3:5 sample to acid ratio, and 30 minutes of reaction time for decrystallization, followed by dilute acid hydrolysis conditions of 12.5% acid and 10% sample at 130°C for 10 minutes. Furthermore, the optimal reaction times of both acid decrystallization and dilute acid hydrolysis were very short, which could make the continuous operation of acid hydrolysis of dairy manure fiber possible.

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Table 4.1. Characteristics of sample manures[a]

	Raw manure	*Pretreated manure*
Dry Matter, wt %	15.50 ± 0.09	14.20 ± 0.05
Crude Protein, % dry basis	16.44 ± 0.53	8.19 ± 0.25
NDF, % dry basis	48.27 ± 0.46	61.17 ± 1.62
ADF, % dry basis	35.80 ± 0.14	47.33 ± 1.70
ADL, % dry basis	13.91 ± 0.45	15.94 ± 0.66
Cellulose (=ADF-ADL), % dry basis	21.89 ± 0.38	31.40 ± 1.05
Hemicellulose(=NDF-ADF), % dry basis	12.47 ± 0.32	13.83 ± 0.79
NDF Ash, % dry basis	6.60 ± 0.21	12.40 ± 0.56
N, % dry basis	2.63 ± 0.09	1.31 ± 0.04
C, % dry basis	45.49 ± 0.30	39.46 ± 1.51
S, % dry basis	0.42	0.56
Ca, % dry basis	2.22	2.71
Mg, % dry basis	0.46	0.38
Na, % dry basis	0.21	0.14
K, % dry basis	2.86	1.17
P, % dry basis	0.48	0.54

a: Data is the average of triplicates with standard deviations (n=3).

Table 4.2. Analysis of variance (ANOVA) table of effects of nitrogen, acid concentration, and reaction time on acid decrystallization

Source term	Degree of freedom		Mean square		F-ratio		P-value[a]	
	Glucose	Sugars from hemicellulose	Glucose	Sugars from hemicellulose	Glucose	Sugars from hemicellulose	Glucose	Sugars from hemicellulose
Nitrogen	1	1	717.28	2274.31	77.11	194.26	<0.0001	<0.0001
Acid concentration	2	2	10021.48	1567.72	1077.29	133.91	<0.0001	<0.0001
Reaction time	3	3	306.97	269.09	33.00	22.98	<0.0001	<0.0001
Time×Nitrogen	3	3	51.20	6.82	5.50	0.58	0.0051	0.6324
Time×Acid conc.	6	6	462.44	26.04	49.71	2.22	<0.0001	0.0757
Acid conc.×Nitrogen	2	2	20.24	18.09	2.18	154	0.1354	0.2334
Acid conc.×Nitrogen×Time	6	6	19.87	22.37	2.14	1.91	0.0862	0.1203
Error	24	24	9.30	11.71				
Total	47	47						

a: term significant at α=0.05

Table 4.3. The highest glucose yield with different decrystallization conditions

Time (minutes)	Acid conc. (wt%)	Nitrogen content (wt%)	Mean of monosaccharide concentration from cellulose (g/L)	Mean of sugar yields from cellulose (g/100 cellulose in manure)
30	75	1.3	15.73	91.28
60	75	1.3	15.17	87.45
30	75	2.6	11.31	88.88

Table 4.4. The highest hemicellulose-sugar yield with different decrystallization conditions

Time (minutes)	Acid conc. (wt%)	Nitrogen content (wt%)	Mean of monosaccharide concentration from hemicellulose (g/L)	Mean of sugar yields from hemicellulose (g/100 hemicellulose in manure)
30	65	1.3	6.74	98.25
60	65	1.3	6.60	95.36

Table 4.5. Analysis of variance (ANOVA) table of effects of sample-acid ratio and reaction time on acid decrystallization

Source term	Degree of freedom		Mean square		F-ratio		P-value[a]	
	Glucose	Sugars from hemicellulose	Glucose	Sugars from hemicellulose	Glucose	Sugars from hemicellulose	Glucose	Sugars from hemicellulose
Time	3	3	46.09	11.36	1.62	0.53	0.2369	0.6675
Sample-acid ratio	2	2	5828.78	1009.57	204.73	47.46	<0.0001	<0.0001
Time×Sample-acid ratio	6	6	290.77	348.06	10.21	16.36	0.0004	<0.0001
Error	12	12	28.47	21.27				
Total	23	23						

a: term significant at α=0.05

Table 4.6. The effect of acid concentration and reaction temperature on glucose yields of acid hydrolysis of decrystallized pretreated manure samples[1, 2,]

Temperature (°C) \ Acid Conc. (wt%)	12.5%	20%
100°C	30.46±0.85	77.94±1.78
120°C	42.56±0.47	70.91±2.83

1. decrystallized pretreated manure samples were obtained by acid decrystallizion using the optimal conditions in section 3.2.
2. data is the average of triplicates with standard deviations (n=3).

Table 4.7. The effect of acid concentration and reaction temperature on hemicellulose-sugar yields of acid hydrolysis of decrystallized pretreated manure samples[1, 2]

Acid Conc.(wt%) / Temperature (°C)	12.5%	20%
100°C	27.08±1.43	89.57±7.10
120°C	29.53±1.02	78.23±10.18

1. decrystallized pretreated manure samples were obtained by acid decrystallizion using the optimal conditions in section 3.2.
2. data is the average of triplicates with standard deviations (n=3).

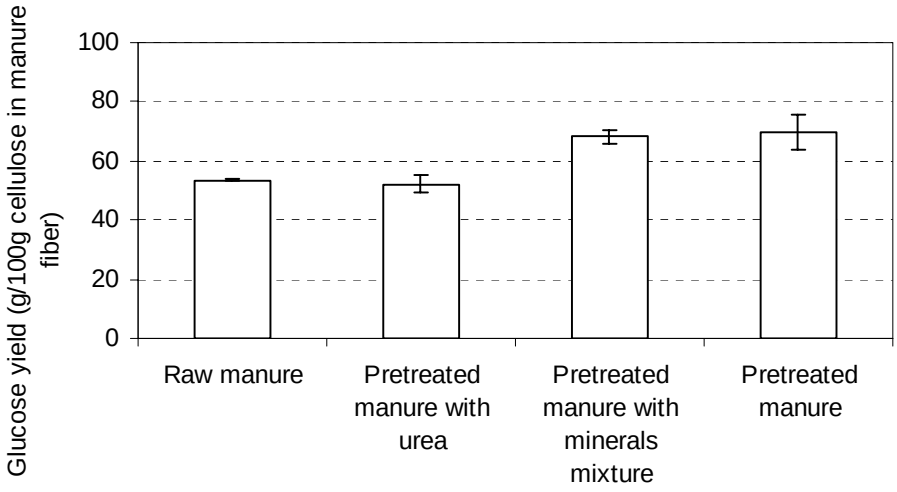


Fig. 4.1(a). Glucose

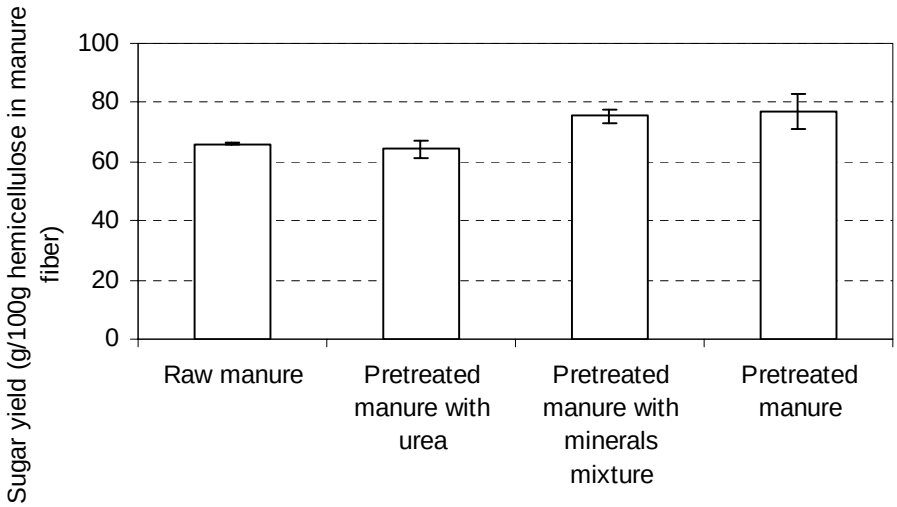


Fig. 4.1(b). Hemicellulose-sugar

Fig. 4.1. Effects of nitrogen, mineral mixture on concentrated acid decrystallization (1): data are presented as the mean of three replicates with standard deviation.

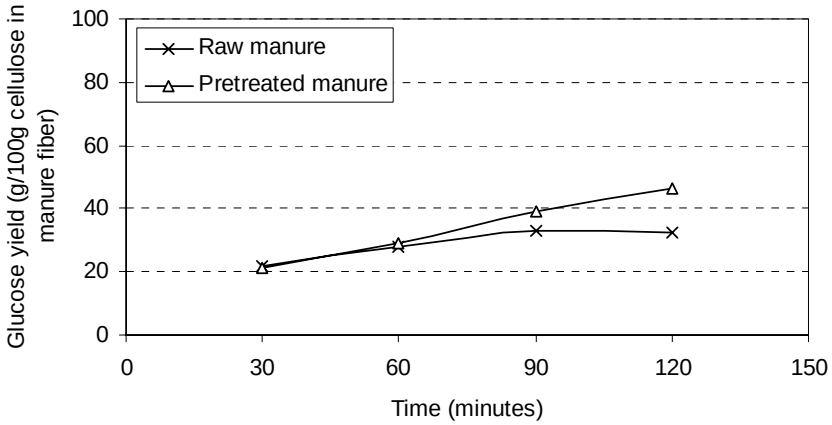


Fig. 4.2(a). 65% acid concentration

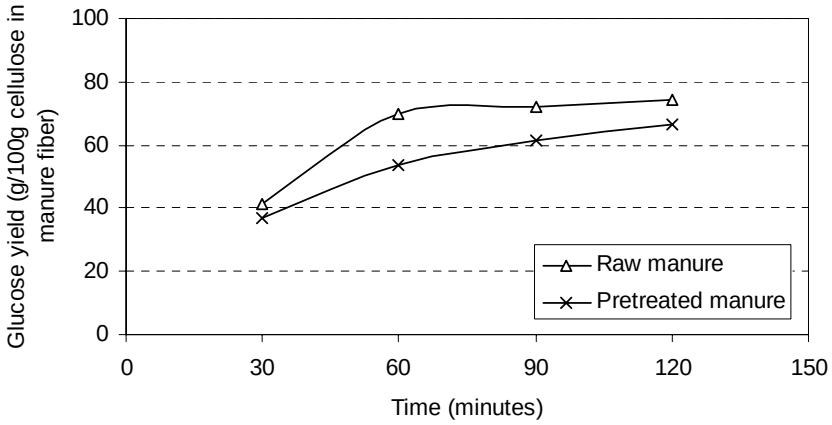


Fig. 4.2(b). 70% acid concentration

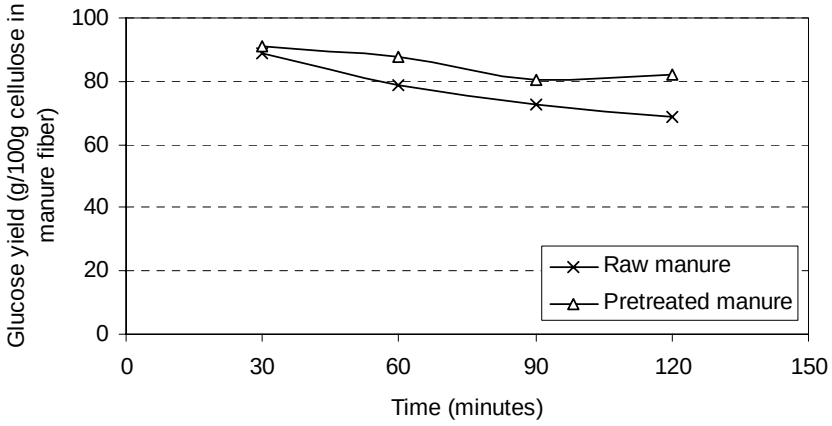


Fig. 4.2(c). 75% acid concentration

Fig. 4.2. Effect of nitrogen, acid concentration and reaction time (at sample-acid ratio of 1:5) on glucose yield of acid decrystallization of raw dairy manure and pretreated manure (1): data are presented as the mean of two replicates.

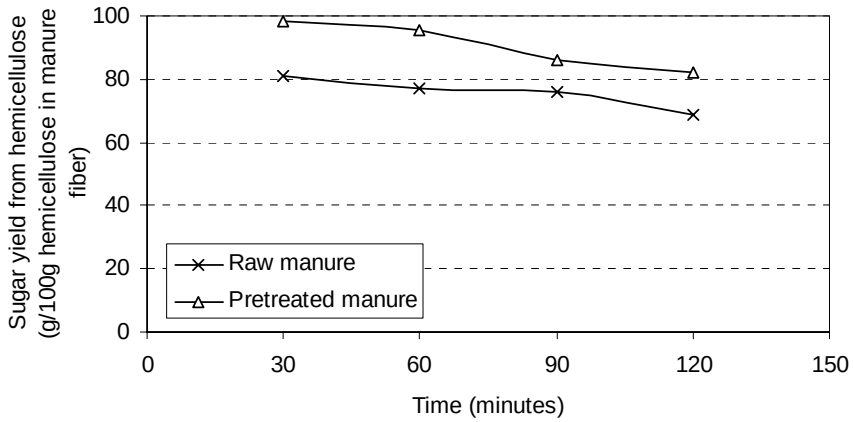


Fig. 4.3(a). 65% acid concentration

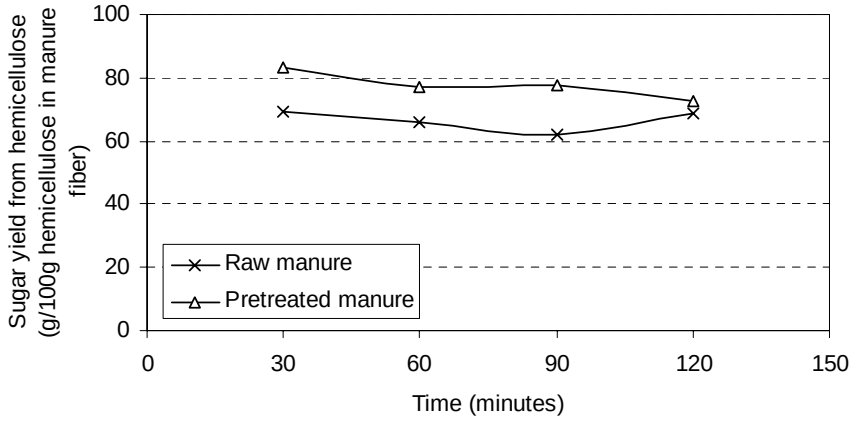


Fig.4.3(b). 70% acid concentration

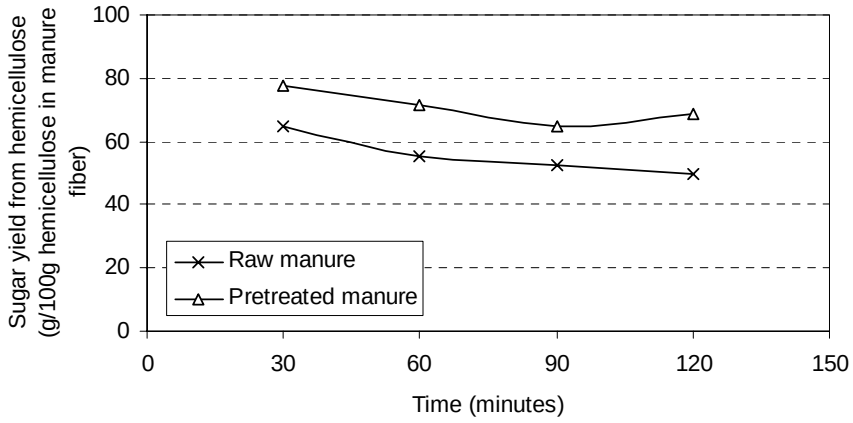


Fig. 4.3(c). 75% acid concentration

Fig. 4.3. Effect of nitrogen, acid concentration and reaction time (at sample-acid ratio of 1:5) on sugar yield from hemicellulose of acid decrystallization of raw dairy manure and pretreated manure. (1): data are presented as the mean of two replicates.

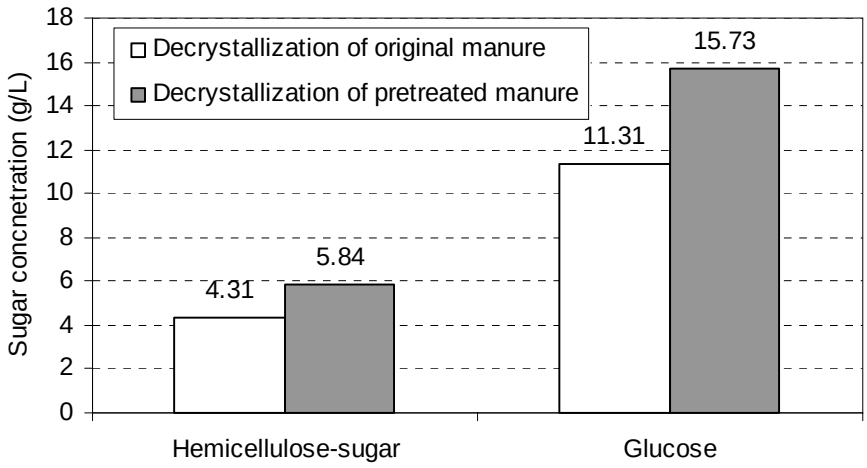


Fig. 4.4. Sugar concentrations of decrystallized raw manure and pretreated manure at 30 minutes decrystallization using 75% acid[1, 2]

1: samples were diluted to acid concentration of 20% and sample concentration of 5%. Sugar concentrations were obtained from the dilute samples hydrolyzed at 100°C for 1 hour.
2: data are presented as the mean of two replicates.

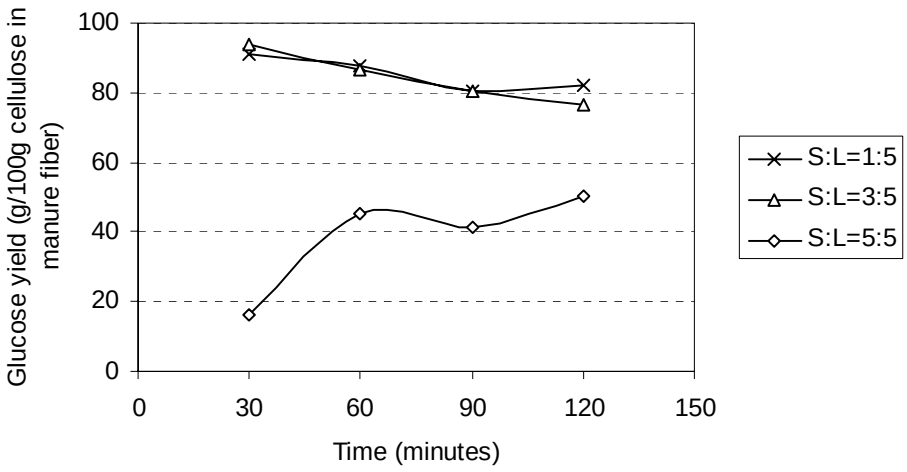


Fig. 4.5(a). Glucose yield from cellulose

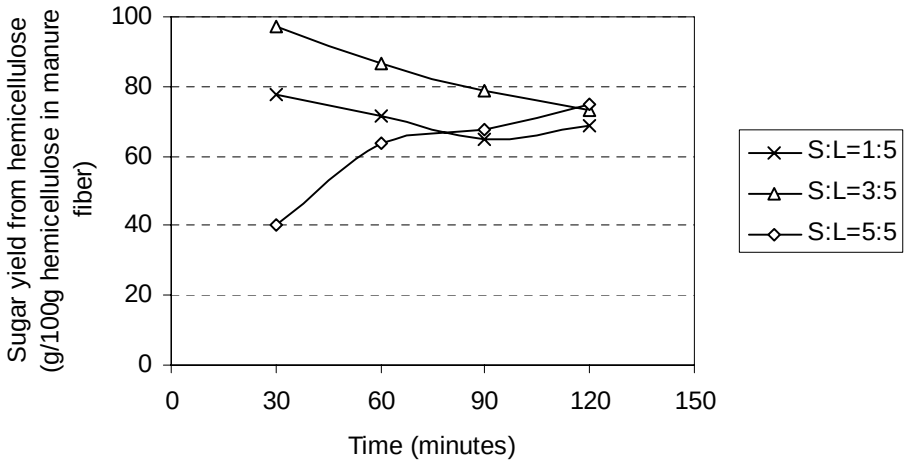


Fig. 4.5(b). Sugar yield from hemicellulose = grams of total monosaccharides (xylose + arabinose + galactose) / g of hemicellulose in raw material

Fig. 4.5. The effect of sample-acid ratio of decrystallization of pretreated dairy manure at 75% acid concentration

(1): S:L means total amount of sample to total amount of acid solution
(2): Data are presented as the mean of two replicates.

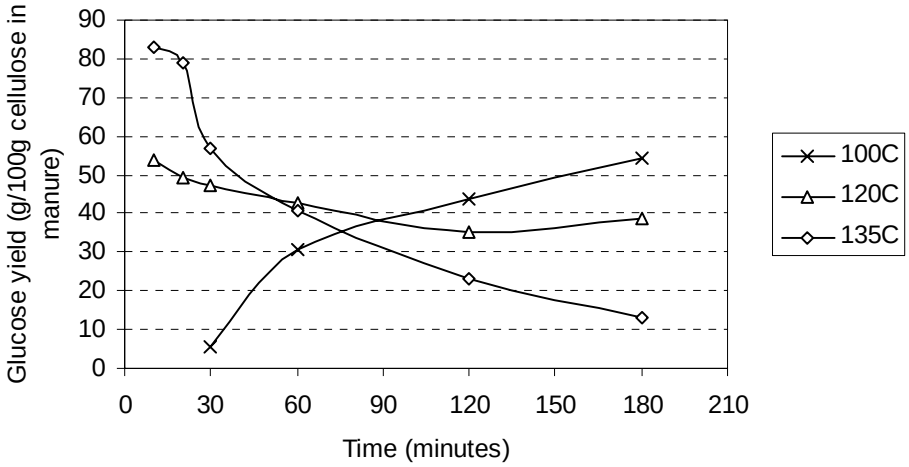


Fig. 4.6(a). Glucose yield

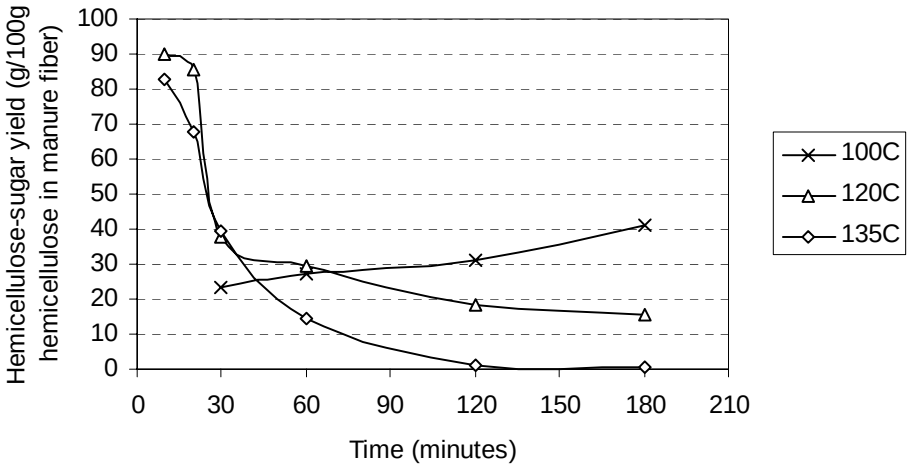
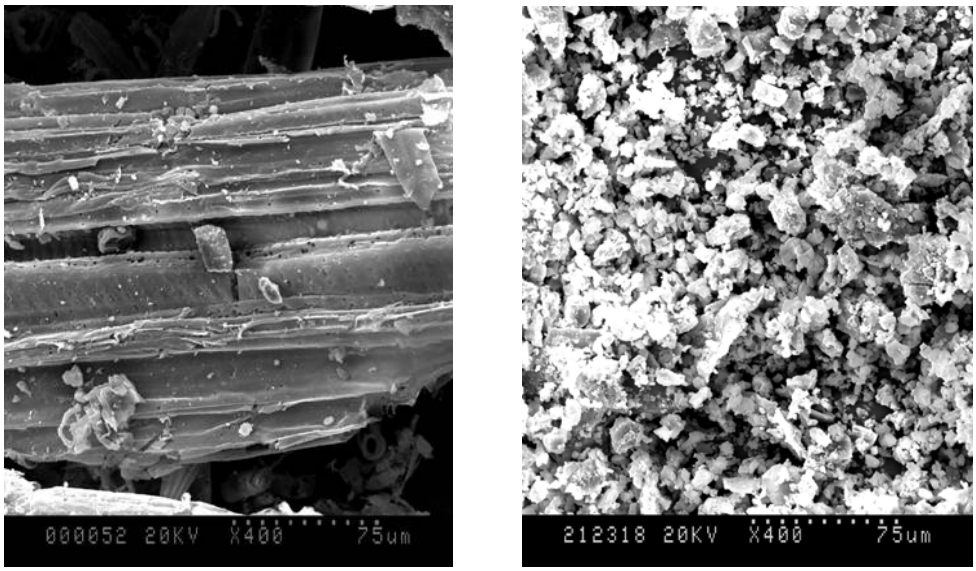


Fig. 4.6(b). Hemicellulose-sugar yield[1]

Fig. 4.6. Effect of different reaction temperatures and time on sugar yields of dilute acid hydrolysis of decrystallized pretreated dairy manure[2]

(1). hemicellulose-sugar yield = grams of total monosaccharides (xylose + arabinose + galactose) / g of hemicellulose in raw material
(2). data are presented as the mean of two replicates.



