EVAN MICHAEL VISSER

STUDIES ON THE PRODUCTION OF BIOETHANOL USING *JATROPHA CURCAS* BIOMASS, A COPRODUCT OF THE BIODIESEL PRODUCTION PROCESS

Dissertation presented to the Universidade Federal de Viçosa, as part of the requirements of the Agricultural Engineering's Graduate Program, for the attainment of the title "Magister Scientiae"

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Do not forsake wisdom, and she will protect you; love her, and she will watch over you. Wisdom is supreme; therefore get wisdom. Though it cost all you have, get understanding.

(Proverbs 4:6-7)

BIOGRAPHY

Evan Michael Visser, son of Jay Lynn Visser and Jeanne Marie Visser, was born on May 25, 1983, in Sioux City, Iowa, USA.

He graduated with a Bachelor of Science in Mechanical Engineering from Iowa State University in May, 2006. In September of 2006, he began his Master's Degree in Agricultural Engineering at the Federal University of Viçosa, Minas Gerais, Brazil, obtaining the title of Master in December of 2008.

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LIST OF ABBREVIATIONS

EJ	exajoule (J 10^{18})
g	gram
Gg	gigagram (g 10^{9})
ha	hectare
kg	kilogram (g 10^{3})
L	liter
Mg	megagram (g 10^{6})
ml	milliliters (L 10^{-3})
PJ	Petajoule (J 10^{15})
t	ton
v/w	volume/weigh (mL/g)
w/w	weight/weigh (g/g)
WIS	water insoluble solids

RESUMO

VISSER, Evan Michael, M.Sc., Universidade Federal de Viçosa, Dezembro de 2008. **Estudos da Produção de Bioetanol usando biomassa de Pinhão Manso, um Co-produto do Processo Produtivo de Biodiesel.** Orientador: Delly Oliveira Filho. Co-orientadores: Valéria Monteze Guimarães, Marcio Arêdes Martins, Marcos Rogério Tótola e Brian Lynn Stewart.

Este trabalho apresenta resultados do estudo relacionado à produção de etanol a partir de fontes lignocelulósicas, em particular, de resíduos do processo de produção de biodiesel. Dados da composição química de diversas fontes lignocelulósicas foram analisados e os resíduos de palma e pinhão-manso foram os que mostraram maior potencial para produção de etanol celulósico, com produtividade igual a 6725 L/ha e 695 L/ha respectivamente. A viabilidade técnica da produção de etanol a partir da torta de pinhão manso por hidrólise enzimática foi avaliada. A torta de pinhão-manso foi submetida à hidrólise enzimática (30 FPU/g de biomassa com excesso de celobiase), utilizando-se três tipos de pré-tratamento (0,2% H_2SO_4, 1,0% NaOH e 1,5% $Ca(OH)_2$) por uma hora a 121°C. As concentrações de açúcar foram medidas por cromatografia liquida de alta eficiência (HPLC) e comparadas com os resultados de concentração dos açúcares redutores determinados pelo método de ácido dinitrossalicílico (DNS). Após 24 h, as concentrações total de glicose e xilose avaliadas por HPLC foram de 3,33, 4,82, 5,80, e 5,55 g/L sem pré-tratamento ou com pré-tratamentos com H_2SO_4, NaOH, e $Ca(OH)_2$, respectivamente. As concentrações de açúcares redutores foram de 7,30, 11,95, 10,24, e 9,34 g/L para as mesmas condições de tratamento, portanto, aproximadamente 100% maiores do que os valores obtidos por HPLC. Visando a produção direta de etanol,

utilizou-se 20 g de casca de pinhão-manso pré-tratada para a etapa de sacarificação e fermentação simultâneas (SSF). Para tal, condições de pré-tratamentos (0,5% H_2SO_4 ou 1,0% NaOH, ambos a 121°C por 1 h), teor de matéria inibidora solúvel (lavados ou não-lavados após pré-tratamento) e concentração de enzimas (15 FPU de celulase/g de biomassa com ou sem um adicional de 4 U de xilanases/g de biomassa) foram utilizados, enquanto a inoculação de leveduras (0,80 g de células secas de *Saccharomyces cerevisiae*/L) foi mantida constante. Após 48h, as concentrações de etanol foram maiores nas amostras submetidas ao pré-tratamento alcalino (Etanol > 7,0 g/L). A adição de xilanases não teve efeito na elevação da concentração de etanol e na produção de xilose. Para as amostras submetidas ao pré-tratamento ácido, a mistura não-lavada apresentou concentrações de etanol inferiores às misturas lavadas (5,39 g/L a 6,07 g/L), porém nas amostras submetidas ao pré-tratamento com NaOH, não houve diferença significativa encontrada entre as amostras lavadas e não-lavadas.

ABSTRACT

VISSER, Evan Michael, M.Sc., Universidade Federal de Viçosa, December, 2008. **Studies on the Production of Bioethanol Using *Jatropha curcas* Biomass, a Coproduct of the Biodiesel Production Process.** Advisor: Delly Oliveira Filho. Co-advisors: Valéria Monteze Guimarães, Marcio Arêdes Martins, Marcos Rogério Tótola and Brian Lynn Steward.

This work presents data and studies related to the production of bioethanol from lignocellulosic sources, in particular, coproducts from the biodiesel production process. Chemical composition data from soybean, castor bean, *Jatropha curcas,* palm kernel, sunflower seed, rapeseed and cottonseed were gathered and palm kernel and Jatropha showed the greatest potentials for cellulose ethanol production with values of 6725 L/ha and 695 L/ha, respectively. The technical feasibility of ethanol production from Jatropha meal via enzymatic hydrolysis was evaluated. Jatropha meal was subjected to enzymatic hydrolysis (30 FPU/g of biomass and an excess of cellobiase), using three types of chemical pretreatments (0.2% H_2SO_4, 1.0% NaOH and 1.5% $Ca(OH)_2$) for 1 h at 121°C. Sugar concentrations were measured by HPLC and compared with results for reducing sugars measured using the dinitrosalicylic acid (DNS) method. After 24 h, the total concentrations of glucose and xylose measured by HPCL were 3.33, 4.82, 5.80 and 5.55 g/L for the untreated, H_2SO_4, NaOH, and $Ca(OH)_2$ pretreatments, respectively. In comparison, reducing sugar concentrations were nearly 100% greater showing values of 7.30, 11.95, 10.24 and 9.34 g/L for the same pretreatments. Simultaneous saccharification and fermentation (SSF) was performed on 20 g of pretreated Jatropha shells for the more consolidated production of ethanol. Pretreatment type (0.5% H_2SO_4 and 1.0 % NaOH at 121°C

for 1 h), water insoluble solids (WIS) (washed or unwashed after pretreatment) and enzyme loading (15 FPU/g biomass of cellulase with or without an additional 4 U/g biomass of xylanase enzymes) were considered while yeast loading (0.80 g/L dry *Saccharomyces cerevisiae* cells) was kept constant. After 48 h, ethanol concentrations were highest in the alkaline pretreated samples (> 7.0 g/L). The addition of xylanase enzymes had no significant effect on ethanol concentration or xylose production. For the acid pretreated samples, the unwashed slurry showed ethanol concentrations inferior to those of the washed sample (5.39 g/L to 6.07 g/L), but in the NaOH pretreated samples, no significant difference was verified between washed and unwashed.

Chapter 1.

INTRODUCTION

1.1. BACKGROUND INFORMATION

According to the Brazilian National Energy Balance (MME 2006), the Brazilian transport fuel demand in 2005 was roughly the equivalent of 429,400 barrels of petroleum per day. This value represents only about three percent of the oil demand of the USA (U.S. Department of Energy 2007). Currently, gasoline sold in Brazil is required by law to contain 23 percent anhydrous ethanol and in 2007, roughly 85% of car sales were flex, being able to run on gasoline, ethanol, or any combination of the two. In many other areas of the world, legislators are mandating small percentages of ethanol be mixed with gasoline to be sold at pumps. As of December 31, 2009, all California refineries will be obligated to blend 10 percent ethanol into their gasoline. Goals for the year 2030 call for 30 percent of U.S. transportation fuels from biomass.