(a) (b)

Fig. 4.7. Scanning electron micrograph of acid decrystallization of pretreated manure (400x)

a: Pretreated dairy manure
b: Solid part of decrystallized pretreated manure from acid decrystallization of 30 minutes, 1:5 sample-acid ratio and 75% acid concentration

CHAPTER FIVE

EFFECTS OF HEMICELLULOSE, LIGNIN ON ENZYMATIC HYDROLYSIS OF CELLULOSE FROM DAIRY MANURE

5.1. Abstract

This study focused on the effect of hemicellulose and lignin on enzymatic hydrolysis of dairy manure and hydrolysis process optimization to improve sugar yield. It was found that hemicellulose and lignin in dairy manure, similar to their role in other lignocellulosic material, were major resistive factors to enzymatic hydrolysis and that the removal of either of them, or for best performance, both of them, improved the enzymatic hydrolysis of manure cellulose. This result combined with scanning electron microscopy (SEM) pictures further supported that the accessibility of cellulose to cellulase was the most important feature to the hydrolysis. Quantitatively, fed-batch enzymatic hydrolysis of fiber without lignin and hemicellulose had a high glucose yield of 52% with respect to the glucose concentration of 17 g/L at a total enzyme loading of 1300 FPU/L and reaction time of 160 hours, which was better than corresponding batch enzymatic hydrolysis.

Key words: cellulose, fed-batch enzymatic hydrolysis, glucose, hemicellulose, yield

5.2. Introduction

Nearly 160 million dry tons of manure is produced annually in the United States. Approximately 55 million dry tons of animal manures are collected for disposal, with about 75% of the total coming from dairy and feed-lot cattle (Council for Agricultural

Science and Technology, 1995). Animal manures are rich in carbohydrate and protein which could be further converted into biobased chemicals, materials and energy. Dairy manure, representing the largest percentage, contains about 12% hemicellulose and 22% cellulose (Liao et al., 2004), which provides a large potential source of carbohydrates that are capable of producing monosaccharides such as glucose, xylose, arabinose and galactose through various hydrolysis processes.

Enzymatic hydrolysis has attracted increasing attention as an alternative to acid hydrolysis for converting lignocellulosic materials to sugars because the process has highly specific yet mild reaction conditions (pH around 5 and temperature less than 50°C) as well as a lack of corrosion problems (Sun et al., 2002). Cellulase is widely used to carry out enzyme hydrolysis of lignocellulosic material such as wood and wheat straw. Cellulase consists of endo 1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and β-glucosidase. The endoglucanase attacks cellulose to create free chain-ends, the exo-glucanase degrades the molecule by removing cellobiose from the free chain-end, and the β-glucosidase produces glucose by breaking down the cellobiose (Bhat et al., 1997). Cellulose in lignocellulosic materials cellulose is physically associated with hemicellulose and physically and chemically associated with lignin (Ladish et al., 1989). The resulting matrix prevents cellulose from being attacked by cellulase (Ladish et al., 1983). There are numerous studies on the effect of this hemicellulose and lignin matrix on enzymatic hydrolysis of cellulose (Fan et al., 1981; Fernandex-Bolanos et al., 2001; Mooney et al., 1998; Kim et al., 2003; Draude et al., 2001). None of these efforts, however, investigated the hydrolysis of cellulose from animal manure, which might be one of the most difficultly digestible cellulosic materials because most of the easily

accessible fiber from forage has already been degraded by cellulolytic enzymes in the animal's digestive system.

There are a number of pretreatment methods, in particular chlorite and dilute acid, which are capable of removing hemicellulose and lignin, respectively (Ahlgren et al., 1970; Roberto et al., 2003). Sodium chlorite has been reported as one of the most effective reagents for the removal of lignin from lignocellulosic materials and is widely used by the paper industry for deligninfication and bleaching. Dilute acid treatment has been shown in our previous work to be effective in completely removing all of the hemicellulose in manure with the least loss of cellulose (Liao et al., 2004).

The objective of this work was to study the effect of hemicellulose and lignin in dairy manure on enzymatic hydrolysis of cellulose, and further optimize the enzymatic hydrolysis process to obtain the best performance. The three treated manure samples investigated in the study were: hemicellulose-free manure fiber from dilute acid treatment, lignin-free manure fiber from sodium chlorite treatment, and manure fiber without lignin and hemicellulose from a combination of dilute acid and chlorite treatments.

5.3. Materials and methods

5.3.1. Materials

Fresh dairy manure was obtained from the Dairy Center of Washington State University. The manure had 15.3% dry matter (DM) with a total carbon content of 46.9 g/100g DM and total nitrogen content of 2.6 g/100g DM. Ten kilograms of original manure was mixed with 5 kg of water and blended for 1 minute to achieve size reduction,

and then 10 kg of the mixture was washed three separate times with 5 kg of water. Solid-liquid separation using a centrifuge at 3,000 rpm for 10 minutes isolated most of the soluble nitrogen and impurities within the liquid fraction. The solid part was collected and dried as manure fiber for hydrolysis. The data for the manure fiber are presented in Table 5.1.

The enzyme, celluclast 1.5 L (Sigma, St. Louis, MO) was used for the study of enzymatic hydrolysis. Celluclast 1.5 L contained 145.5 FPU/g solution of cellulase (Filter paper activity unit, FPU). One unit of FPU is defined by the Commission on Biotechnology, IUPAC as the enzyme amount, which releases 1 μmol of glucose equivalents from Whatman No. 1 filter paper in 1 minute (Ghose, 1987).

5.3.2. Pretreatment

Hemicellulose-free manure fiber was obtained by applying optimized dilute acid treatment from a previous study on manure fiber (Liao et al., 2004). The treatment was conducted using 1% sulfuric acid and 5% substrate concentration at 135°C for 2 hours. After dilute acid treatment, the samples were filtered through Whatman No. 5 filter paper and washed by water until the pH of solution was around 4. After the washed solid part was dried at 100°C overnight the hemicellulose-free manure fiber sample was ready to be hydrolyzed.

Chlorite treatment was used to degrade and extract lignin from the manure fiber (Ahlgren et al., 1970). The substrate concentration was 5%. The chemical mass ratio was 0.3 g sodium chlorite and 0.1 ml glacial acetic acid per g of manure fiber. The sample was treated at 70°C for an hour. The ensuing liquid-solid separation step was carried out

using a No. 120 standard screen with the solid part being washed by 1000 ml of water. After being dried at 100°C the lignin-free manure fiber sample was obtained.

Manure fiber without hemicellulose and lignin was obtained by applying both the dilute acid treatment and chlorite treatment. The acid treatment was used first, followed by chlorite treatment. Each individual step was the same as described before.

5.3.3. The effect of hemicellulose, lignin on enzymatic hydrolysis

Dry sample (2.5 g) from each different type of manure fiber was mixed with 50 ml of cellulase solution. Two enzyme loadings (650 FPU/L and 1000 FPU/L) were studied. The pH of enzymatic hydrolysis was 4.8 and the reaction temperature was 50°C (Wen et al., 2004). Aliquot samples were taken at 3 hour intervals for the first 10 hours and 24 hour intervals for the rest of the 96 hours.

5.3.4. Enzymatic hydrolysis of ensuring treated manure fiber

The same ratio of dry sample to enzyme solution was used in this part of the study. The effect of enzyme loading was studied at four levels of 650 FPU/L, 800 FPU/L, 1000 FPU/L and 1300 FPU/L. A pH of 4.8, reaction temperature of 50°C, and reaction time of 144 hours were employed.

5.3.5. Fed-batch enzymatic hydrolysis of ensuring treated manure fiber

The enzymatic hydrolysis of treated manure fiber started at a level of 650 FPU/L with another 650 FPU/L enzyme solution added into the solution at 24 hours. In evaluating the performance, the fed-batch enzymatic hydrolysis was compared with two

separate batch hydrolyses with 650 FPU/L enzyme and 1300 FPU/L enzyme, respectively.

5.3.6. Statistical analysis

An Analysis of Variance (ANOVA) of sugar yields was tested for each individual experiment mentioned above using the Statistical Analysis System (SAS 8.0) program.

5.3.7. Analytical methods

Enzyme activities during hydrolysis were measured according to the recommendation for dilute enzyme solution by the IUPAC committee (Ghose, 1987). 0.5 ml enzyme solution was added into 1ml of 0.05 M sodium-citrate buffer (pH 4.8) contained in a strip of 50 mg Whatman No.1 filter paper. The solution was incubated in a shaker at 50°C for an hour. The enzyme unit was defined as the total amount of glucose release per minute from 0.5 ml enzyme solution.

Fiber data including neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed using the reflux apparatus (Goering et al., 1970). NDF was used to estimate the total cellulosic materials (cellulose, hemicellulose, lignin, and insoluble ash) while ADF was used to estimate the concentration of lignin and cellulose. Hemicellulose was determined by the difference (%NDF-%ADF). The content of each monosaccharide in the hydrolyzed solution was determined using an ion chromatography (IC) from Dionex (Liao et al., 2004). Carbon and nitrogen contents in solid samples were measured by automated combustion techniques. The LECO CNS-2000 was used to measure the total carbon and total nitrogen of manure samples. The

structure changes during the acid hydrolysis were qualitatively studied using a scanning electron microscope (SEM) from HITACHI (Liao et al., 2004).

5.4. Results and discussion

5.4.1. Effects of hemicellulose and lignin on enzymatic hydrolysis of manure fiber

Dilute acid and chlorite treatments not only removed the corresponding hemicellulose and lignin, but also significantly increased the cellulose content at the same time (Table 5.2). After treatments, hemicellulose and cellulose contents in lignin-free manure fiber reached 18% and 48%, respectively, and hemicellulose-free manure fiber had 43% of cellulose and 29% of lignin. Meanwhile, the combination of dilute acid and chlorite treatment removed most of the hemicellulose and lignin, making the manure fiber without hemicellulose and lignin have the highest cellulose content at 66%.

Enzymatic hydrolysis of differently treated manure fiber demonstrates that removing either hemicellulose or lignin, especially both, was beneficial for improving glucose yield of the hydrolysis (Fig. 5.1). Compared to manure fiber, all three of the treated manure fibers showed much higher glucose yields from both of the enzyme loading levels of 650 FPU/L and 1000 FPU/L. At the low enzyme loading level of 650 FPU/L, hydrolysis of hemicellulose-free manure fiber performed better than the other two treated fibers during the first 48 hours. After 48 hours, the performance of hydrolysis of manure fiber without hemicellulose and lignin became the best among the four hydrolyses. The same trend occurred at the higher enzyme loading of 1000 FPU/L although the glucose yield without hemicellulose and lignin passed the others in just 24 hours. The comparison of sugar concentrations and sugar yields of these two enzyme

loading levels at the end of the reaction is presented in Fig. 5.2. The highest values of glucose concentration and yield of 14 g/L and 42%, respectively, were from the hydrolysis of fiber without hemicellulose and lignin, although all three pretreatments showed improvements based upon the control. Additionally, glucose production for all three pretreatments increased as enzyme loading increased and at a rate that was higher than was observed in the control. In particular, fiber without hemicellulose and lignin showed the largest increase of around 15% which was almost 3 times higher than the increases of the other two treated samples.

The enzyme activity changes of each individual sample were then studied in an attempt to better understand the influences of hemicellulose and lignin on the enzymes during the hydrolysis (Fig. 5.3). Since enzymatic hydrolysis of manure fiber is a type of heterogeneous system, the functional enzyme should be those absorbed on the surface of the fiber. This means, the more enzyme activity in the liquid, the less enzyme absorbed by the fiber. Fig. 5.3 shows that enzyme in liquid from hydrolysis of fiber without hemicellulose and lignin had the lowest enzyme activity. This fact, combined with the observation that cellulase had a slight decay during the retention time of 160 hours (Data not shown), led to the conclusion that hydrolysis of fiber without hemicellulose and lignin had more enzyme absorbed by the cellulose than the other treated manure fibers. This result was consistent with the observations of glucose yields in Fig. 5.1. It showed that the more enzyme was absorbed by fiber, the more glucose was produced. This also indicated that either hemicellulose or lignin might partially inhibit the enzyme physically or chemically and preventing cellulose from being attacked. Fig. 5.3 also shows that the aqueous enzyme activities within lignin-free fiber and hemicellulose-free fiber had no

significant difference ($P>0.05$) at the individual reaction time during the hydrolysis (except at 12 hours and 24 hours). This means that almost the same amount of enzyme was absorbed by both samples. However, as previously discussed, hemicellulose-free fiber had higher glucose yields than lignin-free fiber (Fig. 5.1). This indicated that the functional enzyme was more readily absorbed in hemicellulose-free fiber than in lignin-free fiber. The reason might be that although lignin-free fiber and hemicellulose-free fiber had similar surface areas to absorb the enzyme, hemicellulose-free fiber might have been slightly more accessible to the enzyme because of the changes made to the structure during the high temperature acid pretreatment used to obtain the pretreated sample. This difference in pretreatment and in particular the temperature at which they were exposed might explain the observation that both samples absorbed the same amount of enzyme, but produced slightly but significantly ($P<0.05$) different amounts of glucose.

Structural changes of different treated manure fibers were observed by SEM in order to better determine how lignin and hemicellulose influence the hydrolysis, physically or chemically (Fig. 5.4). The difference in fiber structure between manure fiber and treated manure fiber are presented in Figs. 5.4a, 5.4b, 5.4c and 5.4d. The micrographs qualitatively show the removal or washing off of a considerable amount of substance from the backbone of the pretreated fiber. However, it was apparent that the main structure of lignin-free and hemicellulose-free fibers was not broken down (Fig. 5.4b, 5.4c). The micrographs, combined with the characteristics of the fibers (Table 5.2), elucidated that either the lignin in hemicellulose-free fiber or the hemicellulose in lignin-free fiber was still attached to the cellulose, which formed the shield to protect those cellulose inside of the fiber. The structure of manure fiber without hemicellulose and

lignin was clearly different from the other two treated fibers with most of the cellulose exposed, which made the fiber more easily attacked by the enzyme. After enzymatic hydrolysis, hydrolyzed lignin-free and hemicellulose-free fibers (Fig. 5.4f, 5.4g) clearly show that the textured valleys were much narrower than before hydrolysis, which may mean part of the cellulose on the outside of the main structure was washed off. However, most of the cellulose inside of the structure was still not touched by the enzyme, so the efficiency of hydrolysis was still low. Hydrolyzed manure fiber without hemicellulose and lignin in Fig. 5.4h shows that the main structure of fiber was completely destroyed; explaining why the glucose yield and concentration reached much higher levels for this pretreatment. These phenomena combined with other results, such as glucose yields and enzyme activity further prove the effects of lignin and hemicellulose on enzymatic hydrolysis of dairy manure are mainly physical.

5.4.2. Effects of enzyme loading on enzymatic hydrolysis of manure fiber without hemicellulose and lignin

Based on the above findings, manure fiber without hemicellulose and lignin was chosen as the substance to study the effect of enzyme loading on enzymatic hydrolysis. Fig. 5.5 shows that glucose yields increase with an increase in enzyme loading. The highest glucose yield of 48% was obtained at an enzyme loading of 1300 FPU/L and reaction time of 144 hours (at a glucose concentration of 16 g/L). However, the increase of the yield was not linearly proportional to the increase in initial enzyme loading since it leveled off from 650 FPU/L to 1300 FPU/L. The results concluded that the efficiency of the enzyme during the hydrolysis decreased following the increase of enzyme loading.

The reason might be that there was much more enzyme than available binding sites on cellulose at the beginning of the reaction. The excess enzyme might merely have been absorbed on the surface of the cellulose with inactive binding sites or stuck on a small pole of crystal structure like the trunk in the middle of Fig. 5.4d, which did not work on degrading the cellulose. If it is true, the process of batch reaction has to be modified in terms of improving the efficiency.

5.4.3. Fed-batch enzymatic hydrolysis of manure fiber without hemicellulose and lignin

Fed-batch reaction as a production technique is widely used in the fermentation industry (Lee et al., 1999; Belem et al., 1998). The advantages of this technique are to increase the productivity and reduce the total reaction time. In this particular case, it could be an effective way to improve the efficiency of enzymatic hydrolysis of manure fiber. The hypothesis was that the first enzyme loading is not only to convert hydrolysis-ready cellulose to glucose but also to enzymatically break down the crystal structure of cellulose that remains after pretreatment in order to diminish the negative aspects of the inactive adsorption and heterogeneous structure of the substrate.

Application of this hypothesis resulted in a fed-batch enzymatic hydrolysis that significantly ($P<0.01$) improved the final glucose yield compared to the control with a high enzyme loading level of 1300 FPU/L (Fig. 5.6). The highest yield of 52% (with respect to a glucose concentration of 17 g/L) was obtained from fed-batch hydrolysis with a reaction time of 168 hours. The analysis of enzyme activity showed that the fed-batch system had more enzyme absorbed than the high enzyme level control (Fig. 5.7).

This means the negative factors of inactive adsorption and structure blockage (accessibility) were partially removed by fed-batch enzymatic hydrolysis resulting in enhanced performance for the enzymatic hydrolysis.

5.5. Conclusion

Hemicellulose and lignin in dairy manure, like other lignocellosic materials, have similar functions as major resistors to enzymatic hydrolysis. The removal of either component partially improved the enzymatic hydrolysis of manure cellulose while removal of both resulted in even greater hydrolysis performance; producing a glucose yield of 42% with respect to the concentration of 14 g/L at a reaction time of 120 hours and enzyme loading of 1000 FPU/L. This result, along with the observations of enzyme activity changes during the hydrolysis, further supports that the accessibility of cellulose to cellulase was the most important structural feature influencing the enzymatic hydrolysis. Finding a suitable pretreatment method to substitute for the standard methods of chlorite and alkaline treatments will be critical for commercial utilization of cellulose in lignocellulosic materials. Furthermore, besides the importance of structural features, process optimization also can effectively help improve enzyme performance. Fed-batch enzymatic hydrolysis of fiber without hemicellulose and lignin at a total enzyme loading of 1300 FPU/L produced 17 g/L glucose in 160 hours with respect to a glucose yield of 52%, which was higher than those from the corresponding batch hydrolyses. This means that fed-batch enzymatic hydrolysis is a promising process for hydrolyzing lignocellulosic materials and further study on optimizing fed-batch enzymatic hydrolysis should be conducted.

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Table 5.1. Characteristics of sample manures[a]

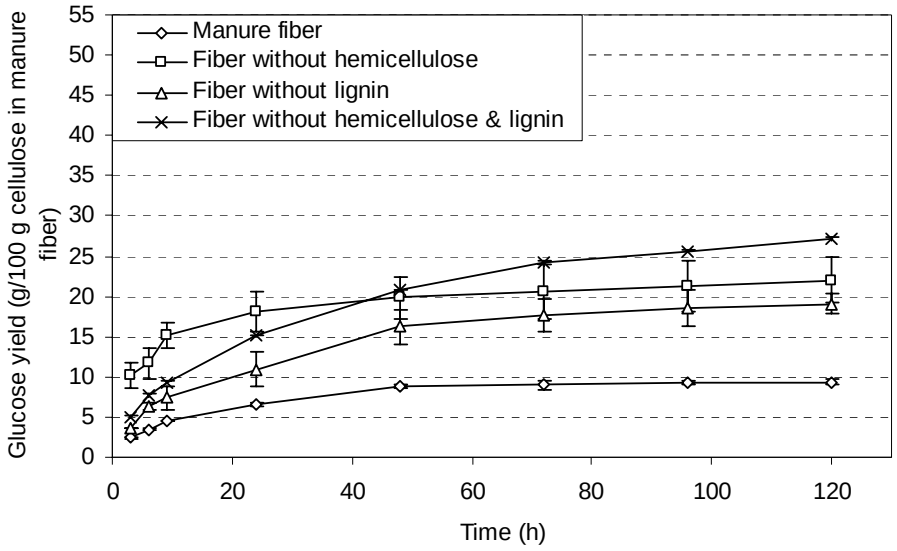
	Manure fiber
Dry Matter, %	14.20 ± 0.05
NDF, % dry basis	61.32 ± 1.02
ADF, % dry basis	47.45 ± 0.56
ADL, % dry basis	16.03 ± 0.17
Cellulose (=ADF-ADL), % dry basis	31.42 ± 0.50
Hemicellulose(=NDF-ADF), % dry basis	13.87 ± 0.60
N, % dry basis	1.23 ± 0.06
C, % dry basis	41.52 ± 1.89

a: Data is the average of triplicates with mean standard deviations (n=3).

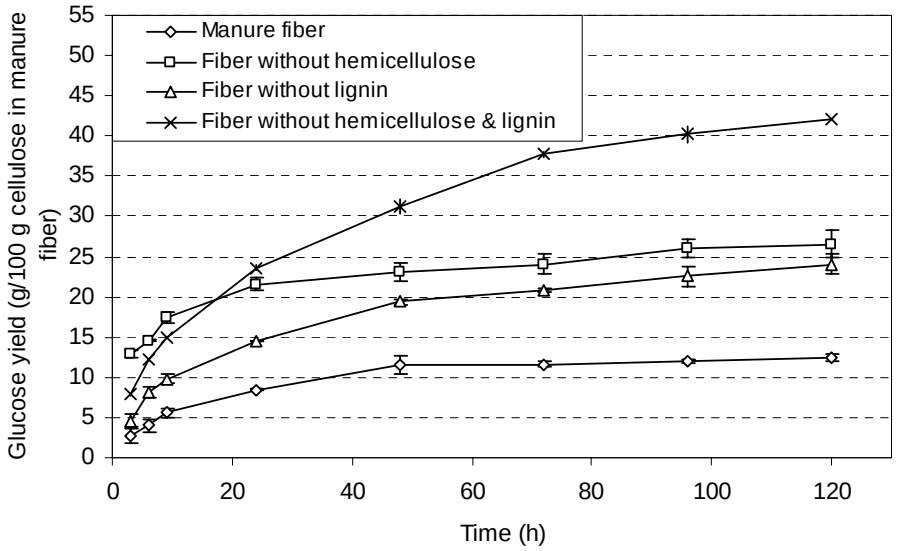
Table 5.2. Fiber characteristics of differently treated manure

	Manure fiber (Control)	Hemicelllulose-free manure fiber	Lignin-free manure fiber	Manure fiber without hemicellulose & lignin
Cellulose, % dry basis	31.42 ± 0.50	42.76 ± 0.18	48.11 ± 0.43	65.95 ± 0.17
Hemicellulose, % dry basis	13.87 ± 0.60	~0	17.80 ± 0.63	~0
Lignin, % dry basis	16.03 ± 0.17	28.85 ± 0.74	3.65 ± 0.48	3.06 ± 0.26

a: Data is the average of triplicates with mean standard deviations (n=3).



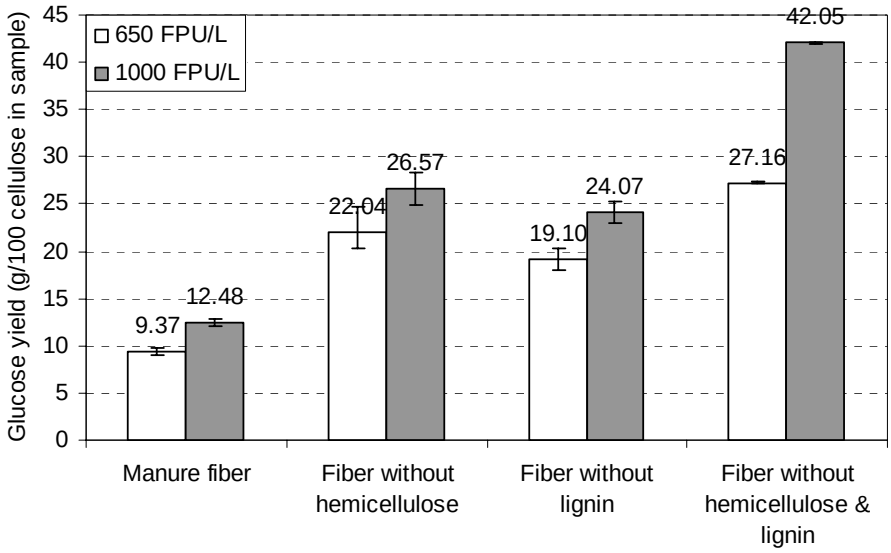
a. 650 FPU/L



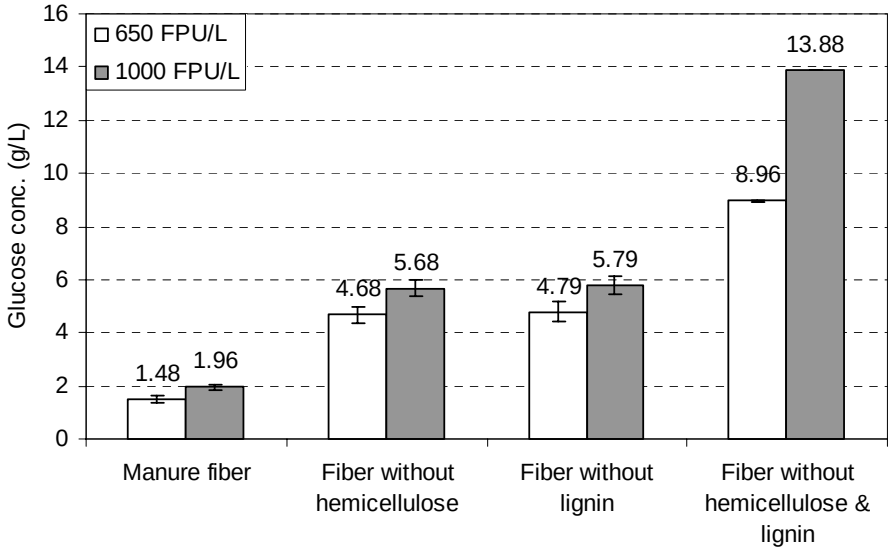
b. 1000 FPU/L

Fig. 5.1. Comparison of enzymatic hydrolysis of differently treated manure fibers

(1): Data are presented as the mean of two replicates and the error bars show the standard deviation



a. Glucose yield



b. Glucose concentration

Fig. 5.2. Glucose concentrations and yields from hydrolysis of differently treated manure fibers

(1): Results were from the point of 120 hours
(2): Data are presented as the mean of two replicates and the error bars show the standard deviation

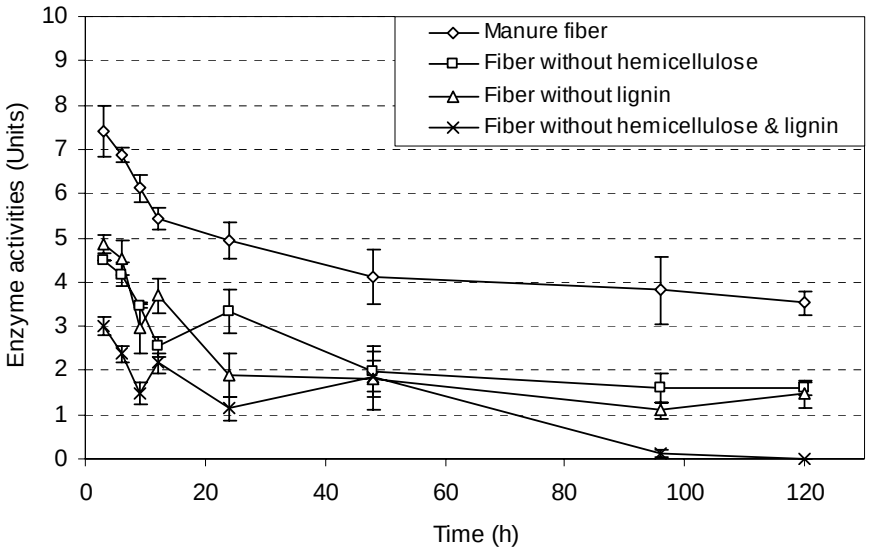


Fig. 5.3. The effect of hemicellulose, lignin on cellulase during the hydrolysis

(1): The initial enzyme activity was 650 FPU/L
(2): All of enzyme activities in the figure are from the liquid part of samples
(3): Data are presented as the mean of two replicates and the error bars show the standard deviation

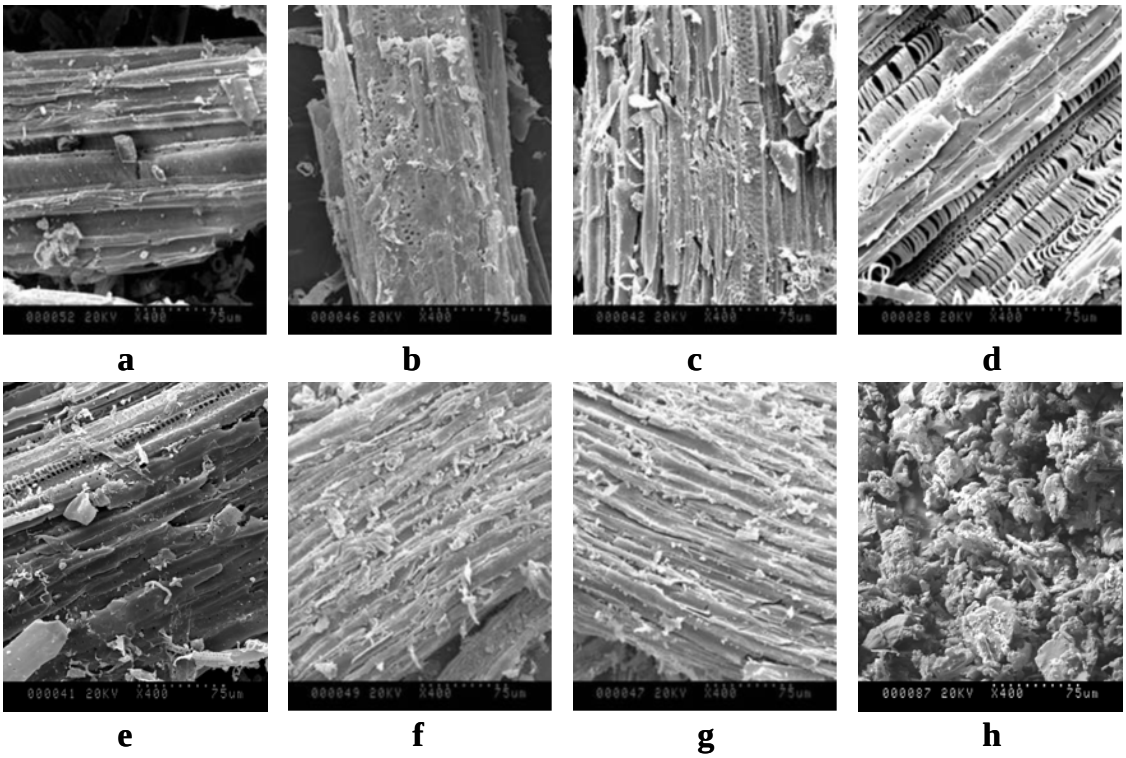


Fig. 5.4. Scanning electron micrograph of hydrolysis of different types of manure samples (400X) from hydrolysis of enzyme loading of 1000 FPU/L

a: Manure fiber, b: Manure fiber without hemicellulose, c: Manure fiber without lignin, d: Manure fiber w/o hemi & lignin, e: Manure fiber after enzymatic hydrolysis, f: Manure fiber without hemicellulose after enzymatic hydrolysis, g: Manure fiber without lignin after enzymatic hydrolysis, h: Manure fiber w/o hemi & lignin after enzymatic hydrolysis.

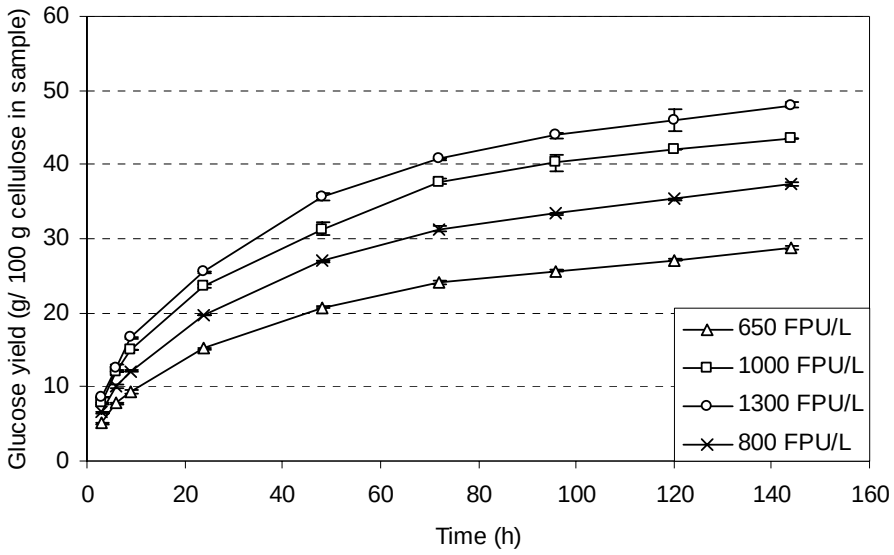


Fig. 5.5. Enzymatic hydrolysis of manure fiber without hemicellulose and lignin

(1): Data are presented as the mean of two replicates and the error bars show the standard deviation

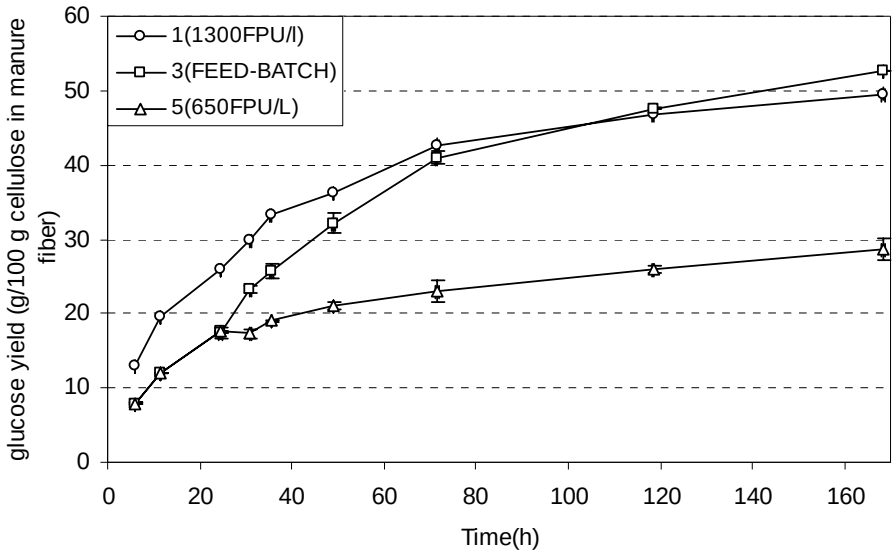


Fig. 5.6. Fed-batch enzymatic hydrolysis

(1): Data are presented as the mean of two replicates and the error bars show the standard deviation

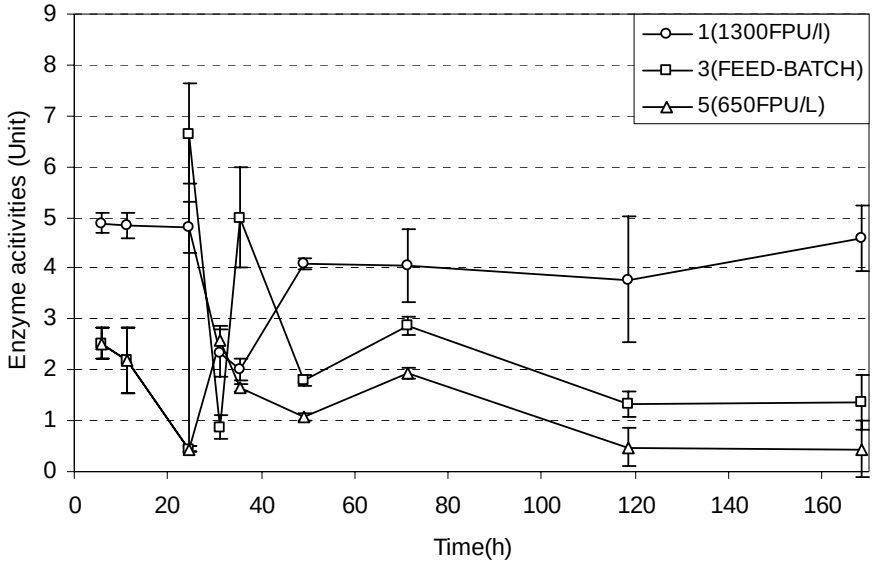


Fig. 5.7. Enzyme activity changes of fed-batch enzymatic hydrolysis

(1): Data are presented as the mean of two replicates and the error bars show the standard deviation

CHAPTER SIX

KINETIC MODELING OF ENZYMATIC HYDROLYSIS OF CELLULOSE IN DIFFERENTLY PRETREATED FIBER FROM A NITROGEN-RICH LIGNOCELLULOSIC MATERIAL – DAIRY MANURE

6.1. Abstract

A kinetic model incorporating dynamic adsorption, hydrolysis process, and production inhibition was developed for enzymatic hydrolysis of differently pretreated fibers from a nitrogen-rich lignocellulosic material – dairy manure. The effects of manure proteins on the enzyme adsorption profile during hydrolysis have been discussed. Enzyme activity, instead of protein concentration, was used to describe the enzymatic hydrolysis in order to avoid the effect of protein concentration in manure. Dynamic enzyme adsorption was modeled based on a Langmiur-type isotherm. A first-order reaction was applied to model the hydrolysis with consideration being given for the competitive inhibition of the final product. The model satisfactorily predicted the behaviors of enzyme adsorption, hydrolysis, and product inhibition for all five sample manure fibers in a batch system that achieved a substrate conversion rate of up to 80%, substrate concentration ranging from 10 to 50 g/L, and an enzyme concentration less than 1300 FPU/L.

Key words: dairy manure, enzymatic hydrolysis, kinetic model, nitrogen-rich lignocellulosic material, pretreatment

6.2. Introduction

Animal manures, as a type of lignocellulosic material, are rich in carbohydrate and protein that could be converted into bio-based chemicals, materials and energy products. Dairy manure, in particular, contains about 12% hemicellulose and 22% cellulose (Liao et al., 2004), which represents a potential source of carbohydrates that are capable of producing monosaccharides of glucose and xylose through various hydrolysis processes such as chemical and enzymatic hydrolysis. Currently, enzymatic hydrolysis is attracting increased attention for converting lignocellulosic materials to sugars because the process has specific and mild reaction conditions (pH around 5 and temperature less than 50°C) as well as a lack of corrosion problems compared to other traditional treatments such as acid hydrolysis (Sun et al., 2002).

The cellulose within lignocellulosic material is physically and chemically associated with hemicellulose and lignin with the resulting matrix structure preventing the cellulose from being attacked by cellulase (Mansfield et al., 1999; Ladisch, 1989; Ladisch et al., 1983; Sinitsyn et al., 1991). Thus, in terms of increasing the efficiency of sugar production, breaking the matrix structure by various pretreatments is necessary prior to enzymatic hydrolysis. The effects of different pretreatments on enzymatic hydrolysis of manure fiber have been reported previously (Liao et al., 2005). However, the kinetic study has not been conducted that describe the relationship between enzyme and treated manure fibers during the hydrolysis.

Numbers of kinetic models of enzymatic cellulose hydrolysis have been developed during the past several decades (Dewey et al., 1982; Gan et al., 2003; Gusakov et al., 1985; Wald et al., 1984; Nidetzky et al., 1993). Most of them focused on raw

materials such as corn stover, straw, and wood (Gonzalez et al., 1989; Kadam et al., 2004; Bernardez et al., 1994), however none have investigated the hydrolysis of cellulose from a nitrogen-rich lignocellulosic material – animal manure. The relatively high protein content of animal manure makes any kinetic study more difficult and complicated. For example, dairy fiber has a nitrogen content of around 0.90 % and even after different pretreatments (dilute acid treatment, sodium chlorite treatment, and dilute alkaline hydrogen peroxide treatment) its nitrogen content still ranges from 0.13 to 0.81%, which is considerably higher than most other lignocellulosic materials (i.e., 0.40 for wheat straw and 0.20% for spruce wood) (Winter et al., 1999). Most of this nitrogen is trapped in the fiber matrix structure, which upon treatment can be released into the hydrolysate and further influence the measurement of enzyme protein concentration during hydrolysis.

Kinetic models of enzymatic hydrolysis of lignocellulosic materials usually incorporate enzyme adsorption and hydrolysis product inhibition (Walker et al., 1991; Lee et al., 1982; Ladisch et al., 1981) with the focus, primarily, being on hydrolysis product inhibition (Chang et al., 2000; Ghose, 1969; Huang, 1975) and little attention being paid to dynamic enzyme adsorption. Wald et al. (1984) and Kadam et al. (2004) applied the Langmuir adsorption equation into their models. However, in their model they assumed that the adsorption equilibrium remained constant throughout the entire course of hydrolysis, which oversimplified the real adsorption that happened during the hydrolysis of cellulose, especially within lignocellulosic materials. Changes in fiber structure and composition during hydrolysis make enzyme adsorption a dynamic process which is subject to change as the hydrolysis progresses. Thus, a model which better incorporates this dynamic enzyme adsorption process along with the concept of

hydrolysis and product inhibition could be helpful in better understanding the behavior of enzymatic hydrolysis of lignocellulosic materials; particularly nitrogen-rich sources such as dairy manure.

The objective of this research was to develop a kinetic model that incorporated dynamic enzyme adsorption and product inhibition with hydrolysis for differently treated dairy manure fibers. More specifically, this study was designed to: (1) understand the effects of the protein content of differently treated fibers on the enzyme adsorption profile, and further determine the parameter that can best reflect the enzyme protein in the system; (2) explore the behavior of enzyme adsorption during the entire hydrolysis course; and (3) develop a model that was able to simultaneously predict the behavior of adsorption, hydrolysis, and product inhibition for differently treated dairy manure fiber.

6.3. Materials and methods

6.3.1. Materials

Fresh dairy manure was obtained from the Dairy Center of Washington State University. The manure had 15.3% dry matter (DM) with a total carbon content of 46.9 g/100g DM and total nitrogen content of 2.6 g/100g DM. Ten kilograms of original manure was mixed with 5 kg of water and blended for 1 minute to achieve size reduction, and then 10 kg of the mixture was washed three separate times with 5 kg of water. Solid-liquid separation was accomplished using a centrifuge at 3,000 rpm for 10 minutes to isolate most of the soluble nitrogen and impurities within the liquid fraction. The solid part was collected and dried as manure fiber for hydrolysis. The data for the manure fiber are presented in Table 6.1.

The enzymes, celluclast 1.5 L and Novozyme-188 (Sigma, St. Louis, MO), were used for the study of enzymatic hydrolysis. Celluclast 1.5 L contained 145.5 FPU/g solution of cellulase (Filter paper activity unit, FPU). One unit of FPU is defined by the Commission on Biotechnology for IUPAC as the enzyme amount that releases 1 μmol of glucose equivalents from Whatman No. 1 filter paper in 1 minute (Ghose, 1987). The β-glucosidase activity of Novozyme-188 was 770 IU/ml. One unit of β-glucosidase activity is defined as the enzyme amount that converts 1 μmol of cellobiose to 2 μmol of glucose in 1 minute (Ghose, 1987). It has been reported that β-glucosidase addition can effectively eliminate the cellulobiose inhibition during the enzymatic hydrolysis (Wen et al., 2004). In terms of maximizing the glucose production, a mixture of two enzymes was used to do the hydrolysis. The ratio of these two enzymes was 2.6 FPU Cellulcast to 1 IU Novozyme-188 (Wen et al., 2004).

6.3.2. Pretreatments

Acid treated manure fiber was obtained by applying optimized dilute acid treatment from a previous study on manure fiber (Liao et al., 2004). The treatment was operated at 1% sulfuric acid, 135°C, and 5% substrate concentration for 2 hours. After dilute acid treatment, the samples were filtered through Whatman No. 5 filter paper and washed with water until the pH of solution reached around 5. After the washed solid was dried at 100°C overnight the acid treated manure fiber sample was ready to be hydrolyzed.

Chlorite treatment was used to delignify the manure fiber (Ahlgren et al., 1970). The substrate concentration was 5%. The chemical mass ratio was 0.3 g sodium chlorite

and 0.1 ml glacial acetic acid per gram of manure fiber. The sample was treated at 70°C for an hour. The ensuing liquid-solid separation step was carried out using a Beckmann centrifuge at 5000 rpm for 5 minutes with three washings of 300 ml water each. After being dried at 100°C the chlorite treated fiber was obtained.

Acid & chlorite treated manure fiber was obtained by applying both the dilute acid treatment and chlorite treatment. The acid treatment was used first, followed by chlorite treatment. Each individual step was the same as described earlier.

Dilute alkaline peroxide treatment was operated at 0.5 % sodium hydroxide, 1% hydrogen peroxide, 150°C, and 5% substrate concentration for 30 minutes. The liquid-solid separation was carried out using a Beckmann centrifuge at 5000 rpm for 5 minutes. Separation was followed by overnight drying at 100°C to achieve a dilute alkaline pretreated fiber ready for hydrolysis.

All dry fibers obtained above were milled using a blender to reduce the particle size. The fibers passed #40 standard screen were used for the study.

6.3.3. Kinetic study of enzyme adsorption and hydrolysis of differently treated manure fiber

Sixteen combinations of four sample concentrations (1, 2, 3, and 4 g/L) and four enzyme concentrations (350, 500, 1000, and 1500 FPU/L) for each fiber sample were carried out for the study. The pH of enzymatic hydrolysis was set at 4.8 and the reaction temperature was 50°C (Wen et al., 2004). Aliquot samples were taken at 3 hour intervals for the first 12 hours and at 24 hour intervals for the rest of the 96 hours. Enzyme

activity, protein concentration, sugar concentration, reaction rate, and substrate concentration were measured at each sampling point.

The parameters for the models were estimated from experimental data using the curve-fit toolbox of Matlab 7.0 (The MathWorks, Natick, MA), The numeric solutions of the model were obtained using the function of ODE45 from the partial differential equation toolbox of Matlab 7.0 (The MathWorks, Natick, MA). The criteria for model discrimination were the mean square error and normal distribution of the residuals. The Matlab codes were presented in Appendix.

6.3.4. Verification of the model

Five grams of dry sample from each different type of manure fiber was mixed with 50 ml of 1300 FPU/L cellulase solution. The reaction conditions were the same as that described in previous section.

6.3.5. Analytical methods

Enzyme activities during hydrolysis were measured according to the recommendation for dilute enzyme solution by the IUPAC committee (Ghose, 1987; Wei et al., 2005). However the method was slightly modified for this study. The hydrolysate was centrifuged at 5000 rpm for 5 minutes to obtain the supernatant. A supernatant of 0.5 ml was then added to 1 ml of 0.05 M sodium-citrate buffer (pH 4.8) containing a strip of Whatman No.1 filter paper (50 mg). The solution was incubated in a shaker at 50°C for an hour. The enzyme unit was defined as the total amount of glucose released per minute per liter enzyme solution (1 unit = 1 g glucose/L/min). The protein concentration in the

supernatant was measured by Bradford reagent using bovine serum albumin as a standard (Bradford, 1976). The molecular mass of the proteins in the supernatant was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Min-PROTEIN 3 Cell.

Fiber data including neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed using the reflux apparatus (Goering et al., 1970). NDF was used to estimate the total cellulosic materials (cellulose, hemicellulose, lignin, and insoluble ash) while ADF was used to estimate the concentration of lignin and cellulose. Hemicellulose was determined by the difference (%NDF-%ADF). The content of monosaccharides and cellobiose in the hydrolyzed solution was determined using an ion chromatograph (IC) from Dionex (Wei et al., 2004). Carbon and nitrogen contents in the solid samples were measured by automated combustion techniques. The LECO CNS-2000 was used to measure the total carbon and total nitrogen of the manure samples.