As fuel demands continue to increase, petroleum reserves lessen and governments mandate the use of biofuels, the need for new fuel sources has prompted the production of ethanol and biodiesel. Environmental preoccupation has also forced politicians and the United Nations (UN) to take more practical actions which includes the more intensive use of renewable energies. Since conventional methods for ethanol and biodiesel production are not sufficient to meed alternative fuel demands, new fuel sources and advances in process efficiencies are needed (Solomon, Barnes, and Halvorsen 2007).

Ethanol produced in the United States, yielded from corn, has often been criticized. Some environmentalists argue that the high energy cost, along with degraded land and polluted water from heavy fertilized fields makes United States ethanol production from corn questionable, especially due to the low energy balance. The production of biofuels from corn starch has shown to be extremely inefficient in terms of energy balance in comparison with sugarcane, where some studies report greater energy consumption than energy production (Hammerschlag 2006). The large corn demand for ethanol is one of the factors that has pushed feed prices higher and may also have an increase prices on all kinds of other food products (Westcott 2007).

The most recent interest in the international biofuels sector is the production of bioethanol from sources of lignocellulosic material, for example, agricultural residues. An alternative or addition to starch-based corn ethanol, cellulosic material is estimated to have the potential to substitute large percentages of the U.S. and world fuel demands using only agricultural residues.

Kim and Dale (2004) reported that corn stover has the potential to produce 38.4 GL (10^9 liters) of bioethanol in North America, even when considering that 60% of this biomass was left in the field to prevent erosion and maintain the fertility of the soil. This represents slightly more than two times the amount of ethanol produced from corn grain, 18.4 GL in the US in 2006 (U.S. Department of Energy 2007). In South America, sugar cane bagasse has the potential to produce 18.1 GL of bioethanol, supposing that once again 60% is returned to the field. It was estimated that the world production of bioethanol is 442 GL, equivalent to approximately 29% of the world consumption of gasoline utilizing only agricultural residues from corn, barley, oats, rice, wheat, sorghum and sugarcane.

The production of biodiesel, via transesterification requires the use of alcohol (ethanol or methanol) as well as catalysts. Internationally, methanol is more often used than ethanol because of its considerably lower cost and greater ease of downstream unreacted alcohol recovery (Haas et al. 2006). In Brazil, ethanol is preferred due to its greater availability in the country.

In order to meet the ethanol needs for biodiesel, ethanol can be made from the biomass left from the oil extraction process. The shells, pressed seeds and other biomass collected during harvest can be used in the production of bioethanol from the contained cellulose. Currently, biodiesel coproducts are mainly used as fertilizers and animal feeds, however, perhaps the biodiesel production process has the potential to be self-sufficient similar to the sugarcane ethanol production process both in chemical consumption as well as in thermal energy, adding value to what are currently process coproducts (Openshaw 2000).

Jatropha curcas is one of the crops being highly studied for the production of biodiesel. Like the castor plant, Jatropha can be grown in

unfavorable climate regions (little rainfall) and unfertile soils (Melo et al. 2006). Because the Jatropha fruit is toxic, the coproducts from oil extraction cannot be used as animal rations, therefore offering a greater opportunity for the production of bioethanol.

The price of biomass grown specifically for the purpose of producing biofuels is by far the greatest expense in the biodiesel production process (Haas et al. 2006). The use of biodiesel coproducts could greatly diminish this cost and make both bioethanol and biodiesel much more competitive, and also reduce the dependence on federal government subsidies.

One of the greatest costs for the bioethanol production is the necessary enzymes. The high price of these enzymes is what currently restricts the production of bioethanol. Recently, the cost of cellulase per liter of ethanol produced has decreased from roughly US$ 2.00 to US$ 0.20 but is still nearly twenty times as expensive as the starch hydrolyzing amylase enzymes used for ethanol production (Somerville 2007). Research on the large scale production of enzyme cocktails capable of hydrolyzing all types of cellulose and hemicellulose and microorganisms capable of rapidly excreting these desired enzymes may make this process more economically attractive.

The bioethanol production potential using coproducts from the biodiesel production process is still unknown, but the large quantities of agricultural residues indicate the demand for large sources of available lignocellulose. Countries located in the tropics, such as Brazil, have large availabilities of biomass from a variety of sources, which can all be used for the production of bioethanol.

Although Brazil is one of the world leaders in biofuels, this should not limit the research conducted on other fuel sources. Cellulose derived bioethanol will open another agricultural market and also allow for the utilization of what is low price waste biomass today. This second generation ethanol produced and used in the biodiesel production process, as depicted in a biorefinery, can reduce biodiesel production costs.

1.2. THESIS STRUCTURE

This work was divided into the following chapters:

Chapter 2 presents the state of the art of bioethanol production, using sources of lignocellulosic biomass. This chapter reviews the processes of pretreatment, enzymatic hydrolysis and fermentation, as well as the consolidation of these processes.

Chapter 3 introduces the bioethanol production potential from various crops grown for the production of vegetable oil, and often destined for biodiesel. The chemical composition of these crops was evaluated for the determination of non-starch polysaccharides which can be transformed into bioethanol.

Chapter 4 presents the results of experimental testing of various chemical pretreatments and their effect on the saccharification of Jatropha meal. The effect of pretreatment and hydrolysis on the protein content was also evaluated due to its significance in animal rations.

Chapter 5 introduces the concept of simultaneous saccharification and fermentation of Jatropha shells. Pretreatments, enzyme loadings and effect of inhibitors produced during pretreatment were all evaluated in different combinations to determine their effect on sugar and ethanol production.

Chapter 6 provides a general conclusion of all completed works and suggestions for further studies.

1.3. REFERENCES

Haas, M. J., A. J. McAloon, W. C. Yee, and T. A. Foglia. 2006. A process model to estimate biodiesel production costs. *Bioresource Technology* 97 (4):671-678.

Hammerschlag, R. 2006. Ethanol's energy return on investment: A survey of the literature 1990-present. *Environmental Science and Technology* 40 (6):1744-1750.

Kim, S., and B. E. Dale. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy* 26 (4):361-375.

Melo, J. C., W. Brander Jr, R. J. A. Campos, J. G. A. Pacheco, A. R. P. Schuler, and L. Stragevitch. 2006. Avaliação Preliminar do Potencial do Pinhão Manso para a Produção de Biodiesel. Paper read at Congresso da Rede Brasileira de Tecnologia de Biodiesel, at Brasilia.

Ministério de Minas e Energia. 2006. Balanço Energetico Nacional (Brazilian National Energy Balance).

Openshaw, K.. 2000. A review of Jatropha curcas: an oil plant of unfulfilled promise. *Biomass and Bioenergy* 19 (1):1-15.

Solomon, B. D., J. R. Barnes, and K. E. Halvorsen. 2007. Grain and cellulosic ethanol: History, economics, and energy policy. *Biomass and Bioenergy* 31 (6):416-425.

Somerville, C.. 2007. Biofuels. *Current Biology* Primer:R115-R119.

U.S. Department of Energy. 2007. Annual Energy Review 2007, edited by Energy Information Administration.

Westcott, P. C. 2007. Ethanol Expansion in the United States - How Will the Agricultural Sector Adjust?, edited by USDA.

Chapter 2.

STATE OF THE ART OF BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC SOURCES

2.1. WHAT IS LIGNOCELLULOSIC BIOMASS?

Lignocellulosic biomass has long been known as a potential raw material for ethanol production. Plant biomass has evolved to resist physical attack on its structural sugars from microbes, making the cost effective transformation from biomass to fermentable sugars more complicated. Expensive enzymes, modest yields, inhibitor production and complex sugar mixtures are the cause of the high costs. Several technologies have already been developed to complete this process, but cost-competitive processes for today's market are still needed.

Cell walls in lignocellulosic biomass can be converted into fermentable sugars and lignin rich solid residues by pretreatment (physical, chemical or biological) and enzymatic hydrolysis (saccharification). Biomass composition plays a large role in the efficiency of this process and genetically modified feedstocks are being investigated in which cell wall polysaccharides are more easily accessible by enzymes.