6.4. Results

6.4.1. Characteristics of differently treated manure fibers

Dilute acid treatment can completely remove the hemicellulose in the fibers while sodium chlorite treatment was very effective at removing the lignin (Table 6.1). Dilute alkaline peroxide treatment has been reported as an environmental friendly and effective method to break down the crystal structure of lignocellulose and further increase the digestibility of the fiber (Gould, 1984; Curreli et al., 1997), although in this study it only partially removed the hemicellulose and lignin (Table 6.1). After treatments, the cellulose concentrations in all four treated manure fibers increased. The cellulose in the dilute acid

and sodium chlorite treated fiber reached 58% and 51%, respectively while the dilute alkaline peroxide treated fiber cellulose concentration reached 65%. Lastly, the combination of dilute acid and chlorite treatment removed almost all of the hemicellulose and lignin, producing a manure fiber with the highest cellulose concentration of 83%. Importantly, the data also demonstrated that there was still a certain amount of nitrogen remaining in the treated fibers (Table 6.1), which, upon further experimentation, could cause an inaccuracy of measurement of enzyme protein during subsequent hydrolysis.

6.4.2. Cellulase adsorption on differently treated manure fibers

The protein concentrations in the reaction solution demonstrate that differently treated fibers had different protein profiles during the hydrolysis (Fig. 6.1). Proteins from hydrolysis of chlorite treated fiber and dilute acid & chlorite treated fiber followed the same trend with the concentration increasing at the beginning of the reaction and then leveling off following an increase of reaction time. In contrast the proteins from hydrolysis of dilute acid treated fiber and alkaline peroxide treated fiber decreased during the hydrolysis. In addition most of the protein concentrations from the reaction solutions of the chlorite treated fiber and dilute acid & chlorite treated fiber were higher than the original enzyme protein concentration of 0.37 g/L. This suggests that some proteins from the fibers were released into the reaction solution and caused an increase of protein concentration in the reaction solution. Meanwhile, SDS-PAGE analysis had shown that some protein not belonging to the enzyme was in the solution (Fig. 6.2). A protein with molecular weight of 36 KDa appeared on the hydrolysis solutions from acid treated fiber, chlorite treated fiber, and acid & chlorite treated fiber at 3 hours reaction time, while

alkaline peroxide treated fiber and enzyme protein control did not have this protein (Fig. 6.2). Thus, protein concentration can not be adopted as the parameter to describe the enzyme concentration for the hydrolysis of nitrogen-rich lignocellulosic materials, although it has been widely used for most of the studies of enzymatic hydrolysis on various lignocellulosic materials.

In terms of the study of the kinetics of enzymatic hydrolysis of differently treated manure fiber, the proper parameter to describe the enzyme has to be determined. The analysis of enzyme activities during the reaction shows that all five hydrolyses followed the same trend that enzyme activities decreased following an increase of reaction time (Fig. 6.3). In addition, the analysis of enzyme stability demonstrates that enzymes between 350 FPU/L and 1300 FPU/L had only a slight decay during the entire reaction time of 96 hours (Fig. 6.4), which could be considered as a constant. This means that enzyme activity, instead of protein concentration, can be used to represent the enzyme concentration.

6.4.3. Kinetic model

The proposed kinetic model is depicted in Fig. 6.5. Enzyme first was adsorbed by different components (cellulose, lignin, and hemicellulose) on the surface of the fiber. Then the enzymes attached on the surface of the cellulose to break down the cellulose to produce sugars. There are two types of bonding sites on the surface of cellulose: active bonding sites and inactive bonding sites with only the enzymes bonded by active bonding sites being functional. Meanwhile, similar with other enzymatic reactions, the hydrolysis reaction can be inhibited by final products of sugars such as cellobiose, glucose, and

xylose. In this particular case, because Novozyme-188 (β-glucosidase) was added into the enzyme system, the β-glucosidase could immediately convert the cellobiose into glucose. As a result, there was no indication of detectable amounts of cellobiose exiting in the reaction systems. Also, since the xylose concentrations from hemicellulose in the solutions were relatively low; its inhibition could be neglected. Thus, glucose was the only inhibitor for enzymatic hydrolysis of differently treated manure fiber.

The entire enzymatic hydrolysis was divided into two parts: enzyme adsorption and hydrolysis. Langmuir-type isotherms have been widely used to study the cellulose-cellulase system (Beldman et al., 1987; Converse et al., 1988; Steiner et al., 1988; Stuart et al., 1984). A Langmuir adsorption model was adopted to describe the enzyme adsorption at every individual reaction duration on differently treated manure fibers (Eq. 1). A competitive model of glucose inhibition was used to simulate the hydrolysis reactions (Eq. 2). The assumptions for the entire model were as follows:

1). There are three major enzymes involved in the conversion of cellulose to glucose: endo-β-glucanase, exo-β-glucanase, and β-glucosidase. In terms of simplifying the model, the enzymes are considered as an enzyme system with different functional groups.

2). Enzyme adsorption at each individual reaction duration is a Langmuir-type isotherm with the adsorption equilibrium being only valid at the individual reaction duration.

3). Competitive enzymatic hydrolysis is a first-order reaction occurring on the surface of cellulose.

4). The structure of the fiber matrix is uniform in terms of enzyme adsorption. In another words, lignin, hemicellulose, and cellulose are evenly distributed in the fiber no matter how the content of each individual component is changed during the hydrolysis (i.e., the composition of a surface for enzyme adsorption is assumed to be the same as the composition of the entire fiber, then the composition of the entire fiber can be used to calculate the concentration of adsorbed enzyme on the fiber surface).

5). Lignin and hemicellulose only influence the enzyme adsorption (i.e., the more lignin and hemicellulose that are in the fiber, the fewer enzymes that are absorbed by cellulose). They do not, however, have an effect on enzyme activities. Enzyme activities are inhibited by the final product of glucose.

6). For each individual fiber at each individual reaction duration the effects of different substrate concentrations and corresponding structure difference on the adsorption were negligible in the experimental range.

7). Available cellulose (C_{eff}) for enzyme hydrolysis is assumed to be related with the ratio of remained cellulose to initial total cellulose in the fiber.

Enzyme adsorption

$$\frac{[E]_0}{[E]_{ad}} = \frac{1}{K}\frac{1}{[S]} + 1 \qquad \text{(Equation 1)}$$

Where:

$[E]_0$: total enzyme activity in the reaction system (unit/L)

$[E]_{ad}$: enzyme activity absorbed by fiber (unit/L)

[S]: substrate concentration (g/L)

K: adsorption constant [$(g/L)^{-1}$]

Enzymatic hydrolysis

$$r = \frac{k[C]_{eff}[EC]}{1 + \frac{[G]}{K_{IG}}} \quad \text{(Equation 2)}$$

Where:

$$[C]_{eff} = (\frac{[C]}{[C]_0})^{\lambda} \cdot [C] \quad \text{(Equation 3)}$$

with:

r: reaction rate (g/L·h)

k: reaction rate constant

$[C]_{eff}$: concentration of cellulose available for enzyme (g/L)

[C]: cellulose concentration (g/L)

$[C]_0$: original cellulose concentration (g/L)

[EC]: concentration of enzyme absorbed by cellulose (unit/L)

[G]: glucose concentration (g/L)

K_{IG}: inhibition constants for glucose (g/L)

λ: constant relating the percentage of cellulose available for enzyme

Mass balances

$$\frac{dG}{dt} = 1.11r \quad \text{(Equation 4)}$$

$$\frac{dC}{dt} = -r \quad \text{(Equation 5)}$$

$$[S] = [C] + [L] + [H] \quad \text{(Equation 6)}$$

$$[E]_{ad} = [EC] + [EL] + [EH] \quad \text{(Equation 7)}$$

Where:

$$[EC] = \frac{[E]_{ad}}{[S]}[C] \quad \text{(Equation 8)}$$

with:

1.11: conversion constant of cellulose to glucose

t: reaction time (hour)

[L]: lignin concentration (g/L)

[H]: hemicellulose concentration (g/L)

[EL]: concentration of enzyme absorbed by lignin (unit/L)

[EH]: concentration of enzyme absorbed by hemicellulose (unit/L)

The parameters in the model illustrate the effects of different functions during the reaction. K reflects a substrate's capability for enzyme adsorption, thus a large value of K means that more enzymes are absorbed by the substrate. The k value is proportional to reaction rate, the higher the k value the higher the hydrolysis reaction rate. K_{IG} is a parameter which describes the inhibition of glucose, with a higher value of K_{IG} indicating a weaker inhibition. λ determines the total amount of available cellulose in the substrate, with a bigger number for λ meaning that there are relatively less cellulose available and ready for hydrolysis.

6.4.4. Determination of parameters of adsorption model for each individual pretreated fiber

The data of K for differently treated fibers shows the effects of pretreatment methods on enzyme adsorption (Fig. 6.6). K values of all five fibers increased following

reaction progress. All four of the treated manure fibers had higher K values than the original manure fiber with acid & chlorite treatment presenting the highest K values.

The data also presents the relationship between enzyme adsorption and factors such as initial enzyme concentration, initial substrate concentration, and reaction duration (Fig. 6.6). Cellulase adsorption has been reported to be influenced by both initial enzyme concentration and initial substrate concentration (Nidetzky et al., 1993). However, a pair-wise comparison showed that enzyme concentration and substrate concentration had no significant ($P>0.05$) influence on most of the sample points except for enzyme adsorption on acid & chlorite treated fiber with initial enzyme concentration of more than 650 FPU/L, substrate concentration less than 25 g/L, and reaction time more than 24 hours. This means that enzyme concentration and substrate concentration had only slight influences on enzyme adsorption of each sample fiber at each individual reaction time in a range of initial enzyme concentrations of 350 FPU/L to 1300 FPU/L and initial substrate concentrations of 10 g/L to 50 g/L. Meanwhile, Fig. 6.6 shows that reaction time had significant ($P<0.05$) impact on adsorption. The results mean that enzyme adsorption of differently treated manure fibers has to be considered as a function of reaction time and fiber structure that is decided by the different pretreatment methods. Thus, an empirical equation (Eq. 9) was proposed in the study to derive that relationship.

$$K = \frac{a \cdot t}{b + t} \qquad \text{(Equation 9)}$$

Where: a and b are the parameters to calculate adsorption constants.

The results show that the empirical equations agreed well with the experimental data (Fig. 6.6). Correlations of r-squares for all five fibers were higher than 0.88. The parameters of the equation (Eq. 9) for differently treated fibers are listed in Table 6.2.

6.4.5. Determination of parameters of hydrolysis model for each individual pretreated fiber

Fig. 6.7 presents the time course of hydrolyses of five sample fibers. There was no cellobiose detected during any of the hydrolyses, which means that addition of the enzyme β–glucosidase accelerated the conversion of cellobiose to glucose, and further eliminated the inhibitory effect of cellobiose on hydrolysis. Meanwhile, since chlorite treated manure fiber and alkaline peroxide treated manure fiber retained some hemicellulose, they produced a small amount of xylose which is a major inhibitor of cellulase (Kadam et al., 2004). However, the concentration of xylose from both hydrolyses were relatively low (Fig. 6.7) and thus the inhibitory effect of xylose would be minimum. Additionally, there were no other monosaccharides detected in the hydrolysates except glucose and xylose. Thus, in the models the final product of glucose was considered as the only inhibitor for the hydrolysis.

The most difficult part of the proposed hydrolysis model was to determine the concentration of available cellulose (C_{eff}) for the binding enzyme. There are several parameters, such as degree of polymerization (DP), crystallinity index, and surface accessibility, available to mechanistically describe the enzyme accessibility of fiber, which could be used to express the total available cellulose for hydrolysis. However, none of them metioned above can be universally used to accurately predict the total amount of cellulose available for the enzyme. Thus, an alternative had to be developed to describe the portion dealing with cellulose. In the heterogeneous system of lignocellulose hydrolysis, the total amount of available cellulose changes as the components and fiber

structure themselves change during hydrolysis. The substrate reactivity (Eqs. 12 & 13) worked very well for modeling of enzymatic hydrolysis of acid treated corn stover (Kadam et al., 2004).

$$R = \alpha \cdot \frac{[C]}{[C]_0} \quad \text{(Equation 12)}$$

$$[C]_{eff} = R \cdot [C] \quad \text{(Equation 13)}$$

Where α is a constant, R is substrate reactivity.

However, in this particular case, the linear relationship did not fit with all the hydrolysis data collected for the differently treated manure fiber. Thus, a modified exponential relationship was adopted for modeling hydrolysis of differently treated manure fiber (Eq. 3). The correlations of the exponential relationship were higher than 0.82. Meanwhile, other parameters such as k and K_{IG} were obtained from the regression of the experimental data.

6.4.6. Model application on prediction of conversion of enzymatic hydrolysis of different treated fiber

The model includes Equations 1-6 and 8-9. The numeric solutions were obtained from these equations. An independent verification experiment was run to test the model. As shown in Fig. 6.8, the models predicted well both the adsorption and hydrolysis behavior of each differently treated manure fiber at higher substrate concentrations than 50 g/L. However, at lower substrate concentrations of 10 g/L, the experimental data of hydrolysis of acid & chlorite treated fiber shows that the total amount of adsorbed enzyme was rapidly decreased to nearly zero after 12 hours of reaction time at enzyme

concentration greater than 650 FPU/L, with the glucose concentration remaining at 9.5 g/L with a conversion rate of 100%. It has been reported that acid & chlorite treatment not only entirely removed fiber components of hemicellulose and lignin but also break down the crystal structure of the fiber (Liao et al., 2005), which makes fiber easier to be enzymatically hydrolyzed as compared to the other three treatments. Once the ratio of enzyme to fiber concentration reached the level of 130 FPU per g acid & chlorite treated substrate the fiber was nearly completely degraded. Consequently the enzyme adsorption disappeared and without adsorption, the Langmiur adsorption and first order hydrolysis based model could not be validated. Thus, the effective range of substrate concentration and enzyme concentration was determined by the validation experiment. The models for manure fiber, acid treated fiber, chlorite treated fiber, and alkaline peroxide treated fiber performed well within a range of substrate concentration corresponding to 10 to 50 g/L and an enzyme concentration of 350 FPU/L to 1300 FPU/L, while the substrate concentration for acid & chlorite treated fiber was narrowed to 20 to 50 g/L at the same range of enzyme concentration.

6.5. Discussion

Proteins existing in nitrogen-rich lignocellulosic materials such as dairy manure make the total protein concentration in solution unsuitable for describing the enzyme concentration in the study on kinetics of enzymatic hydrolysis. Most of the proteins in this type of material exist in a form capable of combining with other components such as cellulose, lignocellulose, and hemicellulose. Even after various severe pretreatments, there was still a relatively large amount of nitrogen remaining in the fiber (Table 6.1).

Different pretreatments had uniquely different effects on nitrogen removal. Alkaline peroxide treatment had the best removal efficiency with around 82% of the total nitrogen removed while other treatments had removal rates ranged from 10% to 70%. Only alkaline peroxide fiber showed no detectable fiber proteins interfering with enzyme protein in the solution (Fig. 6.1 & 2). The other four fibers gradually released proteins into the solution during the hydrolysis (Fig. 6.1), and made the protein concentration unsuitable to accurately reflect the enzyme concentration.

Besides protein concentration, enzyme activity was demonstrated as a suitable replacement to describe the enzyme concentration. The main reason why most researchers prefer protein concentration to enzyme concentration is the complication of enzyme analysis and the concern about thermal deactivation of the enzyme during the hydrolysis which makes enzyme activity unable to reflect the actual enzyme protein concentration. However, this study showed that the enzyme was rather stable during the entire hydrolysis (Fig. 6.4), which is consistent with results from Ooshima who studied enzyme adsorption on pretreated wood (Ooshima et al., 1990). The application of enzyme activity as a description of the enzyme concentration effectively predicted the adsorption and hydrolysis. This proved that enzyme activity can be used as an alternative to protein concentration for the study of enzymatic hydrolysis; especially for the hydrolysis of nitrogen-rich lignocellulosic materials.

Enzyme adsorption is influenced by the physical and chemical properties of fiber. It has been widely reported that the cellulose-cellulase system fits well with Langmiur adsorption (Zhang, et al., 2004). However, Langmiur adsorption is an equilibrium-type isotherm while hydrolysis is a dynamic process. This means that the adsorption

equilibrium is changed during hydrolysis, which leads to a corresponding change in the adsorption constant in the Langmiur equations. Most of the studies on enzymatic hydrolysis of cellulose which use a two-step approach of adsorption and hydrolysis made the simplification that the change of adsorption during the hydrolysis is negligible and therefore Langmiur adsorption is in equilibrium during the entire hydrolysis (Kadam et al., 2004; Wald et al., 1984). Our result shows that the adsorption equilibriums of five sample fibers were significantly ($P<0.05$) changed during the hydrolysis (Fig. 6.6). This indicates that change of adsorption has to be considered in the models. However, since the adsorption on lignocellulosics materials is complicated (especially once the hydrolysis is taking place and factors such as lignin content, hemicellulose content, hydrophilic/hydrophobic property, and the matrix structure of cellulose, hemicellulose and lignin were correspondently changed) it was difficult to develop a mechanistic model to describe the dynamic adsorption process of the cellulase-lignocellulosics system. Thus, in this study an empirical equation (Eq. 9) was developed to describe the change of adsorption constant with respect to reaction time and type of fiber, which made it possible to develop an integrated model including dynamic enzyme adsorption and hydrolysis.

The model was also able to evaluate the pretreatment methods (Fig. 6.6, Table 6.3). Since the more the enzymes are adsorbed by the fiber the more effective the pretreatment method is, acid & chlorite treatment and alkaline peroxide treatment were the best treatments among the types studied (Fig. 6.6). This suggests that disrupting the matrix structure and removing lignin and hemicellulose were beneficial to enzyme penetration and adsorption, and further improved the hydrolysis. As for hydrolysis, the

data demonstrated that acid & chlorite treated fiber had the highest k value of 1.22, and the lowest values of K_{IG} and λ of 0.011 and 0.87, respectively (Table 6.3). This indicates that the hydrolysis of acid & chlorite treated fiber had a much higher production rate, and more available cellulose for enzyme, and stronger inhibition of glucose. Considering both adsorption and hydrolysis, acid & chlorite treatment resulted in improved yields among the four treatments. However, if environmental and economic factors are considered, then dilute alkaline peroxide treatment was the most suitable method for pretreatment.

6.6. Conclusion

This derived model which integrates both the dynamic adsorption and hydrolysis was effective at predicting both the enzyme adsorption and hydrolysis for differently treated manure fiber. The model gave a qualitative answer to questions such as what influences different treated fiber had on enzymatic hydrolysis, and how much improvement would be obtained through modification of the pretreatment and enzymatic hydrolysis. The approach is not only applicable for modeling of hydrolysis of nitrogen-rich lignocellulosic materials but is also suitable for other lignocellulosic materials. The inclusion of a dynamic adsorption explored the relationship between adsorbed enzyme and hydrolysis of lignocellulosic materials, which might be able to provide some new perspectives toward better understanding and designing enzymatic hydrolysis of lignocellulosic materials.

6.7. References

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Table 6.1. Characteristics of differently treated manure fibers

	Manure fiber (Control)	Dilute acid treated manure fiber	Sodium chlorite treated manure fiber	Dilute acid & sodium chlorite treated manure fiber	Dilute alkaline peroxide treated manure fiber
Cellulose, % dry basis	41.03 ± 0.50	57.67 ± 0.18	51.34 ± 0.43	82.50 ± 1.07	64.54 ± 1.54
Hemicellulose, % dry basis	18.19 ± 0.60	~0	21.25 ± 0.63	~0	7.25 ± 0.21
Lignin, % dry basis	19.07 ± 0.17	34.73 ± 0.74	2.12 ± 0.17	3.33 ± 0.26	16.95 ± 0.37
Total nitrogen, % dry basis	0.90 ± 0.11	0.81 ± 0.05	0.39 ± 0.07	0.27 ± 0.02	0.14 ± 0.05

(1). Data is the average of triplicates with mean standard deviations (n=3).

Table. 6.2. Parameters for calculation of adsorption constant (K) of differently treated fibers

Parameters	a	b
Manure fiber	0.051	3.70
Acid treated manure fiber	0.160	12.39
Chlorite treated manure fiber	0.084	3.69
Acid & chlorite treated fiber	0.960	26.96
Alkaline peroxide treated fiber	0.360	14.41

Table 6.3. Parameters for hydrolysis models of different fibers

Parameters	k	λ	K_{IG}
Manure fiber	0.0008	4.64	0.42
Acid treated manure fiber	0.0020	3.79	0.69
Chlorite treated manure fiber	0.0030	4.11	0.51
Acid & chlorite treated manure fiber	1.2200	0.87	0.01
Alkaline peroxide treated manure fiber	0.0130	3.18	5.10

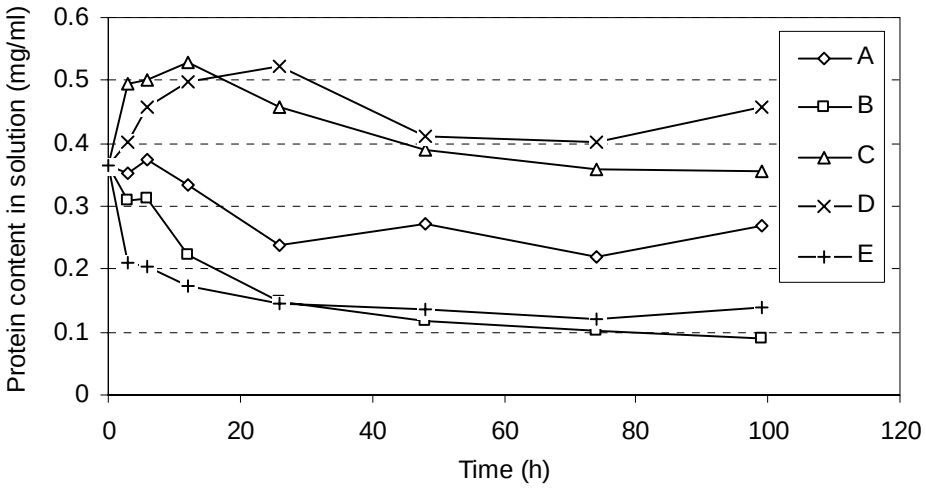


Fig. 6.1. Protein content in solution from hydrolysis of different treated manure fiber

(1). The initial enzyme loading was 1300 FPU/L and the initial solid concentration was 2.5%.
(2). A, manure fiber; B, fiber from dilute acid treatment; C, fiber from sodium chlorite treatment; D, fiber from sodium chlorite & dilute acid treatment; E, fiber from dilute alkaline peroxide treatment.

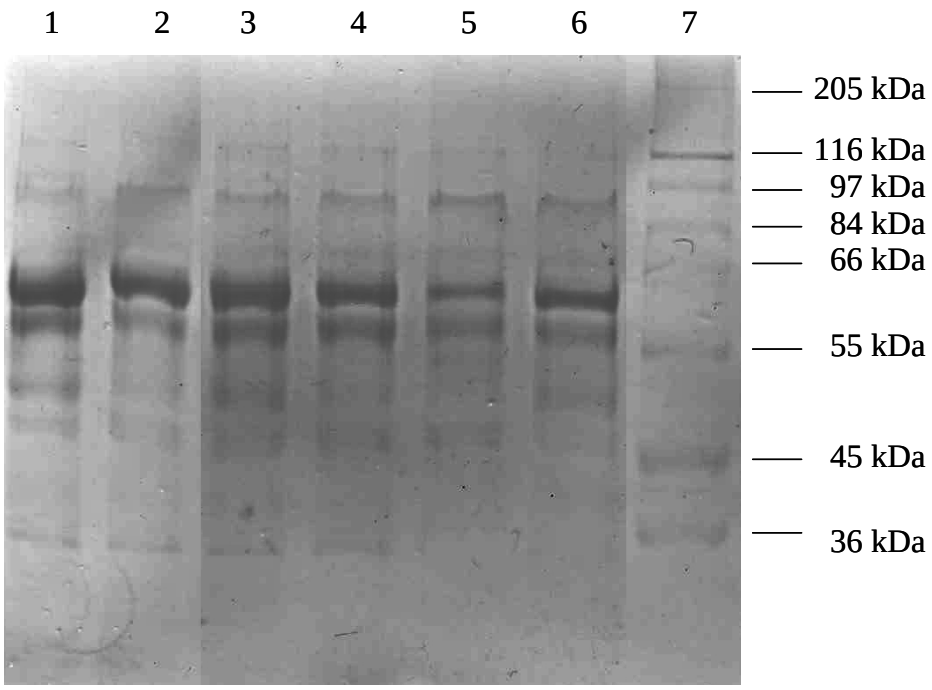


Fig. 6.2. Analysis of proteins in solution by SDS-PAGE electrophoresis

(1). The initial enzyme loading was 1300 FPU/L with respect to a protein concentration of 0.365 g/L, and the initial solid concentration was 2.5%.
(2). Lane 1, proteins from hydrolysis of manure fiber at 3 hours of reaction time; Lane 2, proteins from hydrolysis of dilute acid treated fiber at 3 hours of reaction time; Lane 3, proteins from hydrolysis of sodium chlorite treated fiber at 3 hours of reaction time; Lane 4, proteins from hydrolysis of dilute acid treated/sodium chlorite treated fiber at 3 hours of reaction time; Lane 5, proteins from hydrolysis of dilute alkaline peroxide treated fiber at 3 hours of reaction time; Lane 6, proteins from the hydrolysate at 0 hour of reaction time; Lane 7, standard proteins.