2.1.1. Structure and Assembly of Cell Walls

Plant cells walls are complex mixtures of components which carry out many functions. The cell walls are intricate assemblies of celluloses, hemicelluloses (i.e., xyloglucans, arabinoxylans, and glucomannans), pectins (i.e., homogalaturonans, rhamnogalacturonan I and II, and xylogalacturonans), lignins, and proteoglycans (e.g., arabinogalactan-proteins, extensins, and proline-rich proteins) (Huntley et al. 2003). Most of the cell wall's mass is in the form of polysaccharides (cellulose and hemicelluloses) followed by lignin, predominantly composed of phenylpropane building blocks. Lignin plays an important role in the strengthening of cell walls by cross-linking polysaccharides, providing structural support to the overall plant body and resistance to moisture and biological attack. Lignin, however, interferes with the enzymatic conversion of polysaccharide components. Better understanding of the cell wall concerning the thousands of gene products which are estimated to participate in synthesis

and deposition and their corresponding enzymes will allow for the improvement and optimization of the biomass to ethanol conversion process.

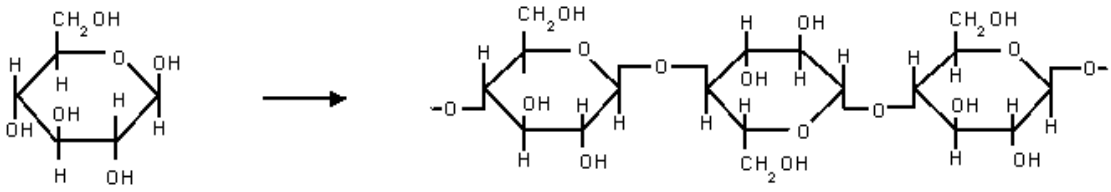

Figure 1. Bonding of glucose molecules which make up cellulose.

Plants can have either primary or secondary cell walls. Primary cell walls are made up of cellulose, consisting of hydrogen-bonded chains of thousands of β-1,4-linked glucose molecules, as well as hemicelluloses and other materials. Microfibrils compose cellulose in higher plants and each contains up to 36 cellulose chains. The cellulose chains are linear collections of thousands of glucose residues; pairs of glucose residues (cellobiose) make up the repeating unit of cellulose (Figure 1).

The crystalline and paracrystalline (amorphous) cellulose core is surrounded by hemicellulose, a branched polymer consisting of pentose (5-carbon) and in some cases hexose (6-carbon) sugars. Hemicelluloses not only cross link individual microfibrils, but in secondary cell walls form covalent associations with lignin, creating a complex aromatic polymer. The crystallinity of cellulose and its association with hemicellulose and lignin are two of the key problems for the efficient conversion of cellulose into fermentable glucose molecules.

Physical modification of native cellulose can inhibit saccharification as it is dehydrated in traditional methods of storage after harvest and during the pretreatment process. For example, enzymatic digestibility can be reduced by lignocellulosic material's reaction to pretreatment processes, such as:

- High mechanical pressure collapses cellulose's natural vascular structure.
- Dilute-acid chemical pretreatments may permit cellulose to reanneal, leading to the "hornification" of cellulose in microfibrils.

- Ambient or elevated temperatures may accelerate denaturation (e.g., the tendency of most (beta-1,4)-pentosans and hexosans to have inverse water-solubility relationships with temperature).
- Some pretreatments may permit lignin to become soluble and "plate out" on cellulose surfaces during the cool-down phase.

These "process-induced" causes of recalcitrance are overcome only through extensive research with various biomass materials.

2.2. ENZYMATIC HYDROLYSIS OF STRUCTURAL POLYSACCHARIDES

2.2.1. Pretreatments

Pretreatment is an essential step in the bioethanol production process required to alter the structure of lignocellulosic biomass to improve enzyme accessibility to cellulose and therefore increase the yield of fermentable sugars (Mosier et al. 2005). Effective pretreatment is characterized by the following criteria: reduce the size of biomass particles, preserve the pentose fractions, limit the formation of degradation products that inhibit fermentation microorganisms, minimize energy demands and limit costs. Other properties such as low pretreatment catalyst cost and facility of catalyst recycle also play a role in the selection of pretreatments.

Unless a large excess of enzymes is used, the enzymatic digestibility of cellulose is low due to its structural characteristics. Pretreatments can be classified as physical (comminution, hydrothermolysis), physico-chemical (steam explosion, ammonia fiber explosion), chemical (acid, alkali, solvents, ozone), or biological (Sun and Cheng 2002). To date, physico-chemical pretreatments are most commonly applied consisting of high temperatures and pressures with dilute acids or bases, typically H_2SO_4 and NaOH.

Acid pretreatments generally aim to remove hemicellulose and expose cellulose for enzymatic hydrolysis (Schell et al. 2003). Dilute acid has been

found to be particularly well suited for agricultural residues such as corn stover. Alkali pretreatment's intent is to remove lignin and various uronic acid substitutions on hemicelluloses, which lower the accessibility of enzymes to cellulose and hemicellulose. Generally, alkaline pretreatments are more effective on agricultural residues and herbaceous crops than on woody material (Silverstein et al. 2007).

2.2.2. Saccharification

Lignocellulosic biomass is a complex structure primarily composed of crystalline cellulose, hydrated hemicelluloses and lignin. The best enzyme cocktails prepared for saccharification of this material to date are synergistic mixtures of primarily cellulose degrading enzymes. Enzyme kinetics has nearly always been the "ensemble average" results from experiments, eliminating the possibility for the study of the reactive site of the individual enzyme. For this reason it is unknown if cellulose recalcitrance is due to enzyme inadequacy, enzyme-substrate mismatch, or both.

Enzymes such as cellulases, hemicellulases and other glycosyl hydrolases (GH) are synthesized by bacteria and fungi synergistically to break down polysaccharides in biomass (Singh et al. 2005). Commercial cellulase solutions are composed of various types of GH, specific to the various types of substrates (i.e. cellulose and xylan) and action patterns (exoenzymes acting from the chain ends, endoenzymes cleaving within the chain, and GH cleaving side-chain branches). Optimization of a single enzyme cocktail requires the detailed understanding of each enzyme's regulation and activity.

Figure 2 shows the function of three types of cellulases and their role in the overall degradation of cellulose. This is the type of reaction which occurs in ruminating chamber of herbivores.

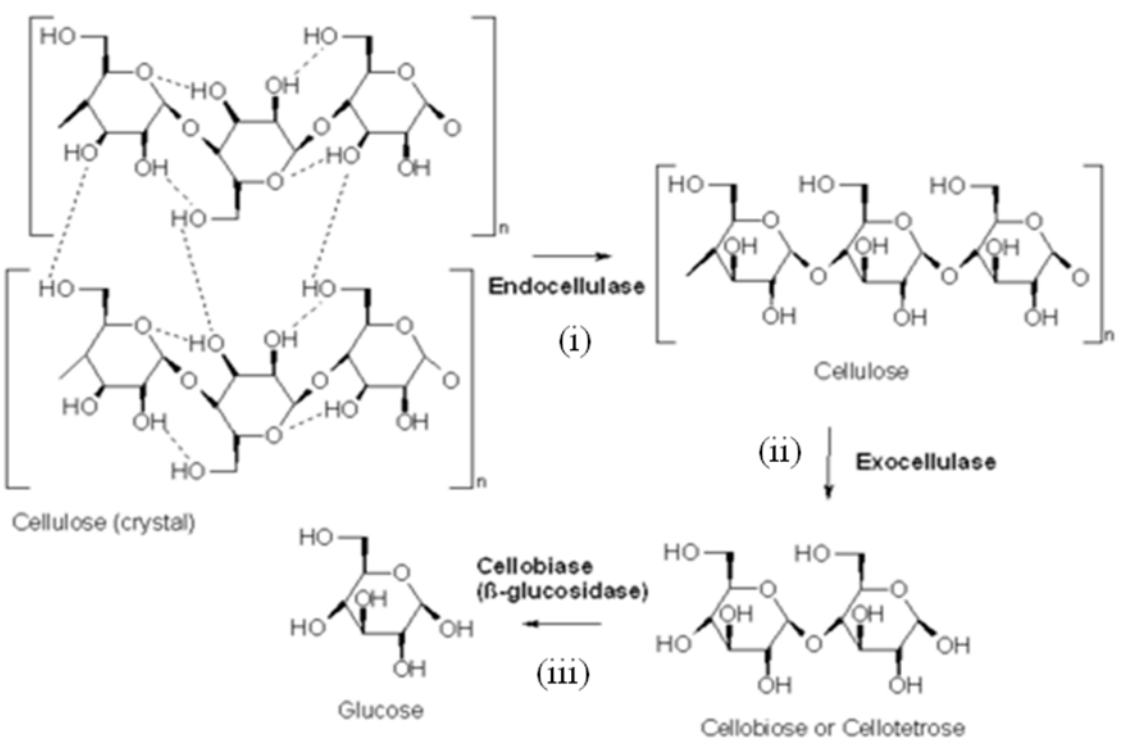

Figure 2. The three types of reaction catalyzed by cellulases: (i) Breakage of the non-covalent interactions present in the crystalline structure of cellulose (endo-cellulase) (ii) Hydrolysis of the individual cellulose fibers to break it into smaller sugars (exo-cellulase) (iii) Hydrolysis of disaccharides and tetrasaccharides into glucose (beta-glucosidase).

The envisioned solution for bioethanol production involves the consolidation of the diverse stages currently required and one of those potential solutions may be the design of an adequate cellulosome (Fierobe et al. 2005). A cellulosome is a large molecular machine that integrates numerous enzymes and functions to break down biomass and therefore reduce enzyme loading required for processing (Bayer et al. 2004).

Better understanding of the utilized feedstock including the cell wall's physical and chemical properties and how certain feature inhibit or facilitate enzyme interactions and saccharification are essential to increase yields and diminish costs (Laureano-Perez et al. 2005). Pretreatment, being the first process applied to the biomass, is critical to both maximize enzyme interaction and minimize inhibitor production. Optimal pretreatment is often determined as a function of the biomass composition. Because these biological systems are

incredibly complex and interconnected, each stage of the process needs to be studied individually, as well as in relation to the overall process.

2.3. SUGAR FERMENTATION TO ETHANOL

Fermentation of sugars by microbes is the most common method for converting sugars from biomass to liquid fuels such as ethanol. This process is well established for some sugars, such as glucose and sucrose which are the produced from both corn and sugarcane. Fermentation of the mixture of sugars present in lignocellulosic biomass is much more complex and requires more robust microorganisms to both improve yields and allow for process simplification through consolidation of process steps.

One of the greatest challenges to the efficient use of biomass-derived sugars is the lack of microorganisms that can grow and function in the demanding environments created by biomass hydrolysis and cellular metabolism. Some of the problems encountered include inhibition by deleterious products formed during biomass hydrolysis, accumulation of alternative products and unwanted microbial growth. Ethanol is one of the greatest inhibitors to fermentation, resulting in low alcohol concentrations and increasing costs of distillation.

Current technology used in the cornstarch and sugarcane industries uses yeasts and is capable of converting glucose at high yields (90%), high alcohol contents (10 to 14 wt %), and reasonable rates (1.5 to 2.5 g/L/h). Recombinant ethanologenic organisms (i.e., yeasts, *E. coli* and *Z. mobilis*) have been developed to ferment both glucose and xylose but are limited to lower alcohol contents (5 to 6 wt %) and slow production rates (< 1.0 g/L/h). The yeast *S. cerevisiae* has been genetically modified to ferment both pentose and hexose sugars and is capable of withstanding a range of temperatures (Nakamura et al. 2008).

Most methods of biomass pretreatment also produce byproducts (e.g., acetate, furfural, lignin, ect.) which are inhibitor to microorganisms. These

byproducts often significantly reduce the growth of microorganisms, sugar metabolism rates and final alcohol concentrations. In all cases, pretreatment has a greater impact on the degradation of pentose polysaccharides than hexose polysaccharides. Co-fermentation of both pentose and hexose sugars in the produced hydrolysate can therefore greatly improve yields since the hydrolysate is predominantly composed of pentose sugars from hemicellulose.

2.4. REFERENCES

Bayer, E. A., J. P. Belaich, Y. Shoham, and R. Lamed. 2004. The Cellulosomes: Multi-Enzyme Machines for Degradation of Plant Cell Wall Polysaccharides. *Annual Review of Microbiology* 58:521.

Fierobe, H. P., F. Mingardon, A. Mechaly, A. Belaich, M. T. Rincon, S. Pages, R. Lamed, C. Tardif, J. P. Belaich, and E. A. Bayer. 2005. Action of Designer Cellulosomes on Homogeneous Versus Complex Substrates: CONTROLLED INCORPORATION OF THREE DISTINCT ENZYMES INTO A DEFINED TRIFUNCTIONAL SCAFFOLDIN. *Journal of Biological Chemistry* 280 (16):16325-16334.

Huntley, SK, D Ellis, M Gilbert, C Chapple, and SD Mansfield. 2003. Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. *Journal of Agricultural and Food Chemistry* 51 (21):6178-6183.

Laureano-Perez, L., F. Teymouri, H. Alizadeh, and B. E. Dale. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. *Applied Biochemistry and Biotechnology* 121:1081-1100.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96 (6):673-686.

Nakamura, N., R. Yamada, S. Katahira, T. Tanaka, H. Fukuda, and A. Kondo. 2008. Effective xylose/cellobiose co-fermentation and ethanol production by xylose-assimilating S. cerevisiae via expression of [beta]-glucosidase on its cell surface. *Enzyme and Microbial Technology* 43 (3):233-236.

Schell, D. J., J. Farmer, M. Newman, and J. D. Mcmillan. 2003. Dilute–Sulfuric Acid Pretreatment of Corn Stover in Pilot-Scale Reactor - Investigation of Yields, Kinetics, and Enzymatic Digestibilities of Solids. *Applied Biochemistry and Biotechnology* 105:69-85.

Silverstein, R. A., Y. Chen, R. R. Sharma-Shivappa, M. D. Boyette, and J. Osborne. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology* 98 (16):3000-3011.

Singh, R., S. Singh, K. D. Trimukhe, K. V. Pandare, K. B. Bastawade, D. V. Gokhale, and A. J. Varma. 2005. Lignin-carbohydrate complexes from sugarcane bagasse: Preparation, purification, and characterization. *Carbohydrate Polymers* 62 (1):57-66.

Sun, Y., and J. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology* 83 (1):1-11.

Chapter 3

BIOETHANOL PRODUCTION POTENTIAL FROM BRAZILIAN BIODIESEL COPRODUCTS: A REVIEW

Submitted to the Biomass and Bioenergy Journal

3.1. INTRODUCTION

Increasing environmental concerns in recent years have encouraged the use of carbon neutral biomass energies. Currently, biomass accounts for 46 EJ (13.4%) of the world's primary energy use per year (International Energy Agency (IEA) Statistics 2005). However, only 30% of biomass energy is used in modern forms (e.g. generation of electricity, steam and liquid bio-fuels) while 70% accounts for traditional (e.g. heating and cooking) uses (Sims et al. 2006). The sustainable use of biomass feedstocks is obtained by using proper technologies and management techniques.

Brazil is one of the world's largest agricultural producers and is especially known for its agricultural expansion potential. Second only to the United States, Brazil is responsible for approximately 16% of world oilseed production (United States Department of Agriculture (USDA 2008). This value considers only the production of soybean, cottonseed, peanut, sunflower and rapeseed production, however, various other oil crops, such as castor bean, palm kernels and *Jatropha curcas*, are proving to be of great potential for vegetable oil production.

World biofuel production is growing exponentially, especially in countries with large agricultural programs. Installed biodiesel production capacity in Brazil has reached approximately 2.5 billion liters per year, equivalent to 82.5 PJ, but still less than 0.2% of the biomass energy consumed in the world (ANP 2007). Vegetable oil extraction for the production of biodiesel rarely yields more than 45% of the weight of the fruit, therefore leaving large amounts of biomass ready for other applications (Pinto et al. 2005).

Biodiesel production requires the mixture of alcohol (methanol, ethanol or buthanol) with triacylglycerides (oils). Complete stoichiometric transesterification requires a 3:1 molar ratio of alcohol to triacylglycerides, however, in practice this ratio must be much greater to achieve a maximal ester yield since the transesterification reaction is reversible (Ma and Hanna 1999). As an average, approximately 200 ml of alcohol are generally needed for each kilogram of oil. The production of ethanol from the lignocellulosic coproducts of

the oil extraction process may be able to meet alcohol demands for biodiesel production or even play a major role in ethanol markets.