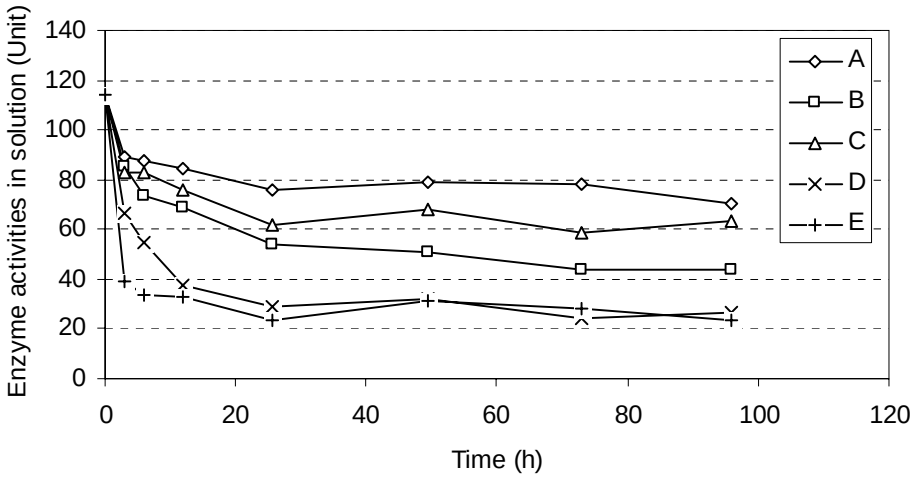


Fig. 6.3. Enzyme activities in solution from hydrolysis of different treated manure fiber

(1). The initial enzyme loading was 1300 FPU/L and the initial solid concentration was 2.5%.
(2). A, manure fiber; B, fiber from dilute acid treatment; C, fiber from sodium chlorite treatment; D, fiber from sodium chlorite & dilute acid treatment; E, fiber from dilute alkaline peroxide treatment.

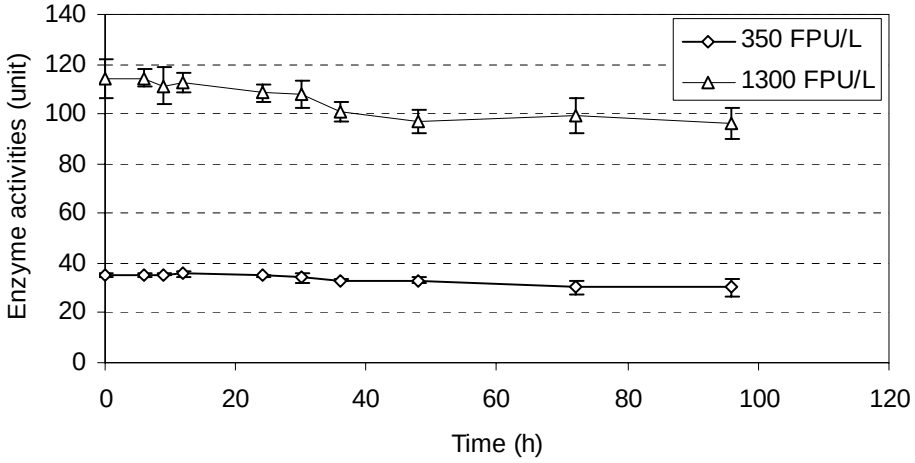


Fig. 6.4. Enzyme stabilities in buffer solution

(1): Data are presented as the mean of triplicates and the error bars show the standard deviation

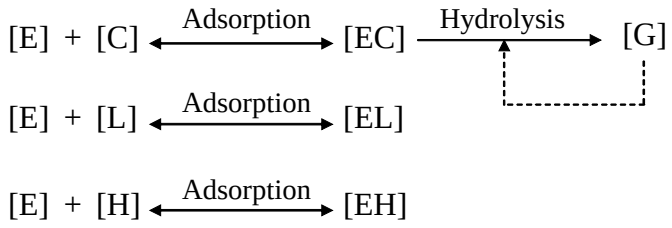


Fig. 6.5. Reaction scheme for proposed kinetic model of enzymatic hydrolysis of manure fiber

(1). Dash line represents the inhibition.

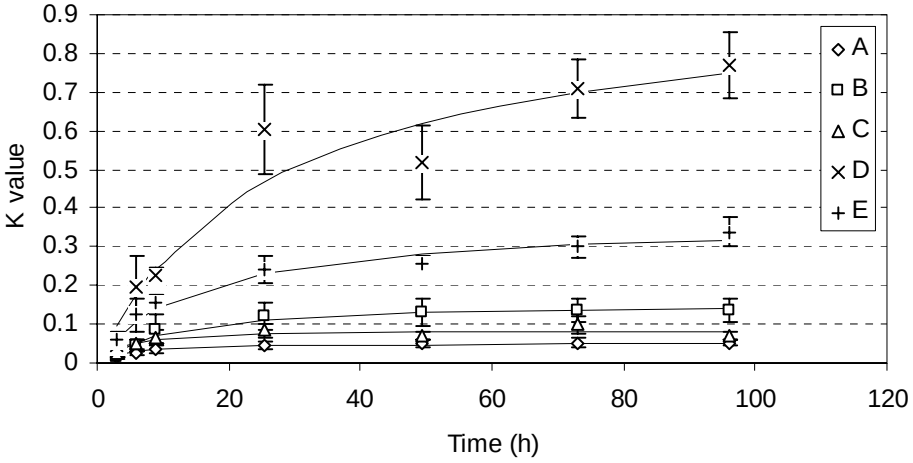
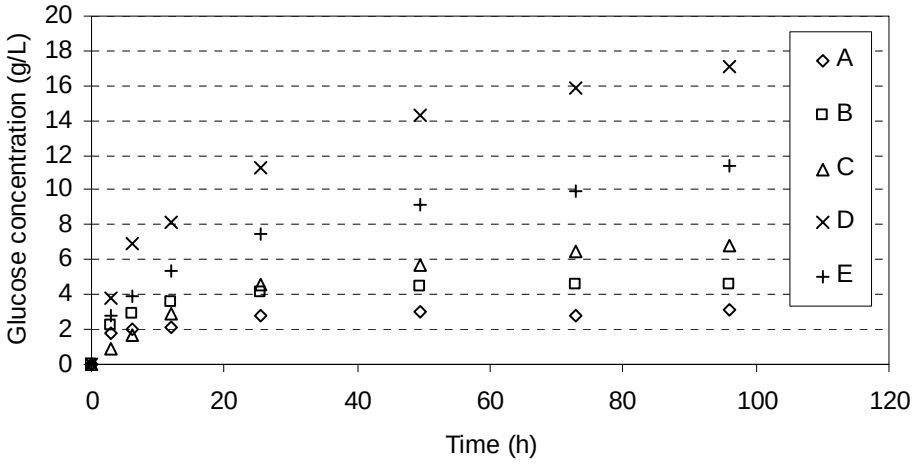
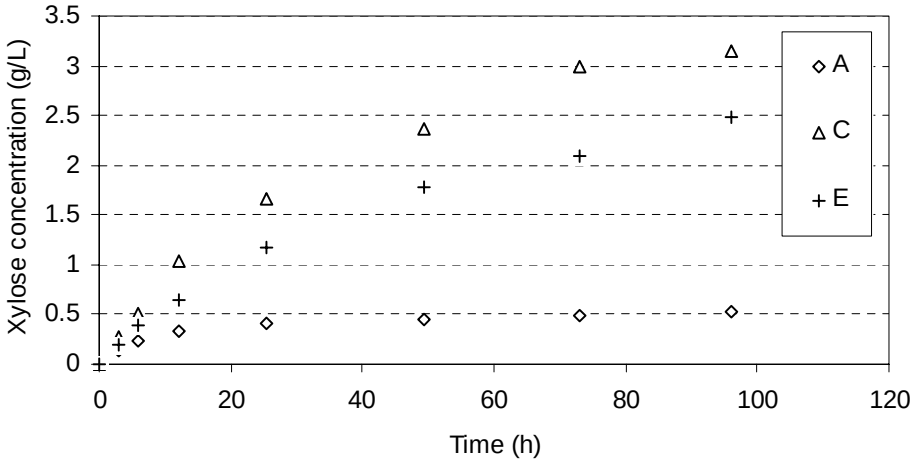


Fig. 6.6. Changes of adsorption constant (K) during the hydrolysis

(1). Data are presented as the mean of 16 sample combinations of 4 enzyme loadings and 4 substrate concentrations, and the error bars show the standard deviation.
(2). A, manure fiber; B, fiber from dilute acid treatment; C, fiber from sodium chlorite treatment; D, fiber from sodium chlorite & dilute acid treatment; E, fiber from dilute alkaline peroxide treatment; -, calculated values from model.



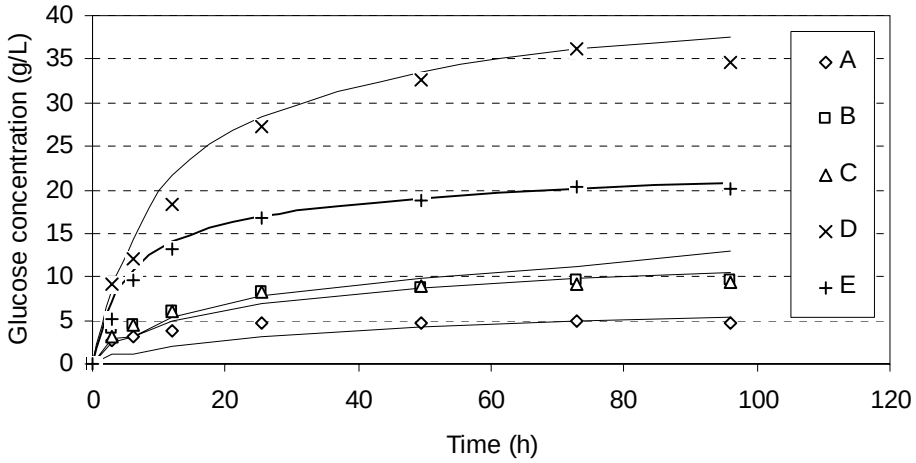
A. Glucose



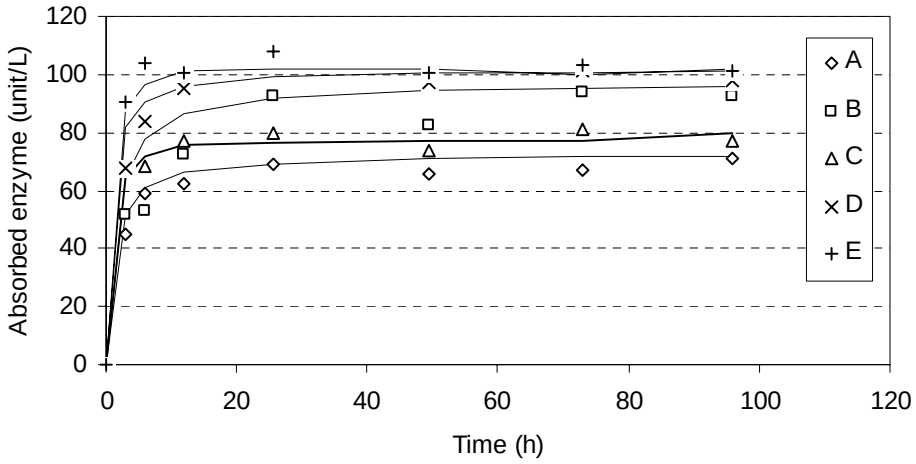
B. Xylose

Fig. 6.7. Sugars from enzymatic hydrolysis of differently treated fiber

(1). The initial enzyme loading was 350 FPU/L, and the initial solid concentration was 50 g/L.
(2). A, manure fiber; B, fiber from dilute acid treatment; C, fiber from sodium chlorite treatment; D, fiber from sodium chlorite & dilute acid treatment; E, fiber from dilute alkaline peroxide treatment.



A. Glucose production



B. Enzyme adsorption

Fig. 6.8. Kinetic model validation

(1). The initial enzyme loading was 1300 FPU/L, and the initial solid concentration was 50 g/L.
(2). A, manure fiber; B, fiber from dilute acid treatment; C, fiber from sodium chlorite treatment; D, fiber from sodium chlorite & dilute acid treatment; E, fiber from dilute alkaline peroxide treatment. -, model prediction.

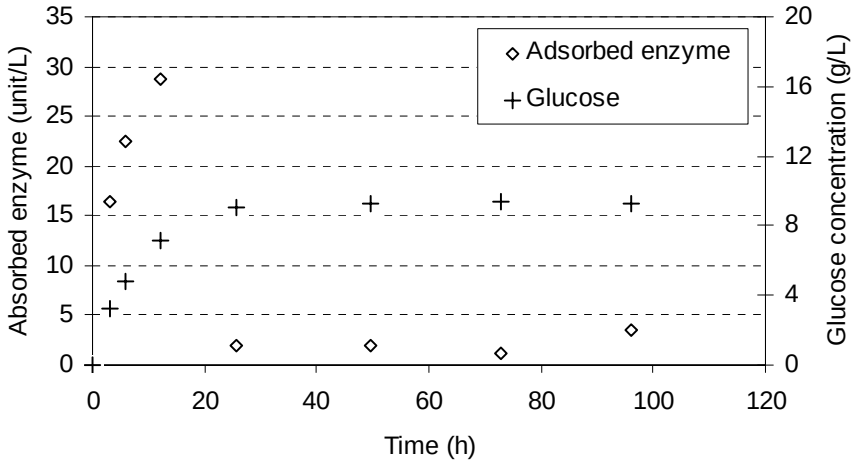


Fig. 6.9. Enzymatic hydrolysis of acid & chlorite treated fiber at the substrate concentration of 10 g/L and the enzyme concentration of 650 FPU/L

CHAPTER SEVEN

A NEW APPROACH OF PELLET FORMATION OF A FILAMENTOUS FUNGUS – *Rhyzopus oryzae*

7.1. Abstract

The effects of inoculum and medium compositions such as potato dextrose broth as carbon source, soybean peptone, calcium carbonate, and metal ions on pellet formation of *Rhizopus oryzae* ATCC 20344 have been studied. It was found that metal ions had significantly negative effects on pellet formation while soybean peptone had positive effects. In addition, potato dextrose broth and calcium carbonate were beneficial to *Rhizopus oryzae* for growing small smooth pellets during the culture. The study also demonstrated that an inoculum size of less than 1.5×10^9 spores/L had no significant influence on pellet formation although it had significant impact on pellet growth. Thus, a new approach to form pellets has been developed using only potato dextrose broth, soybean peptone, and calcium carbonate. The pellet size could be controlled by adjusting inoculum size and the concentration of potato dextrose broth, soybean peptone, and calcium carbonate in the medium.

Keywords: calcium carbonate, fungal pellet, inoculum size, metal ions, potato dextrose broth, *Rhizopus oryzae*

7.2. Introduction

Filamentous fungal fermentation is widely used to commercially produce useful products such as organic acids, enzymes, antibiotics, and the cholesterol lowering drugs (Statins) (Cao et al., 1996; Casas Lopez et al., 2004; Chahal, 1985; Hang, 1989; Papagianni, 2004; Schuurmans et al., 1956; Steel et al., 1954). Fungi can be grown in submerged cultures by several different morphological forms: suspended mycelia, clumps, or pellets (Metz et al., 1977). Many studies have discussed the advantages and disadvantages of growth morphologies in terms of different product (Calam, 1976; Konig et al., 1982; Martin et al., 1952). It has been concluded that the fungal growth in pellet form is a favorable alternative to benefit most fungal fermentations since it not only makes fungal biomass reuse possible but also significantly improves the culture rheology that results in better mass and oxygen transfer into the biomass, and lower energy consumption for aeration and agitation (Van Sjijdam et al., 1980).

The change of fungal morphology is mainly influenced by medium composition, inoculum, pH, medium shear, additives (polymers, surfactants, and chelators), culture temperature, and medium viscosity (Metz et al., 1977; Nielsen et al., 1996; Papagianni, 2004; Znidarsic et al., 1998). For individual strains, each factor has different influence to the growth morphologies; some strains such as *Rhizopus* sp. need strong agitation to form pellets, while some strains such as *Penicillium chrysogenum* require high pH to form pellets (Metz et al., 1977). Thus, the study on fungal pellet formation is limited at the level of the individual strain.

Strains of *Rhizopus oryzae* have the ability to produce fumaric acid, lactic acid, pectinase, amylogucosidase, and α-amlyase (Cao et al., 1996; Hang, 1989; Papagianni,

2004; Soccol et al., 1994). Zhou et al. (2000) investigated the effects of different metal ions (Mg^{2+}, Zn^{2+}, and Fe^{2+}) and pH on the pellet formation of *R. oryzae* ATCC 20344 under glucose as a carbon source and urea as a nitrogen source. Byrne et al. (1989a; 1989b) studied the effects of glucose concentration, peptone concentration, pH, and some additives on the pellet formation of *R. oryzae* ATCC 10260. However, the comprehensive investigation of the effects of medium composition and inoculum on the pellet formation and growth has not been fulfilled. This article is focused on investigating the roles of the factors such as carbon source, nitrogen source, metal ions, buffer, and inoculum on pellet formation; and on the development of a new approach to form *Rhizopus oryzae* pellets.

7.3. Material and methods

7.3.1. Microorganism

R. oryzae ATCC 20344 was obtained from the American Type Culture Collection (Manassas, VA). The strain was first cultured on potato dextrose agar (Difco) slants, and further propagated on potato dextrose agar in 500 ml Erlenmeyer flasks to form spores. The culture temperature was 25°C. The spores were washed from the agar with sterile distilled water, and collected as a spore suspension for the study. The spore concentration of the suspension was 7.5×10^7 spores/ml.

7.3.2. Pellet formation

The factors examined for pellet formation were carbon source, nitrogen source, mineral ions, and buffer. A 2^4 full factorial design with replicates was carried out (Table 7.1). Two different carbon sources (Potato dextrose broth (PDB) and glucose) under two

levels of nitrogen (with and without), mineral ions (with and without), and buffer (with and without) were studied. The glucose concentration in the carbon sources was 20 g/L. The nitrogen source was soybean peptone (Sigma) with a concentration of 6 g/L. The mineral ions included: 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$. The buffer was $CaCO_3$ (6 g/L). The cultures were performed at 27°C for 48 hr on a rotary shaker at 190 rpm.

7.3.3. Effects of inoculum size on pellet formation and growth

Four spore concentrations (1.5×10^8, 3.75×10^8, 7.5×10^8, and 1.5×10^9 spores/L) were run on the selected media which were identified in the previous section as favorable media to form pellets. The cultures were carried out under the same conditions described in the previous section.

7.3.4. Pellet growth

A completely randomized design (CRD) with three replicates on three main factors (PDB, nitrogen source, and buffer) determined from previous sections on pellet formation was used to study the pellet growth. Four PDB concentrations (4, 12, 24, 36 g/L) and four peptone concentrations (1, 3, 6, 9 g/L) were studied at two different levels of buffer (3, 9 g/L). All media were dissolved using deionized water (without mineral ions). The spore concentration for all runs was fixed at 3.6×10^8 spores/L. The culture conditions were same as that from previous sections.

7.3.5. Statistical analysis

The effects of carbon source, nitrogen, mineral ions, and buffer on pellet formation were compared using the special property of factorial designs whose effects can be simply estimated by the differences in average response values between the high and low codes of each factor. A ranked list that presented the relative importance among factors was formed by comparison. The list is given in the Pareto chart (Haaland, 1989).

The effects of various factors on pellet growth were analyzed with General Lineal Model (GLM) using the Statistical Analysis System program 8.0 (SAS institute Inc. NC). ANOVA tables of different responses (pellet number, pellet size, and biomass dry weight) were obtained from analyses, which were used to evaluate the factors.

7.3.6. Analytical methods

The morphology of the cultures was determined by examining submerged cultures dispersed on Petri dishes. An Olympus microscope (Tokyo, Japan) was used to observe the pellet morphology and measure the size of the pellets. The pH value was measured with a portable pH meter (Oakton pH 110, Fisher Scientific, U.S.A.). Dry biomass was determined by washing the pellet mycelia with 6N HCL to neutralize excess $CaCO_3$ attached in the pellets, and then washing to pH 6 with DI water. The washed biomass was dried at 100ºC over night before weight analysis.

7.4. Results

7.4.1. Pellet formation

The effects of carbon source, nitrogen source, metal ions, and buffer on fungal morphology were presented in Table 7.2. Fungal morphologies varied from different combination of factors (Fig. 7.1). There were only four runs (No. 3, 4, 11, 12) which were able to form pellets. In order to conduct statistical analysis on the qualitative variable of morphologies, the quantitative value has to be assigned to describe it. The uniform pellet form of fungal biomass was represented by the value of 1, and other non-pellet forms such as clump, less/non-growth, and non-uniform pellet/clump were represented by the value of 0. The analysis demonstrated that peptone, metal ions, and their combined interaction together had 100% of total effect, which means that peptone and metal ions were the two main factors on pellet formation (Fig. 7.2). The data also showed that peptone had a positive effect (33% of total effect) on pellet formation, while metal ions and the interaction of metal ions and peptone (33% each of total effect) had negative effects.

Statistical analysis concluded that the other two factors of carbon source and buffer were not the main factors on pellet formation. However, both of them had significant influences on fungal pellet growth. Pellets from cultures with calcium carbonate and PDB had an average diameter of 1.98 mm and 3.99 g biomass, while pellets from corresponding cultures without calcium carbonate only had 1.03 mm and 3.16 g, respectively (Fig. 7.3). Pellets cultured on glucose had the same trend as those on PDB (Fig. 7.3). In addition, pellets cultured on both PDB and glucose with calcium carbonate were much smoother than those without calcium carbonate (Fig. 7.4). As for

carbon sources, on the media with calcium carbonate the size of pellets cultured on PDB was 1.98 mm which was smaller than the 2.57 mm from cultures on glucose, and PDB medium produced 1.58 g/L more biomass than the glucose medium (Fig. 7.3). The same trend was on the media without calcium carbonate (Fig. 7.3). In terms of effects of carbon sources on fungal pellets, PDB was more beneficial to producing more biomass and pellets with a smaller size compared to glucose. The detail studies on effects of PDB and calcium carbonate were described in the section of pellet growth.

The results also demonstrated that pH differences caused by different components in the culture medium among experimental runs had no significant influence on pellet formation compared to other factors (Table 7.2). Experiments No. 3 and 4 formed pellets at final pH 6.81 and 3.77, respectively. Experiments No. 11 and 12 formed pellets at pH values of 5.88 and 3.43, respectively.

7.4.2. Effects of inoculum size on pellet formation and growth

Spore concentration did not influence pellet formation. All four different spore concentrations (1.5×10^8, 3.75×10^8, 7.5×10^8, and 1.5×10^9 spores/L) formed smooth pellets on both PDB pellet-formed culture medium (24 g/L PDB, 6 g/L soybean peptone, 6 g/L $CaCO_3$) and glucose pellet-formed culture medium (20 g/L glucose, 6 g/L soybean peptone, 6 g/L $CaCO_3$). Pellet numbers and total amount of biomass increased, and pellet size decreased following the increase of spore concentration no matter what type of carbon source the cultures were on (Fig. 7.5). The changes of total pellet numbers and pellet size with the increase of inoculum concentration was much more significant than the change of biomass. This could be explained because the biomass increase in the

certain range of inoculum was mainly controlled by the nutrients rather than spore concentration.

7.4.3. Pellet growth

Previous sections concluded that mineral ions were the negative factor on pellet formation, peptone had a positive effect, calcium carbonate was important for smooth pellet formation, inoculum size has no influence on pellet formation, and PDB as carbon source produced more biomass and pellets than glucose although both of them have no significant difference on pellet formation. Thus, the study of pellet growth in this section has been focused on factors of PDB, peptone, and calcium carbonate at a fixed spore concentration.

The effects of these factors on changes of pellet number have been presented in Fig. 7.6. The statistical analysis showed that calcium carbonate, PDB, peptone, as well as all two-ways and three-ways interactions of them had a significant ($P<0.05$) influence on the total number of pellets (Table 7.3). At low concentration of calcium carbonate, pellet number from all cultures increased with the increase of PDB concentration (Fig. 7.6a). At low PDB concentrations, high peptone concentrations produced more pellets than low peptone concentrations. Following the increase of PDB concentration, the total amount of pellets from the cultures with low peptone content increased much faster than those cultures with high peptone content. At high concentration of calcium carbonate, media with low peptone content had the same trend with corresponding ones at low concentration of calcium carbonate (Fig. 7.6b). For cultures on 6 g/L and 9 g/L peptone, pellets number decreased with the changes of PDB concentration (Fig. 7.6b). In the

experimental range the highest pellet number of 140,000 was reached at 35 g/L PDB and 1 g/L peptone.

Pellet size was also significantly ($P<0.05$) influenced by the factors of PDB, peptone, calcium carbonate, and their interactions except for the interaction of PDB and peptone (Table 7.3). In addition, the standard deviation of pellet size at each point was relatively larger compared to other parameters of pellet number and biomass concentration, which means that it was very difficult to observe the differences between different combinations of medium components only based on the mean values. Thus, in terms of accurately describing the changes of pellet size a pair-wise comparison using least squares means (lsmeans) was conducted to statistically interpret the effects of the various factors on pellet size. At low calcium carbonate content of 3 g/L, the statistical analysis indicated that the following: 1) Pellets became smaller following the increase of PDB concentration (Fig. 7.7a); 2) There were no significant ($P>0.05$) differences on pellet size among the three peptone concentrations of 1, 3, and 6 g/L at a PDB concentration of 4 g/L while 9 g/L peptone produced significantly ($P<0.05$) smaller size than the other three; 3) For higher PDB concentrations of 12, 24, and 36 g/L, there were no significant ($P>0.05$) differences on pellet size among the four peptone concentrations at each individual PDB concentration. At a high calcium carbonate content of 9 g/L, the same analysis demonstrated that there were no significant ($P>0.05$) differences among the four PDB concentrations at the same peptone concentration, which means that pellet size only changed with respect to the change of peptone concentration (Fig. 7.7b). The analysis also showed that at low peptone concentrations pellet size increased with increase of peptone concentration. However, once peptone concentrations reached 6 g/L

there were no significant ($P>0.05$) differences on pellet size between peptone concentrations of 6 g/L and 9 g/L (Fig. 7.7b). Meanwhile, the analysis showed that most of pellets from high calcium carbonate content were significantly ($P<0.05$) smaller than pellets from low calcium carbonate content at each individual PDB concentration except for pellets from four combinations of PDB concentrations of 24 and 36 g/L, peptone concentrations of 6 and 9 g/L at high calcium carbonate content of 9 g/L.

All three factors and one interaction of PDB and peptone had significant ($P<0.05$) effects on the fungal biomass concentration (Table 7.3). The biomass concentration increased following the increase of PDB, peptone, and calcium carbonate (Fig. 7.8). The highest biomass concentration of 4.70 g/L was reached with the culture on 36 g/L PDB, 9 g/L peptone, and 9 g/L calcium carbonate.

7.5. Discussion

Metal ions are an important factor in the metabolism of *R. oryzae*. The organism utilized the energy more efficiently when metal ions were added to the medium, which made for a relatively fast and abundant fungal growth (Foster et al., 1939). It has been shown by our study that the cultures with metal ions produced more biomass than those without metal ions (Table 7.2). Meanwhile, in terms of fungal morphology, metal ions in the media made *R. oryzae* difficult to form the pellet as well, since the fungus grew so fast on the media with metal ions; the filaments tangled each other and leaned to clumpy morphology (Fig. 7.1a). Thus, in order to form *R. orzyae* pellet, metal ions had to be eliminated in the culture media.

The pH of medium has also been reported as a very important factor for various fungi to form pellets. Generally, pH could change the surface properties of fungi, further influencing the pellet formation. Different strains have different sensibility to pH value (Metz et al., 1977). However, for this particular strain of *R. oryzae*, the results showed that there were no significant differences on pellet formation within the pH range of 3 to 7, which means that this strain was not as sensitive to pH as some other strains such as *Aspergillus niger, Penicillium chrysogenum* (Galbraith et al., 1969; Pirt et al., 1959; Steel et al., 1954).

Inoculum size is generally recognized as of great importance in the process of fungal pellet formation. Generally, the interaction of hyphae is considered as the main mechanism for forming clump. At the early stage of growth, the higher the inoculum size, the more interaction with the hyphae, and the greater the possibility the clump would be formed. Thus, it has been concluded by other researchers that low inoculum concentrations are beneficial for pellet production (Foster, 1949). However, the maximum inoculum size varied from strain to strain (Metz et al., 1977). Most studies on pellet formation of strains of *Rhizopus* were conducted at relatively low concentrations (less than 10^7 spores/L) (Byrne et al., 1989a; Byrne et al, 1989b; Znidarisic et al., 1998; Zndiarsic et al., 2000). Our study on the effects of inoculum on *R. oryzae* pellet formation demonstrated that there were no significant ($P>0.05$) influences on pellet formation once the inoculum concentration was increased up to 10^9 spores/L. The result elucidated that the particular strain of *R. oryzae* is able to prevent hyhpae growth from forming clumps at relatively higher inoculum concentrations compared to other strain in the genus *Rhizopus*.

However, inoculum concentration did have significant influences on pellet growth (Fig. 7.5).

Peptone as nitrogen source was one of two main factors on fungal pellet formation based on the statistical analysis. Peptone had a positive effect on pellet formation mainly because nitrogen was the limiting factor on the growth of *R. oryzae* (Foster et al., 1939). Meanwhile, the type of nitrogen compound also has a considerable influence on fungal pellet formation (Pirt et al., 1959). A study of different nitrogen sources on *R. oryzae* ATCC 20344 showed that peptone produced much smaller, more unique, and heavier pellets than other nitrogen sources such as urea (W. Liao and S. Chen, unpublished data). Peptone had influences on pellet growth as well. It strongly interacted with the other factors such as PDB concentration and calcium carbonate to influence the pellet growth. For example, higher peptone concentrations were beneficial to produce more pellets at low PDB concentrations while vice versa at high PDB concentrations (Fig. 7.6a).

Although the carbon source was not a main factor on pellet formation, it influenced pellet growth. The results concluded that PDB is a better carbon source for the pellet formation of *R. oryzae* compared to glucose. PDB has been widely used as a good nutrient source for fungal and yeast cultures. PDB contains mainly glucose, some vitamins, and a little nitrogen. The effects of PDB on fungal morphology have not been reported to date. It has been found in this study that PDB had a large impact on pellet growth such as pellet size, total biomass, and total amount of pellets. This might suggest that the vitamins in PDB might be the main substances causing the difference on fungal

growth. Additional work is required to determine the effects of PDB components on pellet growth.

Calcium carbonate, as a buffer, prevents pH from dropping into the low pH range of 2-3 which is not favorable for the biomass accumulation (Znidarsic et al., 1998). Total amount of biomass from a medium with calcium carbonate were significantly higher than those without it (Fig. 7.3). In addition, during fungal pellet formation, calcium carbonate is not only a pH buffer, but it also supplies Ca^{2+} ions. It has been reported that calcium ions were usually recognized to induce mycelial aggregation during fungal growth (Jackson et al., 1993), which has been proved by this study that media with calcium carbonate produced smoother and larger pellets than those without calcium carbonate (Fig. 7.4).

7.6. Conclusion

A new, simple culture medium for growing pellets of *R. oryzae* ATCC 20344 has developed in this study. The fungal pellets were formed from the culture on a medium with only three components of PDB, soybean peptone, and calcium carbonate without any additives such as metal ions, polymers etc. Different sizes, numbers, and density of pellets were obtained through adjusting the inoculum size and the concentrations of these three components in the medium. This suggests that the favorable size pellet for different fermentation productions could be successfully achieved. Further studies on the effects of other trace components in PDB, such as vitamins, on pellet formation and the use of potato hydrolysate as a substitute for PDB on pellet formation are currently under investigation in our laboratory.

7.7. References

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Table 7.1. 2^4 full factorial design with replicates for pellet formation [a]

Run	Factors			
	Carbon source	Nitrogen source	Mineral ions	Buffer
1	PDB (+1)	Yes(+1)	Yes(+1)	Yes(+1)
2	PDB(+1)	Yes(+1)	Yes(+1)	No(-1)
3	PDB(+1)	Yes(+1)	No(-1)	Yes(+1)
4	PDB(+1)	Yes(+1)	No(-1)	No(-1)
5	PDB(+1)	No(-1)	Yes(+1)	Yes(+1)
6	PDB(+1)	No(-1)	Yes(+1)	No(-1)
7	PDB(+1)	No(-1)	No(-1)	Yes(+1)
8	PDB(+1)	No(-1)	No(-1)	No(-1)
9	Glucose(-1)	Yes(+1)	Yes(+1)	Yes(+1)
10	Glucose(-1)	Yes(+1)	Yes(+1)	No(-1)
11	Glucose(-1)	Yes(+1)	No(-1)	Yes(+1)
12	Glucose(-1)	Yes(+1)	No(-1)	No(-1)
13	Glucose(-1)	No(-1)	Yes(+1)	Yes(+1)
14	Glucose(-1)	No(-1)	Yes(+1)	No(-1)
15	Glucose(-1)	No(-1)	No(-1)	Yes(+1)
16	Glucose(-1)	No(-1)	No(-1)	No(-1)

a: Code values are in the parentheses.

Table 7.2. Experimental results from 2^4 full factorial design [a]

Run	Fungal morphology [b]	Pellet size (mm) [c]	Biomass (g dry matter/L)	Initial pH	Final pH
1	Clump (0)	-	6.344	6.06	7.10
2	Clump (0)	-	3.620	5.76	7.13
3	Uniform pellet (1)	1.98±0.41	3.992	6.84	6.81
4	Uniform pellet (1)	1.03±0.15	3.158	5.99	3.77
5	Clump (0)	-	2.818	5.58	5.59
6	Non-uniform pellet & clump (0)	-	2.318	4.65	3.11
7	Non-uniform pellet & clump (0)	-	2.366	6.58	6.21
8	Non-uniform pellet & clump (0)	-	0.912	4.93	3.31
9	Clump (0)	-	4.574	6.44	7.18
10	Clump (0)	-	3.030	6.20	6.11
11	Uniform pellet (1)	2.57±0.22	2.408	7.42	5.88
12	Uniform pellet (1)	1.47±0.35	1.842	7.13	3.43
13	Non-growth (0)	-	0.008	5.43	5.84
14	Non-growth (0)	-	0.008	4.64	4.52
15	Less-growth (0)	-	0.016	6.81	6.74
16	Less-growth (0)	-	0.036	6.05	6.09

a. All data except pellet size are the mean of two replicates, pellet size is the mean of 200 replicates with standard deviation at α=0.05.
b. Code values are in the parentheses. (1) is the value to represent the pellet, (0) is the value to represent the non-pellet forms such as clump, non-growth etc.
c. "-" means non-pellet.

Table 7.3. Analysis of variance (ANOVA) table of effects of PDB, peptone, and CaCO3 on pellet growth

Source term	Degree of freedom			Mean square			F-ratio			P-value [a]		
	Pellets No.	Pellet diameter	Biomass	Pellets No.	Pellet diameter	Biomass	Pellets No.	Pellet diameter	Biomass	Pellets No.	Pellet diameter	Biomass
PDB	3	3	3	2.74×10^{10}	1.47	42.63	1198.91	16.56	1809.28	<0.0001	<0.0001	<0.0001
Peptone	3	3	3	1.60×10^{9}	0.60	8.71	69.99	6.74	369.58	<0.0001	0.0004	<0.0001
$CaCO_3$	2	2	2	2.72×10^{10}	3.00	4.06	1190.54	33.81	172.12	<0.0001	<0.0001	<0.0001
PDB × Peptone	9	9	9	3.15×10^{9}	0.16	0.38	137.85	1.79	16.19	<0.0001	0.0795	<0.0001
PDB × $CaCO_3$	6	6	6	5.08×10^{9}	0.61	1.31×10^{-2}	222.06	6.88	0.56	<0.0001	<0.0001	0.7632
Peptone × $CaCO_3$	6	6	6	4.43×10^{8}	0.36	1.10×10^{-2}	19.39	4.06	0.47	<0.0001	0.0011	0.8303
PDB × Peptone × $CaCO_3$	18	18	18	1.50×10^{8}	1.53×10^{-3}	1.20×10^{-2}	6.57	0.02	0.51	<0.0001	1.0000	0.9487
Error	96	96	96	2.29×10^{7}	8.9×10^{-2}	2.35×10^{-2}						
Total	144	144	144									

a: Term significant at α=0.05.

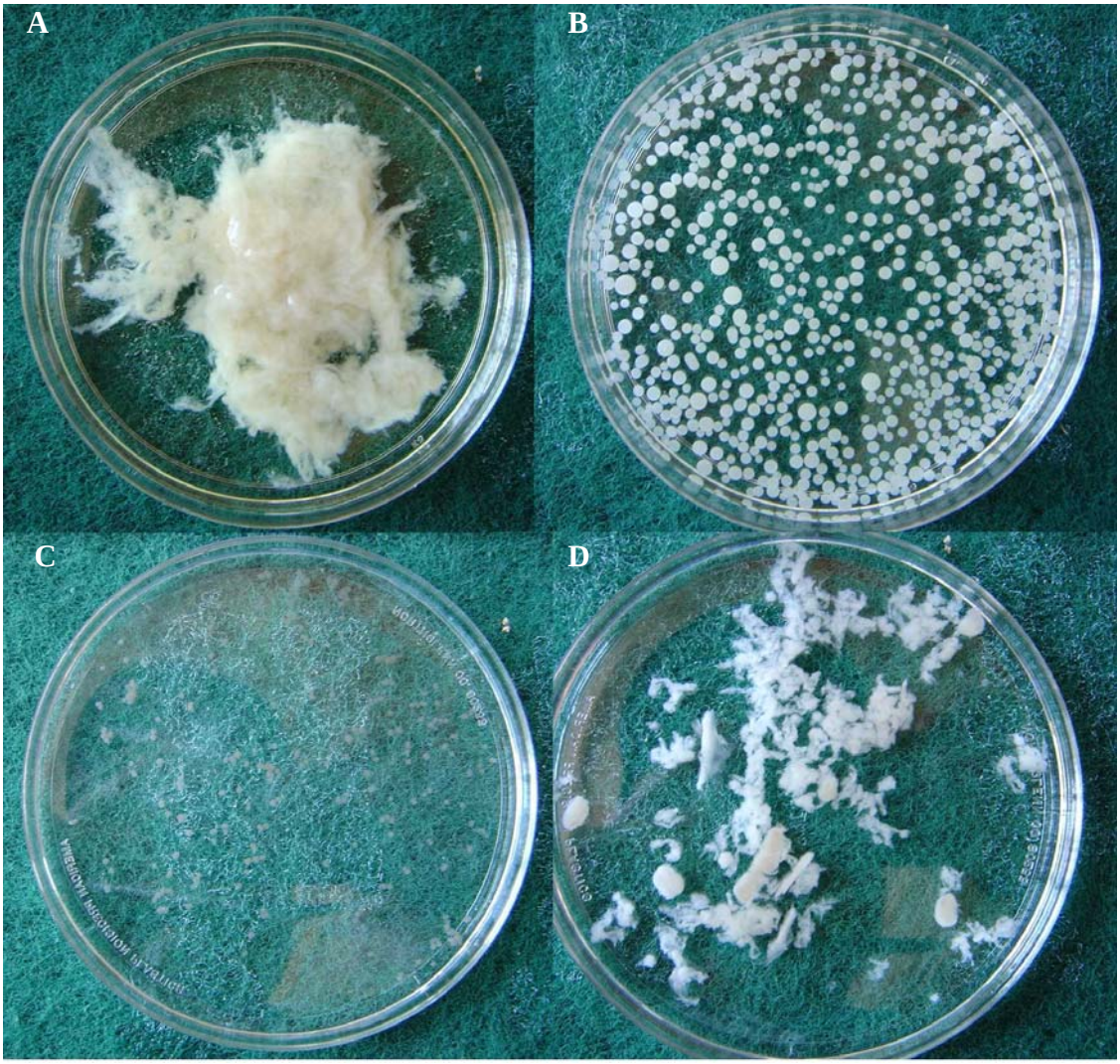


Fig. 7.1. Morphology of fungal biomass

A: Clump from Run 2;
B: Pellet from Run 3;
C: Less-growth from Run 15;
D: Non-uniform pellet & clump

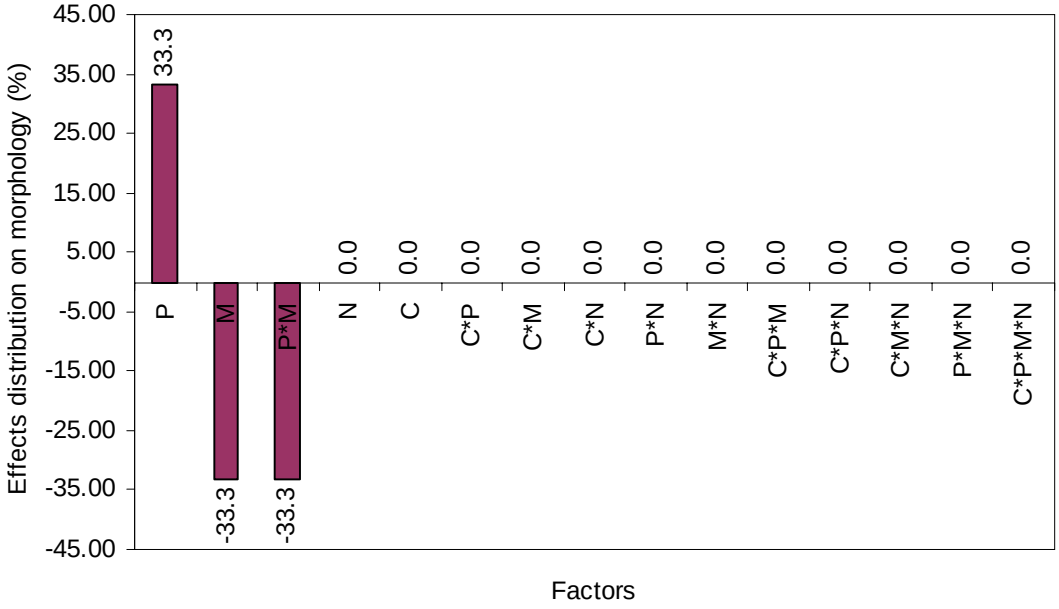


Fig. 7.2. Pareto charts of effects of medium composition on pellet formation

Where P is peptone, M is mineral ions, N is buffer, C is carbon source.

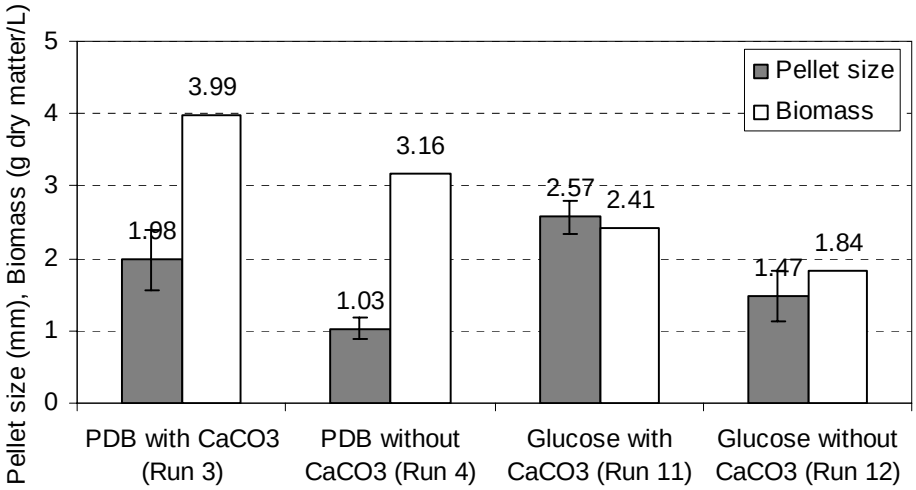


Fig. 7.3. Effects of carbon source and buffer on pellet morphology during pellet formation.

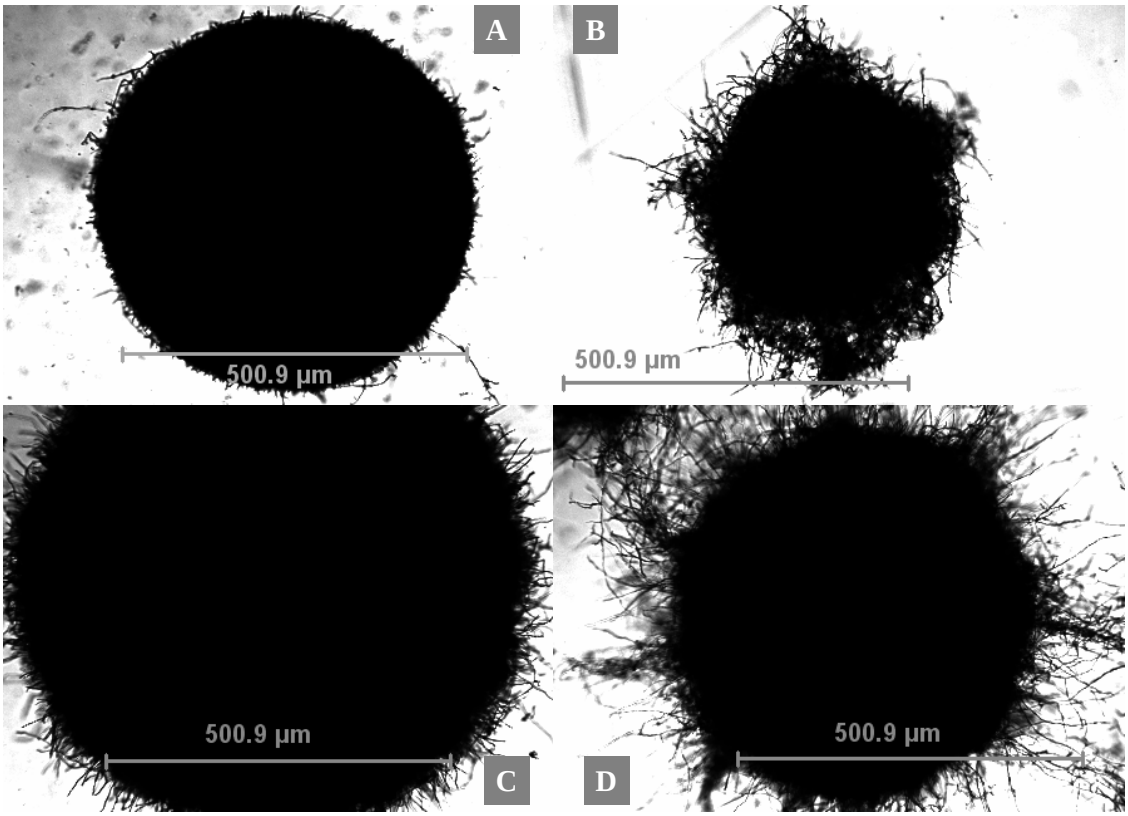
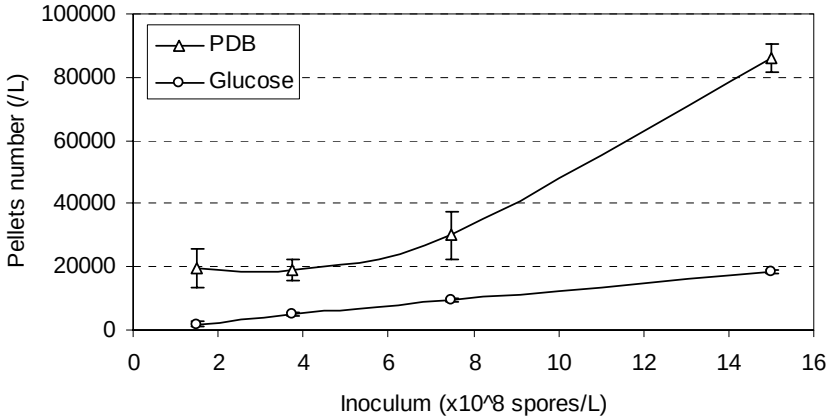
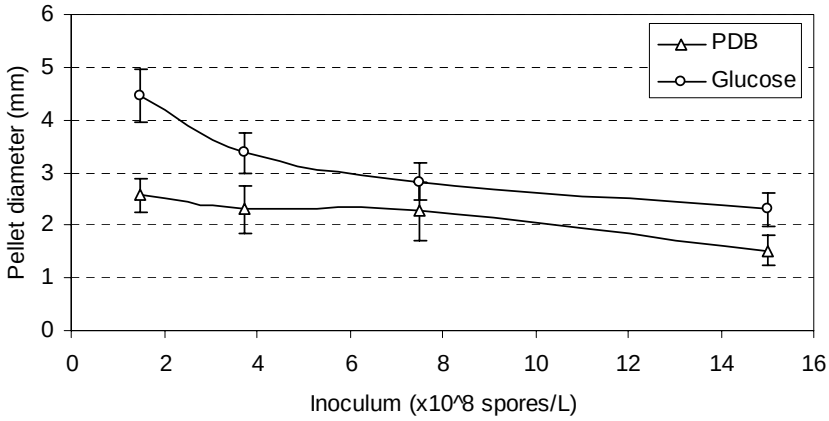