Ethanol produced from cellulose has vast opportunities to greatly increase worldwide production. Combined usage of both grain and plant, for example corn kernel and stover, greatly increases ethanol yields per area planted (Kim and Dale 2004). In the same way, oil plants intended for biodiesel production are capable of ethanol production using the shells and pressed seeds discarded during oil extraction destined for biodiesel production. In many cases, expeller or pressed cake meals from oil extraction plants are used as high protein animal nourishments. However, the polysaccharides found in soybean meal and other expeller meals are poorly utilized by monogastric animals and the enzymatic degradation of these polysaccharides may also improve animal digestibility (Huisman, Schols, and Voragen 1998).

This study estimated how much bioethanol can potentially be produced from residues of oil extraction destined for the biodiesel process. These biodiesel crops include soybean, castor bean, *Jatropha curcas*, palm kernel, sunflower seed, rapeseed and cotton seed. Yields per hectare were based on Brazilian crop harvest statistics and estimates. In many cases oil extraction coproducts are used as animal feeds due to their high protein content; however, this study estimates the potential for ethanol production using these same coproducts.

3.2. CROPS AND ETHANOL YIELD

The data for crop yields and cultivated area in 2007 was obtained from the Brazilian Institute of Geography and Statistics and the United States Department of Agriculture´s World Agriculture Production Report (United States Department of Agriculture 2008; Brazilian Institute of Geography and Statistics 2007). Oil content of each of the studied crops was provided by Pinto et al. (2005).

Nearly all oil crops are rich in protein and offer valuable feeds for animal nutrition. If protein degradation can be avoided, during the bioethanol production process, these coproducts may have even greater nutritional values. In some

cases, fiber in the form of large polysaccharide molecules is not easily digested by livestock and therefore is not fed pure. In the case of Castor beans and Jatropha, the seeds are highly toxic and cannot be used as an animal ration without detoxification.

Figure 3 presents the oil percentage (percent mass of oil/dry mass of the seed) and yield (harvested tons/ha) of the oil crops under study (Pinto et al. 2005; United States Department of Agriculture 2008; Brazilian Institute of Geography and Statistics 2007).

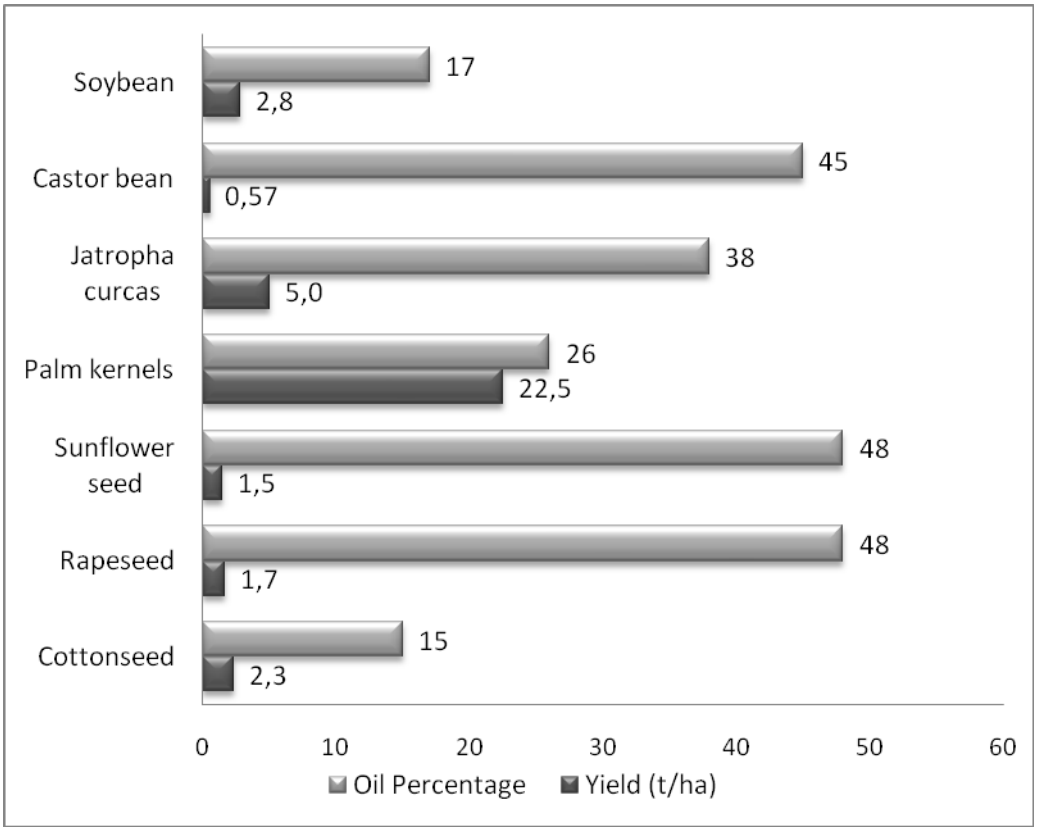

Figure 3 Oil percentages and yield of oil crops.

3.3. CONVERSION TECHNIQUES – BIOETHANOL AND BIODIESEL

Ethanol production from lignocellulosic sources requires the breakdown of cellulose and hemicellulose into fermentable sugars, the process known as hydrolysis. The US Department of Energy's theoretical yield calculator estimated values of ethanol production from cellulose and hemicelluloses as 654.5 L/Mg and 669.8 L/Mg respectively (US Department of Energy 2006) These values were based on the conversion factors of 1.11 kg of C6 sugar/kg of polymeric sugar, 1.136 kg of C5 sugar/kg of C5 polymeric sugar and 0.51 kg of ethanol can be produced per kg of sugar. In this study, an average value of 662 L/Mg was used, the average of production from both pentose and hexose polysaccharides.

The production of biodiesel by the transesterification process requires catalysts and alcohol (methanol or ethanol) as could be seen in Figure 4. In Brazil, transesterification is typically performed using ethanol due to its abundance and ease of acquisition (Ferrari, Oliveira, and Scabio 2005; MME 2006). The amount of alcohol required is dependent on the type of catalyst used and can range from the alcohol/oil stoichiometric relationship of 3:1 to an excess ratio of 30:1 (alcohol-oil molar ratio), however, when using alkali catalysts, a typical alcohol-oil molar ratio is 6:1 (Marchetti, Miguel, and Errazu 2007). It is important to note that the excess ethanol, over the stoichiometric alcohol-oil molar ratio (3:1), is recovered in the process. Berchmans and Hirata (2008) found the optimal methanol to oil ratio to be 24% (w/w) for the production of biodiesel from *Jatropha curcas* oil. For this study, the stoichiometric relationship was used to determine the amount of alcohol necessary for the transesterification process, equivalent to approximatly 20% (v/w). This value of 20% is based on the stoichiometric relationship of alcohol to oil and from averaging vegetable oil densities; biodiesel density was assumed to be 0.88 g/ml. Biodiesel production efficiency was assumed to be 100% (1 liter of oil produces 1 liter of biodiesel).

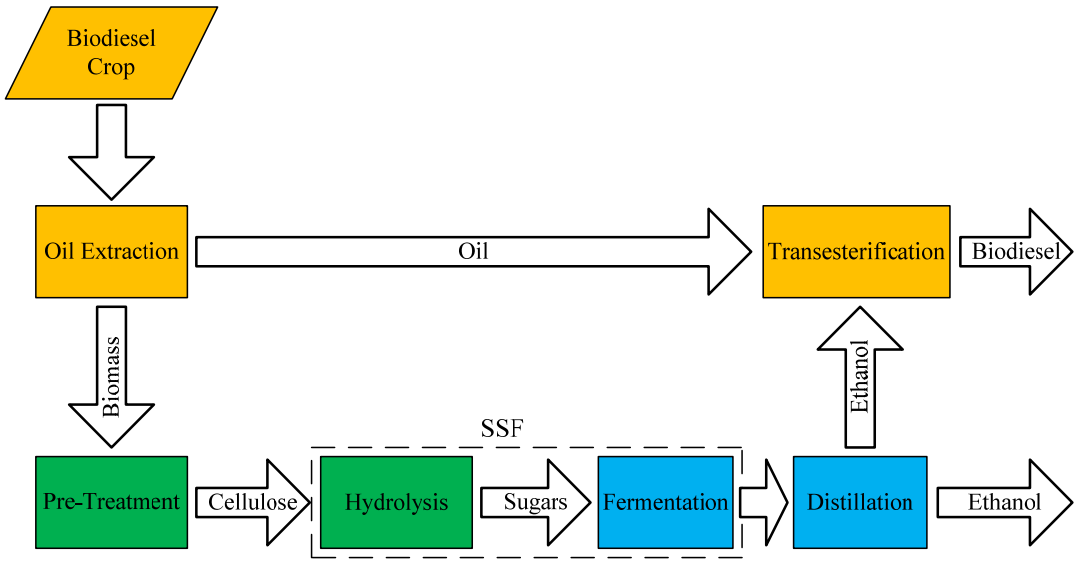

Figure 4. Biorefinery flow diagram.