Fig. 7.4. Surface structure of fungal pellets from different cultural conditions

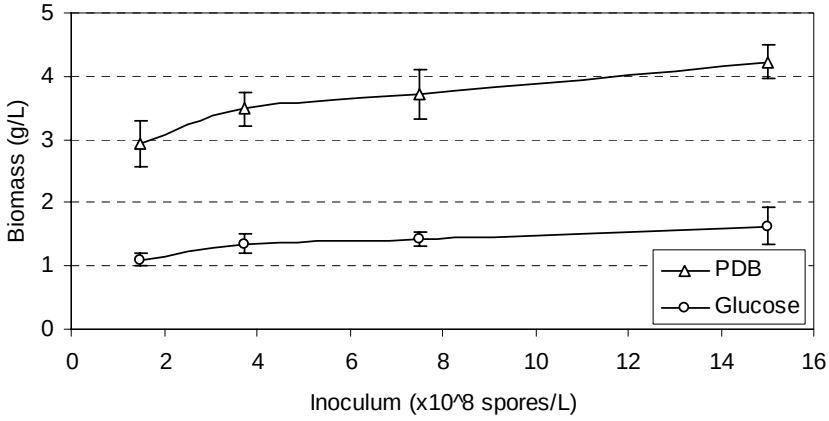
A: Run 3 (PDB with $CaCO_3$)
B: Run 4 (PDB without $CaCO_3$)
C: Run 11 (glucose with $CaCO_3$)
D: Run 12 (glucose without $CaCO_3$)



a. Pellet number

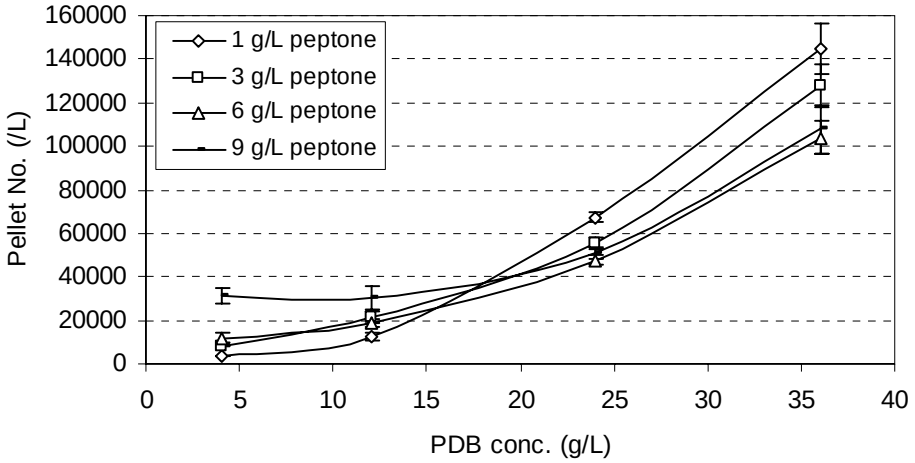


b. Pellet size

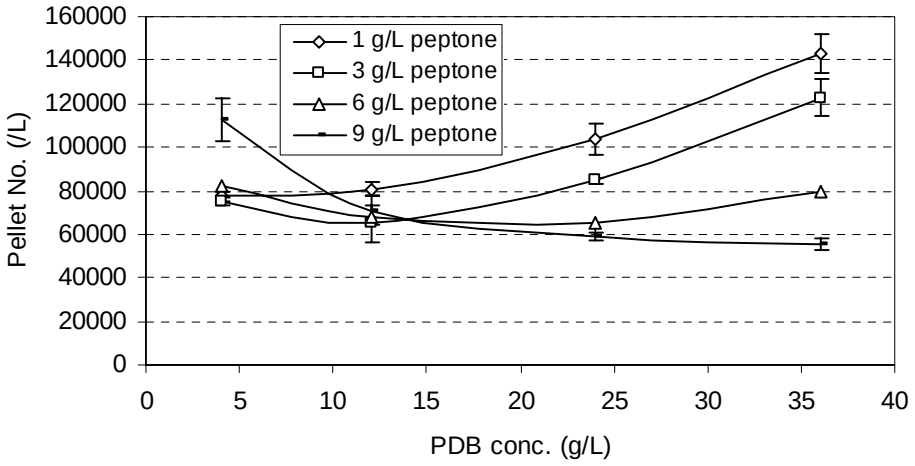


c. Biomass

Fig. 7.5. Effects of inoculum on pellet formation

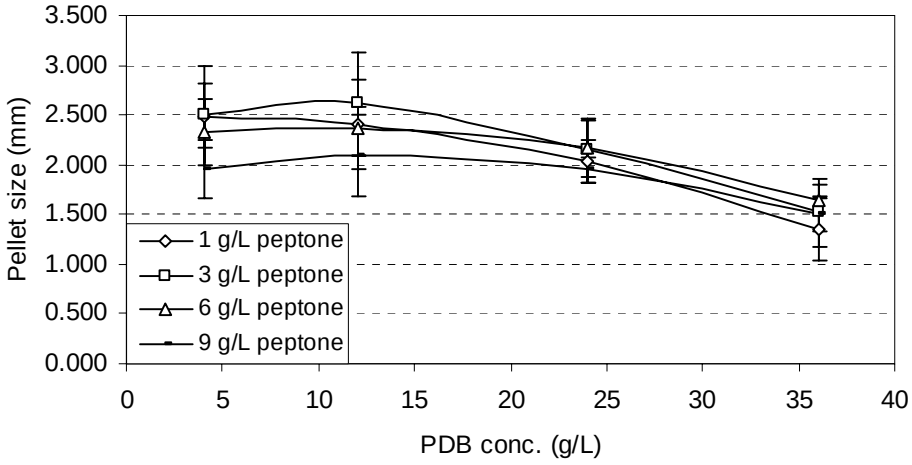


a. 3 g/L $CaCO_3$

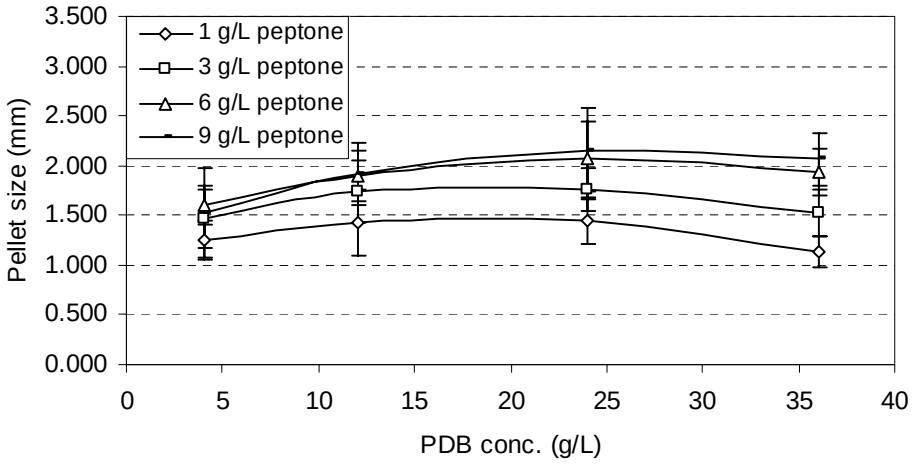


b. 9 g/L $CaCO_3$

Fig. 7.6. Effects of PDB concentration, $CaCO_3$ concentration, and peptone concentration on pellets number during pellet growth

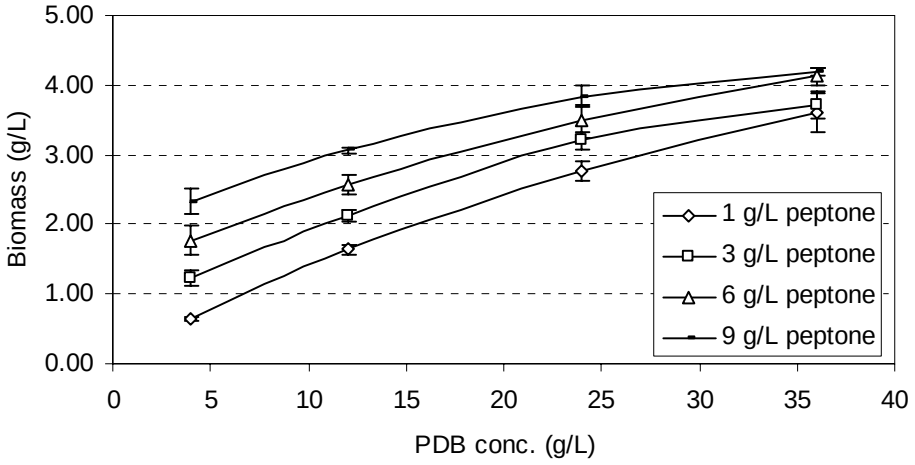


a. 3 g/L $CaCO_3$

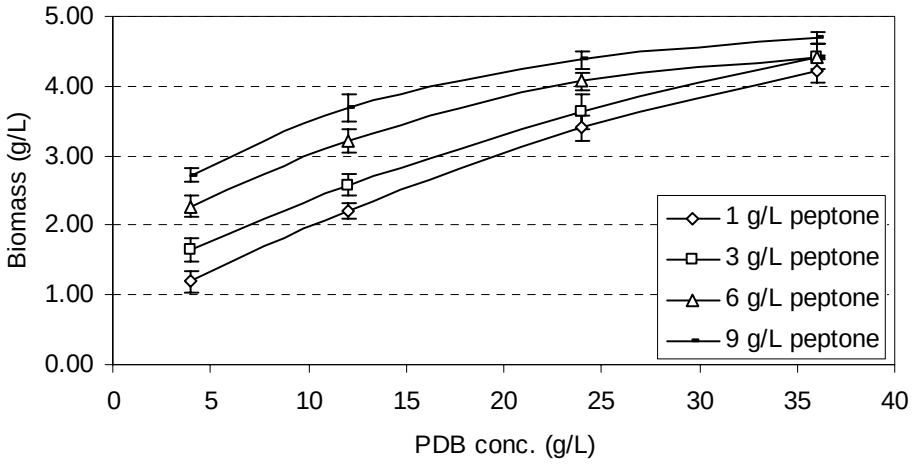


b. 9 g/L $CaCO_3$

Fig. 7.7. Effects of PDB concentration, $CaCO_3$ concentration, and peptone concentration on the size of pellets during pellet growth



a. 3 g/L $CaCO_3$



b. 9 g/L $CaCO_3$

Fig. 7.8. Effects of PDB concentration, $CaCO_3$ concentration, and peptone concentration on the biomass yield [a]

a: biomass yield = total biomass/PDB

CHAPTER EIGHT

CO-PRODUCTION OF FUMARIC ACID AND CHITIN FROM A NITROGEN-RICH LIGNOCELLULOSIC MATERIAL – ANIMAL MANURE – USING A PELLETIZED FILAMENTOUS FUNGUS – *Rhizopus oryzae*

8.1. Abstract

Fumaric acid is widely used as a food additive for flavor and preservation. *Rhizopus oryzae* ATCC 20344 is a fungus known for good fumaric acid production. It also has been reported that the fungal biomass has high chitin content. This study investigated the possibility of producing fumaric acid and chitin via *R. oryzae* fermentation of dairy manure. Co-production of valuable bio-based chemicals such as fumaric acid and chitin could make the utilization of manure more efficient and more profitable. A three step fermentation process was developed which effectively utilized the nitrogen as well as the carbohydrate sources within the manure. These steps were: culturing of pellet seed ; biomass cultivation on liquid manure to produce both biomass and chitin; and fumaric acid production on the hydrolysate from the manure fiber. Under the identified optimal conditions, the fermentation system produced 25 g/L fumaric acid and 5.50 g/L biomass that contained 0.21 g chitin/g biomass.

Key words: dairy manure, chitin, fumaric acid, glucosamine, *Rhizopus oryzae*

8.2. Introduction

Animal manures rich in carbohydrates and protein are potential sources of feedstock for production of renewable bio-based chemicals. There are nearly 160 million dry tons of manure produced annually in the United States (Council for Agricultural Science and Technology, 1995). This provides a large source of biomass that can be used to produce bio-based chemicals, materials, and energy products such as organic acids, chitin, and biogas. Environmentally friendly processes for converting manure to such renewable, bio-based high value chemicals could change manure from a disposal problem to an important biomass resource for chemical and energy production.

Fumaric acid is a four-carbon unsaturated dicarboxylic acid and is widely used as a food acidulant and beverage ingredient. Because of its double bond and two carboxylic groups, fumaric acid has many potential industrial applications, ranging from the manufacture of synthetic resins and biodegradable polymers to the production of intermediates for chemical syntheses (Tao et al., 1993). In turn, chitin, a linear polysaccharide of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranosal residues, where each individual residue is N-acetyl-D-glucosamine, is the second most abundant biopolymer after cellulose in the biosphere.. As a natural biopolymer, chitan has found uses as a coagulating agent in water treatment, a plant seed coating agent in agriculture, and as an absorbent in biomaterials within the biomedical industry (Yusof et al., 2001).

The mycelia fungi *Rhizopus oryzae* has been shown to be the most productive microorganism in regard to fumaric acid production (Foster et al., 1939a; Rhodes et al., 1962; Osmani et al., 1985; Peleg et al, 1989). In addition, the resulting fungal biomass has relatively high chitin content, ranging from 10% to 90% (Carlile, 2001). This

suggests that co-production of fumaric acid and chitin could be possible from one fermentation process.

It has been reported that fumaric acid production by *Rhizopus oryzae* is limited by nitrogen (Foster et al., 1939a; Rhodes et al., 1959; Rhodes et al., 1962), and that a nitrogen presence leads to increased fungal growth potentially at the expense of fumaric acid production. Therefore, most fumaric acid fermentation processes consist of three steps: 1) seed culture; 2) fungal biomass cultivation with nitrogen; and 3) fumaric acid production with limited nitrogen (Kenealy et al., 1986; Zhou et al., 2002; Romano et al., 1967). This general fumaric acid production process, specifically the co-production of fumaric acid and chitin, is uniquely adaptable and advantageous for use within an animal manure platform For example, dairy manure contains 12% hemicellulose, 22% cellulose, and 18% crude protein (Liao et al. 2004), representing a large potential source of carbohydrates to be used as a carbon source in the second step as well as proteins to be used as a nitrogen source in the third step. In addition, 50-60% of the crude protein in dairy manure is soluble. This means that a liquid/solid separation could be used to obtain a nitrogen-rich liquid stream that could be used as a nitrogen source to grow fungal biomass and attain chitin accumulation, while the manure solid stream, containing mostly carbohydrates in the form of cellulose and hemicellulose, could be converted by various pretreatment methods into monosaccharides for use as the carbon source in fumaric acid production.

However, use of a fungal fermentative process for organic acid production has some challenges. These include the production of cotton-like mycelia and mycelia-associated mass transfer and oxygen transfer difficulties, which cause reactor control

concerns as well as little possibility for fungal biomass reuse. The above factors ultimately lead to low efficiency and yield of organic acid in the fermentation process (Yin et al., 1998). Growing fungi in pellet form however, can significantly improve the mass transfer condition and reactor performance and consequently benefit organic acid production. The pellet formation of the fungus *Rhizopus oryzae* ATCC 20344 has been reported in a previous study (Liao et al., 2006a)

Fumaric acid production from starch-based materials such as molasses, potato, corn flour, and cassava using *R. oryzae* fermentation were investigated (Carta et al., 1999; Moresi et al., 1992). However, little research has been reported on co-production of fumaric acid and chitin from lignocellulosic materials. The goal of this research was to develop a novel process using pelletized filamentous fungus *Rhizopus oryzae* ATCC 20344 to simultaneously produce fumaric acid and chitin from a nitrogen-rich lignocelllulosic material — dairy manure. More specifically, the research studied: 1) the effects of manure nitrogen on biomass and chitin production in the cultivation step and 2) the effects of sugar solutions, obtained from manure fiber using different pretreatments, on fumaric acid production.

8.3. Methods and Materials

8.3.1. Material and preparation

Fresh dairy manure was obtained from the Dairy and Beef Centers of Washington State University. The raw manure was composed of 14.3% dry matter (DM) with a total carbon content of 44.3g/100g DM and total nitrogen content of 2.7g/100g DM. Ten kg of the raw manure sample was mixed with 20 kg of water and then blended for 1 minute to

achieve size reduction. The target manure samples were obtained using a centrifuge at 3,000 rpm for 10 minutes. The supernatant was manure liquid, and the solid portion was manure fiber. The data describing the manure liquid and fiber are shown in Tables 8.1 and 8.2.

Manure fiber was hydrolyzed using two different pretreatments to produce monosaccharides. A concentrated acid treatment as a first pretreatment was performed based on a previous study (Liao et al., 2006b) while an enzymatic hydrolysis with a dilute alkaline peroxide treatment was the other pretreatment method used to convert manure fiber into monosaccharides (Liao et al., 2006c). The hydrolysate data is showed in Table 8.3. The hydrolysates were used as the carbon source for fumaric acid production.

8.3.2. Microorganism and spore culture method

The fungus *Rhizopus oryzae* ATCC 20344 was obtained from the American Type Culture Collection (Manassas, VA). The fungus was first grown on potato-dextrose agar (PDA) (Difco, 213400) slants at 30°C for 7 days. For the experiments, the fungal spores in the slant were suspended in sterilized water maintained at 4^{o}C. For storage, the spores were placed in 20% glycerol solution at –80°C.

8.3.3. Pelletized seed culture

Pelletized seed was produced according to a procedure reported in a previous study (Liao et al., 2006a). The culture medium contained 24 g/L of Potato Dextrose Broth (PDB), 6 g/L of peptone, and 6 g/L of $CaCO_3$. The medium was sterilized at 121°C for 15 minutes prior to inoculation. The spore concentration was 1.5×10^{8} spores/L. The seed

culture process was carried out in a 125 ml flask containing 50 ml of medium at 27°C on a rotary shaker at 190 rpm. The culture time was shortened to 24 hours.

8.3.4. Biomass cultivation

8.3.4.1. Effects of manure liquid as an alternative nitrogen source used on fungal biomass and chitin during cultivation

Three nitrogen sources, manure liquid, soybean peptone, and urea were tested. Glucose concentration in the medium was 50 g/L. The concentration of manure liquid, soybean peptone, and urea were 50%, 5g/L, and 1 g/L, respectively, all with a nitrogen content of 0.425 g/L.

Since it has been reported that mineral ions are important for fungal growth (Foster et al., 1939b), mineral ions of 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$ were added into the soybean peptone and urea cultures. Calcium carbonate (6 g/L), as a buffer, was added to maintain a pH of 5 during the culture process. All media were sterilized at 121°C for 15 minutes. The inoculum was 0.48 g/L pelletized seeds. The cultures were incubated in 250 ml flasks containing 100 ml of medium at 30°C for 48 hours on a rotary shaker at 190 rpm.

8.3.4.2. Effects of pellet size on fumaric acid production

Two pellet sizes (1.2 mm and 2.4 mm) were used based on a previous study (Liao et al., 2006a). The medium for fumaric acid production contained 100 g/L glucose, 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$. The cultures were

processed at 30°C for 96 hours in a 1 L New Brunswick BioFlo 110 stirred reactor (New Brunswick Scientific, NJ).

8.3.4.3. Fungal biomass cultivation using manure liquid as a nitrogen and mineral source

The experiment was carried out according to a Completely Randomized Design (CRD) with three replicates of 8 culture combinations. Two glucose concentrations (20g/L and 50 g/L) and four manure liquid concentration (10%, 15%, 25%, and 50%) were studied in flask culture at a fixed temperature of 30°C. The seed for all cultures was inoculated into the flasks at a fixed concentration of 0.48 g dry biomass/L. The cultures were incubated in 250 ml flasks containing 100 ml of culture medium. The other conditions were the same as described in the previous section, 8.3.4.1.

8.3.5. Fumaric acid production

8.3.5.1. Effects of inoculum size on fumaric acid production using glucose as a carbon source

Three replicates of six cultivated biomass concentrations (0.60, 0.90, 2.45, 5.32, and 6.01 g dry biomass/L) were inoculated to the media which contained 100 g/L of glucose, 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$, and 60 g/L of $CaCO_3$. The cultures were incubated in 250 ml flasks containing 100 ml of culture medium. The culture conditions were the same as described in section 8.3.4.1.

8.3.5.2. Effects of different hydrolysates of manure fiber on fumaric acid production

A 1 L New Brunswick Bioflo 110 stirred reactor (New Brunswick Scientific, NJ) equipped with a pH controller was used to carry out the fumaric acid production study. Three media were examined to produce fumaric acid, which were glucose, hydrolysate from the acid treatment, and hydrolysate from the enzymatic treatment. For all three media, mineral ions of 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$ were added to meet the needs of fungal metabolism. For the hydrolysates, additional glucose was added making the total glucose concentration for all three media 100 g/L while calcium carbonate was used to keep the pH at 5. The aeration rate was 1 VVM (Volume/Volume/Minute). The fermentations were run at 30°C at 200 rpm for 6 days.

8.3. 6. Statistical analysis

A pair wise comparison using the Statistical Analysis System program 8.0 (SAS institute Inc. NC) was conducted to identify the effects of manure liquid on the cultivation of fungal biomass and chitin, and the differences of fumaric acid production from various sized inoculums.

8.3.7. Analytical methods

The fumarate in the broth was analyzed using a Dionex DX-500 system (Sunnyvale, CA, USA) (Liu, 2005) including an AS11-HC (4mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler. Dry biomass was determined by washing the pellet mycelia with 6N HCl and

then washing to a pH 6 with DI water. The washed biomass was dried at 100°C overnight before weighing. The diameter of seed pellets was determined using an Olympus microphotograph system (Tokyo, Japan). Glucosamine as the major constituent of chitin was used to express the total amount of chitin in the biomass. Glucosamine was analyzed by a modified method of hydrochloric acid hydrolysis (Muzzarelli et al., 1985). A 0.25 biomass mixed with 25 ml 6 N HCl solution was hydrolyzed at 100C for 4 hours. The glucosamine in the hydrolysate was separated on CarboPac PA 10 guard (4x50 mm) and analytical (4x250 mm) columns at room temperature (approximately 25°C) using a Dionex DX-500 system (Sunnyvale, CA, USA).

8.4. Results and Discussion

8.4.1. Characteristics of manure samples

The characteristics of the manure liquid and manure fiber used in the study are listed in Tables 8.1 and 8.2. The liquid manure had a relatively high nitrogen concentration of 0.85 g/L, and contained some minerals such as magnesium, potassium, and calcium (Table 8.1), which is important because both the nitrogen and the mineral ions are required elements for growth and metabolism of the *Rhizopus oryzae strain* (Foster et al., 1938). This suggests that manure liquid might be a sufficient nutrient source for fungal cultivation.

Meanwhile, manure fiber also had a relatively high cellulose and hemicellulose content (Table 8.2). It is well known that cellulose and hemicellulose can be converted to monosaccharides via hydrolysis. The hydrolysate from the manure fiber could then be further used as carbon source for fumaric acid production. Two hydrolysis methods were

used in the study: concentrated acid hydrolysis and enzymatic hydrolysis with a dilute alkaline peroxide treatment. The data for the hydrolysate from the two different hydrolysis processes are presented in Table 8.3. Acid hydrolysis and enzymatic hydrolysis produced 26 g/L and 21 g/L of glucose, respectively, which unfortunately were both lower than the concentration of 100 g/L that is widely used by most fermentation processes to produce fumaric acid (Cao et al., 1996). Thus, in terms of maintaining glucose at a favorable concentration for the process, additional glucose was added to the hydrolysates to obtain the glucose concentration of 100 g/L.