The crops studied were soybean, castor bean, *Jatropha curcas*, African palm kernel, sunflower seed, rapeseed and cotton seed. Protein, carbohydrate and lignin data used for the calculations of ethanol production potential were encountered in various scientific articles (Malathi and Devegowda 2001; Robb et al. 1974; Openshaw 2000; Carvalho et al. 2006; Martínez-Herrera et al. 2006; Evangelista et al. 2004; Bandeira et al. 2004; Villamide and San Juan 1998; Irshaid, Harb, and Titi 2003; Soliva et al. 2005; Arana et al. 2001) based on the nutritional values of animal meals. Lignin concentration was subtracted from the total value of neutral detergent fiber (NDF) for the calculation of available carbohydrates. Bioethanol production potential was determined based on the quantities of holocellulose (cellulose and hemicellulose) found in the oil extraction coproducts and conversion was performed using the theoretical values for enzymatic hydrolysis and fermentation.

3.4. RESULTS

3.4.1. Soybeans

More than 50 Tg of soybeans are produced in Brazil each year where yields average about 2.8 Mg/ha (Brazilian Institute of Geography and Statistics 2007). Large quantities of soy products are exported each year being more than 27 Tg of soybeans, 13 Tg of soybean meal and 2 Tg of soy oil (Brazilian Association of Vegetable Oil Industries 2008).

Soy is responsible for 90% of the vegetable oil produced in Brazil and biodiesel production is one of the largest consumers of soy oil. The low oil content of soybeans makes it unfavorable as a source of biodiesel, however, soy is the only oil crop which is presently produced on an extremely large scale and is also capable of producing the necessary bioethanol for biodiesel production using oil extraction coproducts.

Soybean meal is largely used as an animal feed because of its high protein content of 38% but also has potential for bioethanol production (Embrapa Soja 1995). The polysaccharide content of soybean meal is approximately 16% (pectosans, hexosans and pectin), however, nearly 13% of the lignin-polysaccharide complex is composed of lignin, unsuitable for bioethanol production (Malathi and Devegowda 2001). From the 16% kg of fibers found in soybean coproducts, 247 liters of bioethanol can be produced, sufficient to meet 257% of alcohol demands for transesterification. If all Brazilian soy coproducts were to be utilized for ethanol production, more than 5 GL of second generation ethanol could be produced, equivalent to 19% of the total ethanol produced in Brazil in 2007 (CONAB 2008).

Table 1. Soybean yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	**2,819 kg/ha**	100 %
Vegetable Oil	480 kg/ha	17 %
Vegetable Oil extraction coproducts	2340 kg/ha	83 %
Proteins	890 kg/ha	38 %
Fibers (cellulose/hemicellulose)	374 kg/ha	16 %
Lignin	280 kg/ha	12 %
Biodiesel	**545 L/ha**	
Alcohol necessary for biodiesel production	96 L/ha	
Bioethanol from fiber	**247 L/ha**	

Protein isolation is a problem faced when considering the indigestibility of polysaccharides found in soybean meal. Enzymatic degradation of nonstarch polysaccharides as proposed by Malathi and Devegowda (2001) breaks down polymeric chains allowing for better digestibility by monogastric animals (Huisman, Schols, and Voragen 1998). In this case, the production of ethanol from existing carbohydrates may also make soybean meal more digestible.

3.4.2. Castor Bean

In 2007, Brazil harvested little over 87 Gg of castor beans for the extraction of oil. It is estimated that in 2008, production will increase by about 80% to 157 Gg (Brazilian Institute of Geography and Statistics 2007). Higher yields will be mostly responsible for the increase; however, biodiesel demand is pulling more land area into castor bean production.

The castor bean plant has been introduced in semi-arid conditions in order to produce oil for biodiesel production and increase regional development. Capable of growing in nearly any conditions, its inferior oil yield is compensated by its ability to thrive under dry, infertile conditions.

Chemical composition of the Guarany castor meal variety was studied by Evangelista et al. (2004). Protein and fiber content in oil extraction coproducts varied slightly depending on the extraction method used; however, average values for crude protein, neutral detergent fibers and acid detergent fibers were 39.72%, 52.07% and 37.32%, respectively. Considering 4.0% lignin, cellulose and hemicellulose respectively comprise approximately 33.2% and 15.9% of the castor meal (Robb et al. 1974). Protein content was also confirmed by Bandeira et al. (2004). The castor bean has one of the highest fiber contents of all oilseeds under study (49%), however, bioethanol production potential is limited to only 103 L/ha because of the low yield of castor beans per hectare (568 kg/ha).

Table 2. Castor Bean yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	568 kg/ha	100 %
Vegetable Oil	250 kg/ha	45 %
Vegetable Oil extraction coproducts	318 kg/ha	56 %
Proteins	126 kg/ha	39.7 %
Fibers (cellulose/hemicellulose)	156 kg/ha	49.1 %
Lignin	13 kg/ha	4.0 %
Biodiesel	**284 L/ha**	
Alcohol necessary for biodiesel production	50 L/ha	
Bioethanol from fiber	**103 L/ha**	

Utilization of castor meal in animal feeding is limited by the presence of ricin, a toxic lectin classified as a type 2 ribosome inactivating protein, found in the castor bean. Its presence eliminated the possibility of castor meal use as an animal ration without detoxification. The U.S. Center for Disease control estimated that 500 micrograms of Ricin can be lethal to humans, the equivalent of only eight castor beans. The presence of this toxin makes ethanol production from castor meal very attractive.

3.4.3. Jatropha curcas

Jatropha curcas is perhaps one of the most promising of the oil crops under study. Capable of growing in various climates, fruit production can range from 0.4 to 12 Mg/ha/y, depending on rainfall for the given region (Openshaw 2000). The perennial shrub yields nuts after 9 to 12 months but full production potential is not reached for 2 to 3 years. Once the shrub reaches its full production, its life cycle is approximately 40 years, making tilling and replanting minimal. For this study an average production yield of 5 Mg per hectare per year was considered.

Fruit from the *Jatropha curcas* plant is composed of seeds and an outer shell. The carbohydrate rich outer shell accounts for approximately 31% of the fruit's total weight while the inner seed, required for oil extraction, accounts for roughly 69% of the fruit's weight (Martínez-Herrera et al. 2006). The outer shell is composed of 53.5% fiber, 36.7% lignin and 7.8% protein while the seed contains a much higher level of protein (38.4%) and lower levels of fiber and lignin (22.9% and 14.2%).

Table 3 considers the use of the entire *Jatropha curcas* fruit. Jatropha meal generally refers to only the byproducts from the inner seed after oil extraction, however, by including the outer shell as one of the raw products for ethanol production, carbohydrate concentrations are increased by more than 50%, doubling ethanol production potential. Nearly 700 liter per hectare of bioethanol can be produced from Jatropha coproducts which are capable of meeting 199% of transesterification alcohol demands for biodiesel produced from Jatropha.

Table 3. *Jatropha curcas* yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	**5,000 kg/ha**	100 %
Vegetable Oil	1,750 kg/ha	38 %
Vegetable Oil extraction coproducts	3,250 kg/ha	62 %
Proteins	939 kg/ha	28.9 %
Fibers (cellulose/hemicelluloses)	1,052 kg/ha	32.4 %
Lignin	689 kg/ha	21.2 %
Biodiesel	**1,990 L/ha**	
Alcohol necessary for biodiesel production	350 L/ha	
Bioethanol from fiber	**695 L/ha**	

Just as the Castor bean plant, *Jatropha curcas* is also highly toxic. Toxalbumins called curcin and cyanic acid are present in all parts of the plant but the highest concentrations are found in the seeds which are highly poisonous, eliminating their use as a protein source for animal rations.

3.4.4. Oil palm

The oil palm tree is the world leader in vegetable oil production due to its exceptionally large fruit yield. Primarily produced in Southeast Asia, Africa and Central America, palm oil production requires humid tropical climates with extensive rainfall. It is estimated that the African Palm yields approximately 22.5 Mg of fruit per hectare; however, three years are required before the first kernels can be harvested and only after five years is the plant's full potential reached (Pinto et al. 2005).

According to Carvalho et al. (2006), neutral detergent fiber (cellulose, hemicelulose, and lignin) accounts for 73.4% of palm kernel meal and the level of acid detergent lignin is only 12.2%. The remaining 61.2% of polysaccharides can be hydrolyzed in fermentable sugars for ethanol production. This fiber content is by far the greatest found among oil crops; and due to the large amount

of harvested palm kernels, nearly 7,000 liters of ethanol can be produced per hectare.

Table 4 presents the composition of palm kernel meal in terms of vegetable oil and oil extraction byproducts. Because palm production is relatively small in Brazil, production values specific to the country are not presented. The exceptionally large potential for ethanol production from the biomass generated per hectare of palm kernels harvested is more than sufficient to meet palm oil biodiesel's alcohol demands (575%).

Table 4. Palm Seeds yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	22,500 kg/ha	100 %
Vegetable Oil	5,850 kg/ha	26 %
Vegetable Oil extraction coproducts	16,650 kg/ha	74 %
Proteins	2,664 kg/ha	16 %
Fibers (cellulose/hemicelluloses)	10,190 kg/ha	61.2 %
Lignin	2,031 kg/ha	12.2 %
Biodiesel	**6,645 L/ha**	
Alcohol necessary for biodiesel production	1,170 L/ha	
Bioethanol from fiber	**6,725 L/ha**	

3.4.5. Sunflower

In 2007, approximately 30 Tg of sunflower seed were harvested worldwide, and Brazil was responsible for 101 Gg of sunflower seed (United States Department of Agriculture 2008; Brazilian Institute of Geography and Statistics 2007). Sunflower seed oil is commonly used for frying in the food industry, and in cosmetic formulations as an emollient.

According to Malathi and Devegowda (2001), nonstarch polysaccharides account for more than 41% of sunflower seed meal. Lignin concentration among these polysaccharides is approximatly 8%, limiting the potential for hydrolyzed

ethanol production (Villamide and San Juan 1998). Sunflower seed meal is often used as an animal feed due to a 31.2% protein content (Irshaid, Harb, and Titi 2003).

Table 5. Sunflower seed yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	1500 kg/ha	100 %
Vegetable Oil	720 kg/ha	48 %
Vegetable Oil extraction coproducts	780 kg/ha	52 %
Proteins	245 kg/ha	31.2 %
Fibers (cellulose/hemicelluloses)	320 kg/ha	41.3 %
Lignin	62 kg/ha	8.0 %
Biodiesel	**820 L/ha**	
Alcohol necessary for biodiesel production	144 L/ha	
Bioethanol from fiber	**210 L/ha**	

The full potential for bioethanol production from sunflower seed meal is 210 liters per harvested hectare, totaling more than 14 ML of potential production in Brazil. Considering the assumptions made for biodiesel production, ethanol produced from sunflower oil extraction coproducts is capable of meeting 146% of transesterification alcohol demands.

3.4.6. Rapeseed

Rapeseed is the preferred biodiesel feedstock grown in Europe where it is primarily used as a winter-cover crop. In 2005, over 46 Tg were harvested worldwide and nearly 35% being grown in Europe (United States Department of Agriculture 2008). In Brazil, rapeseed is produced in small quantities in the southern regions where temperatures are more temperate. Production is so little that the Brazilian Institute of Geography and Statistics (IBGE) doesn't include

rapeseed production in their annual agricultural production values (Brazilian Institute of Geography and Statistics 2007). World production yields are estimated at about 1.7 Mg/ha, and this value was used for the calculation of bioethanol production potential in the current study (United States Department of Agriculture 2008).

Rapeseed meal, with a protein content of 40%, is often used as an animal nutrition supplement in Europe and India (Rashid and Anwar 2008). The amount of nonstarch polysaccharides available for hydrolysis was estimated to be about 40% according to Malathi and Devegowda (2001). Soliva et al. (2005) confirmed fiber and protein quantities found in rapeseed meal and also quantified the amount of lignin as 9.1% of the total mass.

The fiber content of rapeseed meal (39.8%) allows for the potential production of 235 liter of bioethanol per hectare. This value represents 144% of the total alcohol required for biodiesel production by transesterification. As in the case of sunflower meal, the high oil content in relation to relatively low yields per hectare and average fiber contents limit bioethanol potential, however, alcohol demands for transesterification are still met.

Table 6. Rapeseed yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	1700 kg/ha	100 %
Vegetable Oil	815 kg/ha	48 %
Vegetable Oil extraction coproducts	885 kg/ha	52 %
Proteins	355 kg/ha	40.0 %
Fibers (cellulose/hemicelluloses)	352 kg/ha	39.8 %
Lignin	80 kg/ha	9.1 %
Biodiesel	**925 L/ha**	
Alcohol necessary for biodiesel production	163 L/ha	
Bioethanol from fiber	**235 L/ha**	

3.4.7. Cottonseed

In 2007, 4.0 Tg of cotton was harvested in Brazil, of which about 2.61 Tg were cottonseed (United States Department of Agriculture 2008; Brazilian Institute of Geography and Statistics 2007). The average yield of cottonseed was calculated to be 2.3 Mg/ha.

Oil extracted from cottonseed is often used for food processing and cottonseed meal is then typically used as a cattle feed. For the given study, all oil extraction coproducts were considered, including both cottonseed meal and hulls. Pinto et al. (2005) estimated the oil content of cottonseed to be 15% of the total weight. Table 7 shows the chemical composition of the coproducts and their content per hectare of cotton produced.

The high fiber content in relation to the low oil content in the seed, 30 to 15% respectively, allows for the production of bioethanol to easily meet the transesterification demands of biodiesel production (574%), as can be seen in Table 7 (Arana et al. 2001).

Table 7. Cotton Seed yield, composition and bioethanol production potential.

	Yield	Composition (% grain and % oil extraction coproduct)
Average yield	2,331 kg/ha	100 %
Vegetable Oil	350 kg/ha	15 %
Vegetable Oil extraction coproducts	1,981 kg/ha	85 %
Proteins	505 kg/ha	25.6 %
Fibers (cellulose/hemicelluloses)	610 kg/ha	30.8 %
Lignin	295 kg/ha	14.9 %
Biodiesel	**380 L/ha**	
Alcohol necessary for biodiesel production	70 L/ha	
Bioethanol from fiber	**402 L/ha**	

3.4.8. Overview

Table 8 provides an overview of the protein, carbohydrate and lignin content of each of the crops studied as well as the ethanol production potential per hectare. The percentage of the alcohol demand for transeterification alcohol demand the production of biodiesel is also included.

Table 8. Composition of oil seed extraction coproducts and bioethanol production potential

	Protein (%)	Carbohydrates (%)	Lignin (%)	Ethanol (L/ha)	Biodiesel ethanol demands met by lignocellulosic ethanol (%)
Soybean	38.0	16.2	12.9	247	257
Castor seed	39.7	49.1	4.0	103	206
Jatropha curcas	38.4	32.4	14.2	695	199
Oil Palm Kernel	16.0	61.2	12.2	6725	575
Sunflower seed	31.2	41.3	8.0	210	146
Rapeseed	40.0	39.8	9.1	235	144
Cottonseed	25.6	30.8	14.9	402	574

Lignin, unsuitable for bioethanol production, has an exceptionally high energy content of 29.54 MJ/kg, similar to that of coal (Cardona Alzate and Sánchez Toro 2006). In the case of *Jatropha curcas*, 689 kg of lignin are produced per hectare, equivalent to approximately 18 GJ/ha. According to Cardona Alzate and Sánchez Toro (2006), energy demands for ethanol distillation are roughly 27.43 MJ per liter of ethanol generated, totaling slightly more than 19 GJ of energy needed for the 695 liters of ethanol which can be produced from Jaropha oil extraction coproducts. Assuming perfect combustion, nearly 95% of energy demands for distillation are met by burning lignin which remains after saccharification.