8.4.2. Fungal biomass cultivation

8.4.2.1. Comparison of effects of different nitrogen sources on fungal cultivation

The different nitrogen sources had a significant ($P<0.05$) influence on fungal cultivation (Table 8.4). The cultivation on soybean peptone produced the highest fungal biomass concentration of 4.51 g/L and also resulted in a chitin biomass of 0.16 g/g and a fumarate concentration of 2.47 g/L. On the other hand, use of urea as a nitrogen source, resulted in a cultivation that had the lowest values of biomass concentration and chitin content at 1.91 g/L, and 0.12 g/g, respectively, although it produced the highest fumarate at 7.41 g/L. The manure liquid produced the highest chitin at 0.19 g/L while also resulting in a biomass of 2.13 g/L and a fumarate concentration of 3.84 g/L. It is apparent then that manure liquid was beneficial for fungi to synthesize more chitin than the other two nitrogen sources. In addition, fumaric acid production from fungal biomass with different pellet sizes shows that the smaller the pellets the more fumaric acid that could be produced (Fig. 8.1). This is because smaller pellets have a higher surface area and

much better mass and oxygen transfer capabilities which makes the environment favorable for acid production. Thus, in terms of the purpose of the biomass cultivation step, which is to produce the most chitin with the smallest pellets while simultaneously producing the least fumarate, the data elucidated that manure liquid can serve as a good alternative nitrogen source and feedstock for fungal biomass growth, even though the production of biomass was higher from soybean peptone.

8.4.2.2. Effects of manure liquid as nitrogen source on fungal biomass and chitin during the cultivation

The cultivations using different percentages of manure liquid at two levels of glucose concentration are shown in Fig. 8.2. At the 50% level of manure liquid, the low glucose concentration of 20 g/L led to a biomass yield of 7.5% with a chitin content of 0.21 g/L and fumarate yield of 6%. As the proportion of manure liquid decreased, the biomass yields and chitin content both decreased. At the 10% level of manure liquid, the statistical analysis by pair wise comparison showed that there were no significant ($P>0.05$) differences in chitin content between the two glucose concentrations, while 20 g/L glucose still had a higher biomass yield of 2.5% than that with 50 g/L glucose. Meanwhile, fumarate yield decreased following an increase of both the glucose concentration and the percentage of manure liquid. Thus, in terms of both biomass and chitin production, 20 g/L glucose and 50% manure liquid were selected to cultivate the fungal biomass for fumaric acid production.

8.4.3. Fumaric acid production

8.4.3.1. Effects of inoculum size on fumaric acid production

Fig. 8.3 shows that during the fumaric acid production step, the fumarate concentration increased following an increase of inoculum size. The fumarate concentration reached 24.8 g/L at an inoculum of 5.32 g/L in 4 days from flask culture. Meanwhile, the statistical pair wise comparison demonstrated that after 5.32 g/L of inoculum, further increases in inoculum size did not significantly ($P>0.05$) improve fumaric acid production. In addition, the data also presents that the fungal biomass almost had no growth without nitrogen source during the step of fumaric acid production (data not shown). These results demonstrate that the maintenance of fungal metabolism does not require the nitrogen source and the fumaric acid production was mainly influenced by the total amount of fungal biomass in the broth. Therefore, an inoculum size of 5.32 g/L was the optimal one for flask culture in producing fumaric acid using glucose as the sole carbon source.

8.4.3.2. Effects of different hydrolysates of manure fiber on fumaric acid production

Fumaric acid production using different hydrolysates of manure fiber was performed in a 1 L stirred reactor. The optimal inoculum size of 5.32 g/L obtained from the previous section was used to carry out the fermentation. The data demonstrated that different hydrolysates had different influence on fumaric acid production (Fig. 8.4). The fumarate concentration from fermentation on glucose reached 28 g/L in 6 days, while the hydrolysate from enzymatic hydrolysis and the hydrolysate from acid pretreatment reached 25 g/L and 18 g/L, respectively. Compared to the fermentations on glucose and

hydrolysate from enzymatic hydrolysis, the fermentation on hydrolysate from acid pretreatment had a much lower fumarate concentration during the entire course of fermentation. It has been reported that acid hydrolysis of lignocellulosic materials, especially concentrated acid hydrolysis, produces some byproducts such as furfural from xylose, hydroxymethylfurfural (HMF) from glucose, and some phenols from lignin while it converts cellulose into the target product of glucose (Larsson et al., 1999; Palmqvist et al., 2000ab). These byproducts could have some inhibition effects on microorganisms. For instance, both furfural and HMF are known to be major inhibitors for yeast during ethanol fermentation (Palmqvist et al, 2000b). The data shows that inhibition did exist on the fungal fermentation as well. Therefore, concentrated acid hydrolysis is not a suitable pretreatment method for converting manure fiber into monosaccharides. Meanwhile, fumarate concentrations from the fermentation of hydrolysate from enzymatic hydrolysis of manure fiber were rather close to those from the control (Fig. 8.4). This result indicated that hydrolysate from enzymatic hydrolysis with a dilute alkaline treatment had almost no inhibitory effect on the fungal fermentation. In addition, enzymatic hydrolysis is a mild process with limited reactor corrosion compared to acid hydrolysis. Thus, enzymatic hydrolysis with dilute alkaline peroxide pretreatment was the suitable option to produce monosaccharides from manure fiber for fumaric acid production.

8.5. Conclusion

Dairy manure can be used as a substrate via *R. oryzae* fermentation to co-produce fumaric acid and chitin. Compared with other nitrogen sources such as urea or soybean peptone, manure liquid was beneficial for chitin accumulation in fungal biomass during

cultivation. Meanwhile, enzymatic hydrolysis with dilute alkaline peroxide treatment was shown to be the preferred pretreatment method to convert manure fiber to monosaccharides that were later used to produce fumaric acid. The optimal conditions for the entire process were as follows: 1) pelletized seed cultured under the optimal conditions obtained in a previous study (Liao et al., 2006); 2) cultivation conducted in a shaker at 30°C and 190 rpm for 48 hours using 50% manure liquid, 20 g/L glucose, and 0.48 g seed/L of inoculum; 3) fumaric acid production carried out at 30°C for 6 days using a mixture of glucose and hydrolysate from enzymatic hydrolysis of fiber using 5.32 g/L pelletized fungal biomass from cultivation. Under the optimal conditions, 25 g/L fumaric acid was produced with a biomass concentration of 5.32 g/L biomass that contained 0.21 g chitin/g biomass.

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Table 8.1. Characteristics of manure liquid[a]

	Manure liquid
N, g/L	0.85 ± 0.09
C, g/L	4.19 ± 0.30
S, g/L	0.04
Ca, g/L	0.22
Mg, g/L	0.05
Na, g/L	0.02
K, g/L	0.29
P, g/L	0.05

a: Data with sign "±" are the average of triplicates with standard deviations (n=3).

Table 8.2. Characteristics of manure fiber[a]

	Manure fiber
Dry Matter, wt %	14.20 ± 0.05
NDF, % dry basis	61.17 ± 1.62
ADF, % dry basis	47.33 ± 1.70
ADL, % dry basis	15.94 ± 0.66
Cellulose (=ADF-ADL), % dry basis	31.40 ± 1.05
Hemicellulose(=NDF-ADF), % dry basis	13.83 ± 0.79
NDF Ash, % dry basis	12.40 ± 0.56
N, % dry basis	1.31 ± 0.04
C, % dry basis	39.46 ± 1.51

a: Data are the average of triplicates with standard deviations (n=3).

Table 8.3. Hydrolysates from manure fiber using different treatments

	Concentrated acid hydrolysis	Enzymatic hydrolysis with dilute alkaline peroxide pretreatment
Glucose, g/L	26.4±1.24	21.24±0.77
Xylose, g/L	12.4±0.61	4.51±0.27
Nitrogen, g/L	0.05	0.02

a: Data with sign "±" are the average of triplicates with standard deviations (n=3).

Table 8.4. Comparison of effects of different nitrogen sources on fungal cultivation[a]

	Biomass (g/L)	Chitin (g/g biomass)	Pellet diameter (mm)[b]	Fumarate (g/L)
Urea	1.91±0.24	0.12±0.02	2.4±0.32	7.41±0.27
Soybean peptone	4.51±0.33	0.16±0.01	2.61±0.49	2.47±0.18
Manure liquid	2.13±0.15	0.19±0.02	1.24±0.31	3.84±0.11

a: Data are the average of 3 replicates with standard deviations (n=3) at α=0.05.
b: The data of pellet diameter are the average of 200 replicates with standard deviation.

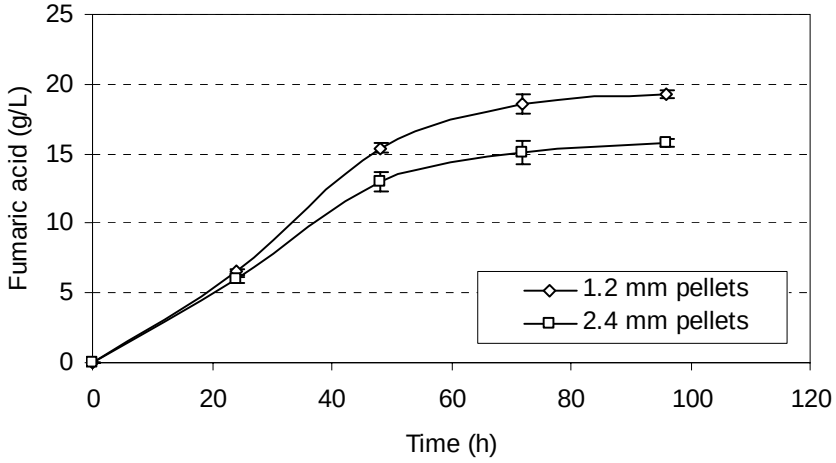
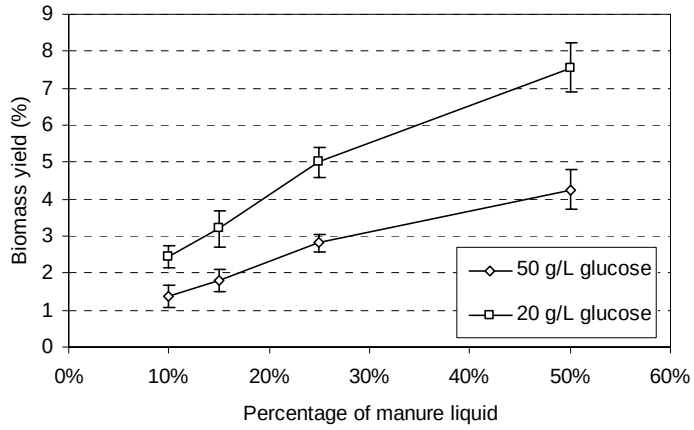
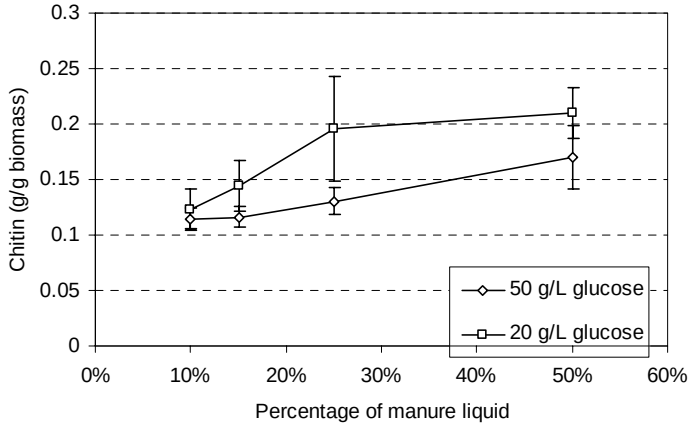


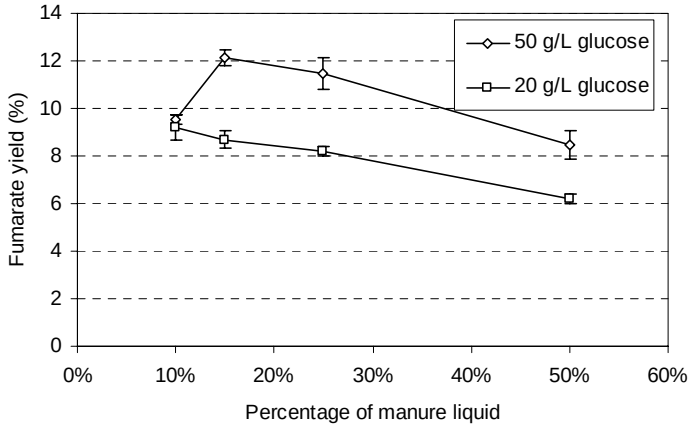
Fig. 8.1. Fumaric acid production using pellets with different size from cultivation (1). Data are presented as the average of 3 replicates with mean standard deviations.



A. Biomass



B. Chitin



C. Fumarate

Fig. 8.2. Effects of manure liquid on fungal cultivation

(1). Data are presented as the average of 3 replicates with mean standard deviations.

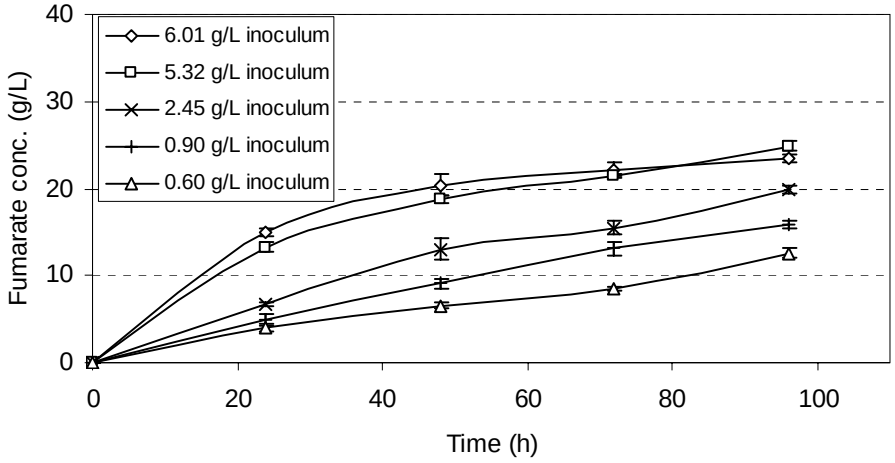


Fig. 8.3. Effects of different amount of biomass on fumaric acid production (1). Data are presented as the average of 3 replicates with mean standard deviations.

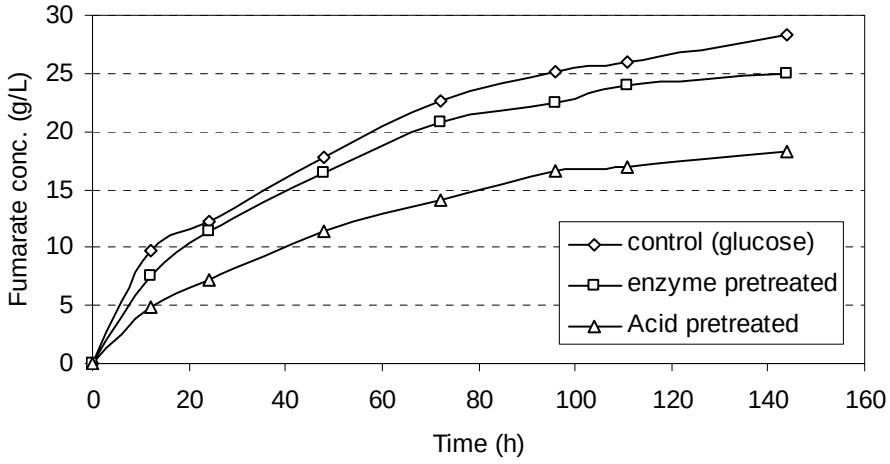


Fig. 8.4. Comparison of fermentations on hydrolysates of manure fiber using different pretreatments

(1). Innoculum was 5.3 g/L.

CHAPTER NINE

GENERAL CONCLUSIONS

In this collection of studies, a novel process was developed to convert dairy manure into the high value chemicals, fumaric acid and chitin, using fungal fermentation combined with several chemical and physical pre-treatments. The process provides an alternative solution to traditional animal manure management practices, producing valuable bioproducts while alleviating environmental concerns. Major conclusions obtained from this research are:

1. The effects of acid concentration, temperature, and reaction time during acid treatment on each individual fiber component such as cellulose, hemicellulose, nitrogen, and lignin were determined during acid treatment. Compared to reaction time and reaction temperature, acid concentration was the most influential factor on all components. The results demonstrate that the degree of break-down of manure fiber could be controlled by adjusting acid concentration along with other reaction conditions such as reaction duration and reaction temperature.
2. Dilute acid treatment under optimal conditions can effectively remove most of the hemicellulose, and disrupt fiber matrix structure, while concentrated acid can hydrolyze both the hemicellulose and cellulose in manure to its corresponding monosaccharides.
3. The nitrogen content in dairy manure was a key component that influenced the hydrolysis of cellulose and hemicellulose. Effective removal of the nitrogen sources

from manure contributed largely to an increase in the sugar yield from both cellulose and hemicellulose, and moderated the reaction conditions.

4. The components of hemicellulose and lignin, and the matrix structure of the manure fiber are major resistors for enzymatic hydrolysis. Either removing hemicellulose and lignin or disrupting the matrix structure greatly improved enzyme accessibility and therefore increased hydrolysis.
5. A dynamic model for enzymatic hydrolysis of the various pretreated manure fibers mechanistically explained what influence various pretreated fiber had on enzymatic hydrolysis and how much improvement could be obtained through modification of the pretreatment process along with adjustment of the conditions of enzymatic hydrolysis. The model accurately predicted both enzyme adsorption and hydrolysis for the treated manure fibers as well.
6. Clump-like morphology was identified as one of the biggest barriers for fungal fermentation, which caused mass transfer and oxygen transfer difficulties, and further influenced the efficiency of the fermentation. This study developed a new, simple culture medium to grow pellets for *R. oryzae* ATCC 20344. Fungal pellets with unique size were formed in the medium that only consists of three components: PDB, soybean peptone, and calcium carbonate. Pellet sizes, numbers, and density could be obtained by adjusting the inoculum size and the concentrations of these three medium components.
7. Fermentation of dairy manure using pelletized fungus demonstrated that manure liquid is a better nitrogen source for chitin accumulation during the fungal biomass cultivation compared against other nitrogen sources including urea and soybean

peptone. Enzymatic hydrolysis of dilute alkaline peroxide treated manure fiber was shown to be the preferred pretreatment method for converting manure fiber to monosaccharides that were subsequently used for fumaric acid production. Under the optimal conditions, 25 g/L fumaric acid and 5.50 g/L biomass with 0.21 g glucosamine/g biomass were obtained from dairy manure.

APPENDIX

MATLAB M-FILES FOR DEVELOPMENT OF KINETIC MODEL OF ENZYMATIC HYDROLYSIS OF DIFFERENTLY TREATED MANURE FIBER

1. Parameters of adsorption model for each individual pretreated fiber

1.1. Determination of adsorption constant (K)

```
%   Calculation the adsorption constant (K)
lam=1;  % define parameter first
options=optimset('Display', 'off', 'TolX', 0.1);
lambda=fminsearch('forAds', lam, options)  % calculate lambda and return the error
%   Where: Lambda represents the adsorption constant (K).
%------------------------------------------------------------------------
function err = forAds(lambda)
%   forAds is used for calculating the parameters of adsorption curve of enzyme
adsorption.
%   forAds(lambda) returns the error between the data and the values computed by the
current function of lambda.
%   forAds represents a function of the Langmiur adsorption:
%                               S=1/((P0/P-1)*lambda)
%   where S is the substrate concentration (g/L); P0 is the original enzyme concentration
(unit/L); P is the enzyme concentration (unit/L).
P = [
%   input the experimental data of enzyme concentration at the individual reaction
duration for the same fiber samples
    ];
S = [
```

```
%   input the experimental data of substrate concentration at the individual reaction
duration for the same fiber samples
  ];
P0 = [
%   input correspondingly original enzyme concentration for the same fiber samples
];
z = zeros(length(P),length(lambda)); % z is the computed value
z(:,1)=1./((P0./P-1).*lambda); % a function of the Langmiur adsorption
plot(P, z, P, S)
drawnow
err = norm(z-S)
```

1.2. Determination of parameters of a and b for calculation of adsorption constant

```
%   Calculation the parameters a and b for the adsorption constant
lam=[1,2];
options=optimset('Display', 'off', 'TolX', 0.1);
lambda=fminsearch('forParaAds', lam, options)
%   Where: the matrix of lambda was used to express the parameters of a, b. Lambda(1)
represents a, and lambda(2) represents b.
%--------------------------------------------------------------------------
function err = forParaAds(lambda)
%   The function of forParaAds is used for calculating the parameters a and b for the
adsorption constant of enzyme adsorption:
%                       K = a*t/(b+t)
```

```
%   where K is the adsorption constant; t is the reaction time; a, b are the parameters.
%   forParaAds(lambda) returns the error between the data and the values computed by
the current function.
t = [
%   input the reaction time
];
K = [
%   input the adsorption constant with respect to corresponding reaction time
];
z = zeros(length(t));
z(:, 1) = lambda(1).*t./(lambda(2)+t);
plot(t, K, t, z)
drawnow
err = norm(z-K);
```

2. Parameters of enzymatic hydrolysis for each individual pretreated fiber

```
% Calculate the parameters of reaction constant (k),
lam=[1,2,3]; % define parameters first
options=optimset('Display','off','TolX',0.1);
lambda=fminsearch('forEnzyPara', lam, options)
%--------------------------------------------------------------------------
function err = forEnzyPara(lambda)
```

```
%   The function of forEnzyPara is used for calculating the parameters of enzymatic
hydrolysis:
%               r = lambda(1).*E.*((S./C).^lambda(3)).*S./(1+G./lambda(2))
%   Where r is the reaction rate as the individual reaction time; E is the adsorbed enzyme;
%   S is the remained cellulose; C is the original cellulose content; G is the glucose
concentration;
%   lambda(1) is the reaction rate constant; lambda(2) is the inhibition constant;
%   lambda(3) is the constant relating the percentage of cellulose available for enzyme.
r = [
%   input the measured reaction rates
];
E = [
%   input corresponding adsorbed enzymatic concentrations
  ];
G = [
%   input corresponding glucose concentrations
];
S = [
%   input corresponding remained cellulose concentrations
];
C = [
%   input initial cellulose concentrations
];
```

```
z = zeros(length(t),length(lambda));
z(:,1)=lambda(1).*E.*((S./C).^lambda(3)).*S./(1+G./lambda(2));
plot(t, z, t, X)
drawnow
err = norm(z-r);
```

3. The model of enzymatic hydrolysis

```
function gluPlot
tspan=[3; 6; 12; 25.5; 49.5; 73; 96];  % reaction durations
y=[c0; g0];  % initial values of cellulose (c0) and glucose (g0) concentrations
[t,y]=ode45(@glucose, tspan, y);  % solve the differential equations for glucose and
substrate
t    % output reaction durations
y   % output the calculated glucose and remained substrate concentrations
g = [
%   input correspondingly measured glucose concentrations
];
plot(t, y, t, g)
%   draw the curves of both calculated glucose concentrations and measured glucose
concentrations
%--------------------------------------------------------------------------
function dydt=glucose(t, y)
lambda=[k, lambda, Kig]; % coefficients for hydrolysis: k, lambda, and Kig
```

```
S = S0; % input the substrate (S0) concentration (g/L).

L = S*l; % L is lignin concentration (g/L); l is lignin content in substrate.

H = S*h; % H is hemicellulose concentration (g/L); h is hemicellulose content in
substrate.

C = S*c; % C is original cellulose concentration (g/L); c is cellulose content in substrate.

k = [a, b]; % input parameters a,b with respect to the individual treated fiber

E = 114.37; % initial adsorbed enzyme activities on whole fiber

K = k(1)*t/(k(2)+t); % calculate the adsorption constant using a and b

dydt = [-
lambda(1)*(y(1)/C)^lambda(2)*y(1)/(1+y(2)/lambda(3))*E/(1/(K*(y(1)+L+H))+1);
1.1*lambda(1)*(y(1)/C)^lambda(2)*y(1)/(1+y(2)/lambda(3))*E/(1/(K*(y(1)+L+H))+1)
]; % differential equations for glucose (y(1)) and remained cellulose (y(2))
```

4. The model of enzyme adsorption

```
function enzymePlot

t = [3; 6; 12; 25.5; 49.5; 73; 96];  % reaction durations

C = [y(2)]; % remained cellulose concentrations obtained from the model of enzymatic
hydrolysis

H = % input correspondingly original hemicellulose concentration

L = % input correspondingly original lignin concentration

S = C+H+L; % calculate the remained substrate concentrations

P0 =  %   input correspondingly original enzyme concentration for the same fiber samples

k = [a, b]; % input parameters a,b with respect to the individual treated fiber
```

```
E = 114.37; % initial adsorbed enzyme activities on whole fiber
K = k(1)*t/(k(2)+t); % calculate the adsorption constant using a and b
P = E/(1/(K*S)+1);
t    % output reaction durations
P   % output the calculated enzyme concentrations
p = [
%   input correspondingly measured adsorbed enzyme concentrations
];
plot(t, P, t, p)
%   draw the curves of both calculated absorbed enzyme and measured absorbed enzyme
concentrations
```