Palm kernel yields three times the amount of lignin per hectare than Jatropha, yielding 60 GJ/ha, however, bioethanol production potential is nearly ten times that of Jatropha, requiring the use of other energy sources for ethanol distillation.

3.5. CONCLUSIONS

Ethanol production potential from oil extraction coproducts is capable of meeting transesterification demands in nearly all the crops studied. Ethanol production from palm kernel coproducts (6,725 L/ha and 575% of the transesterification alcohol demand) is much larger than all other coproducts and can be very competitive for the production of cellulose based ethanol. *Jatropha curcas* and castor bean, both of which are toxic, are also capable of producing 199 and 206% of transesterification alcohol demands, creating new markets for these byproducts.

Brazilian agricultural production has the potential for large expansion and is already an exporter of biofuels (biodiesel and ethanol). Based on data provided by the Brazilian Institute of Geography and Statistics (Brazilian Institute of Geography and Statistics 2007), less than 7% of the total land area of Brazil is utilized for crop production. In contrast, 19.1% of US and 33.9% of German soils are considered arable and permanent cropland (World Resources Institute 2007).

Perhaps one of the largest challenges facing the production of ethanol from lignocellulose is the high cost of enzymes. In the case of bioethanol production from oil crop byproducts, biomass is already harvested and transported to oil extraction facilities. In this case, no extra costs are required for the collection of biomass, compensating the high prices of enzymes.

Demands for biofuels, both biodiesel and ethanol, are forcing producers to look for new feedstocks. Oil crops which produce large quantities of both vegetable oil and lignocellulosic biomass may provide the solution to this problem.

3.6. REFERENCES

Agência Nacional de Petróleo. 2007. Authorized Capacity of Biodiesel Production Plants.

Arana, M., E. DePeters, J. Fadel, J. Pareas, H. Perez-Monti, N. Ohanesian, M. Etchebarne, C. Hamilton, R. Hinders, M. Maloney, C. Old, and T. Riordan. 2001. Comparing cotton by-products for dairy feed rations. *Dairy Buisness Communications*, http://www.dairybusiness.com/western/Oct00/byproducts3.htm.

Bandeira, D. A., W. V. Cartaxo, L. S. Severino, and N. E. de M. Beltrão. 2004. Resíduo industrial da mamona como fonte alternativa na alimentação animal. Paper read at I Congresso Brasileiro de Mamona, at Campina Grande - PB.

Berchmans, H. J., and S. Hirata. 2008. Biodiesel production from crude Jatropha curcas L. seed oil with a high content of free fatty acids. *Bioresource Technology* 99 (6):1716-1721.

Brazilian Association of Vegetable Oil Industries. 2008. Soybean Exports.

Brazilian Institute of Geography and Statistics. 2007. Systematic Survey of Agricultural Production.

Brazilian National Energy Balance. 2006. edited by Department of Mines and Energy.

Cardona Alzate, C. A., and O. J. Sánchez Toro. 2006. Energy consumption analysis of integrated flowsheets for production of fuel ethanol from lignocellulosic biomass. *Energy* 31 (13):2447-2459.

Carvalho, L. P. F., A. R. J. Cabrita, R. J. Dewhurst, T. E. J. Vicente, Z. M. C. Lopes, and A. J. M. Fonseca. 2006. Evaluation of Palm Kernel Meal and Corn Distillers Grains in Corn Silage-Based Diets for Lactating Dairy Cows. *Journal of Dairy Science* 89 (7):2705-2715.

Companhia Nacional de Abastecimento. 2008. Sugarcane harvest.

Embrapa Soja. 1995. Average chemical composition of soybeans

Evangelista, A. R., J. G. de Abreu, A. J. Peron, A. C. Fraga, and P. C. Neto. 2004. Avaliação da composição química de tortas de mamona e amendoim obtidas por diferentes métodos de extração de óleo. Paper read at I Congresso Brasileiro de Mamona, at Campina Grande - PB.

Ferrari, R. A., V. da Silva Oliveira, and A. Scabio. 2005. Biodiesel from soybean: characterization and consumption in an energy generator. *Química Nova* 28 (1):19-23.

Huisman, M. M. H., H. A. Schols, and A. G. J. Voragen. 1998. Cell wall polysaccharides from soybean (Glycine max.) meal. Isolation and characterisation. *Carbohydrate Polymers* 37 (1):87-95.

International Energy Agency (IEA) Statistics. 2005. Renewables Information with 2004 Data

Irshaid, R. H., M. Y. Harb, and H. H. Titi. 2003. Replacing soybean meal with sunflower seed meal in the ration of Awassi ewes and lambs. *Small Ruminant Research* 50 (1-2):109-116.

Kim, S., and B. E. Dale. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy* 26 (4):361-375.

Ma, F., and M. A. Hanna. 1999. Biodiesel production: a review. *Bioresource Technology* 70 (1):1-15.

Malathi, V., and G. Devegowda. 2001. In vitro evaluation of nonstarch polysaccharide digestibility of feed ingredients by enzymes. *Poultry Science* 80 (3):302-305.

Marchetti, J. M., V. U. Miguel, and A. F. Errazu. 2007. Possible methods for biodiesel production. *Renewable and Sustainable Energy Reviews* 11 (6):1300-1311.

Martínez-Herrera, J., P. Siddhuraju, G. Francis, G. Dávila-Ortíz, and K. Becker. 2006. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of Jatropha curcas L. from Mexico. *Food Chemistry* 96 (1):80-89.

Openshaw, K. 2000. A review of Jatropha curcas: an oil plant of unfulfilled promise. *Biomass and Bioenergy* 19 (1):1-15.

Pinto, A. C., L. L. N. Guarieiro, M. J. C. Rezende, N. M. Ribeiro, E. A. Torres, W. A. Lopes, P. A. de P. Pereira, and J. B. de Andrade. 2005. Biodiesel: an overview. 16:1313-1330.

Rashid, U., and F. Anwar. 2008. Production of biodiesel through optimized alkaline-catalyzed transesterification of rapeseed oil. *Fuel* 87 (3):265-273.

Robb, J. G., R. C. Laben, H. G. Walker, Jr., and V. Herring. 1974. Castor Meal in Dairy Rations. *Journal of Dairy Science* 57 (4):443-450.

Sims, R. E. H., A. Hastings, B. Schlamadinger, G. Taylor, and P. Smith. 2006. Energy crops: current status and future prospects. 12 (11):2054-2076.

Soliva, C. R., M. Kreuzer, N. Foidl, G. Foidl, A. Machmüller, and H. D. Hess. 2005. Feeding value of whole and extracted Moringa oleifera leaves for ruminants and their effects on ruminal fermentation in vitro. *Animal Feed Science and Technology* 118 (1-2):47-62.

United States Department of Agriculture. 2008. World Agricultural Production - Foreign Agricultural Service.

United States Department of Agriculture (USDA). 2008. World Agricultural Production - Foreign Agricultural Service.

US Department of Energy. 2006. Theoretical Ethanol Yield Calculator 2006.

Villamide, M. J., and L. D. San Juan. 1998. Effect of chemical composition of sunflower seed meal on its true metabolizable energy and amino acid digestibility. *Poultry Science* 77 (12):1884-1892.

World Resources Institute. 2007. Agriculture and Food: Country Profiles.

Chapter 4

CHEMICAL PRETREATMENT METHODS FOR THE PRODUCTION OF ETHANOL FROM *JATROPHA CURCAS* MEAL BY SACCHARIFICATION

Expanded abstract published in the conference proceedings of *ENZITEC 2008*

4.1. INTRODUCTION

Growing concerns over the environmental impact of fossil fuels has led to the extensive biofuel research across the world. New biomass sources are constantly being considered due to the large potential for ethanol production from cellulose (Sims et al. 2006). Although the high costs of enzymes are blamed for the economic infeasibility of cellulosic ethanol production, the harvest and transport of biomass are also very costly. When biodiesel crops are harvested and transported for oil extraction, large quantities of biomass are produced which may be suitable as cellulose sources for ethanol production.

Jatropha curcas is one of the oil plants considered to have the greatest potential for biodiesel production in various countries around the world, including Brazil. Its high oil yield (nearly four times that of soybeans) and ability to grow in a diverse range of tropical and subtropical climates, make it appealing for biodiesel production. Growing conditions, especially the amount water available to the plant play a significant role in the productivity of the shrub and composition of the seeds (Abou Kheira and Atta 2008). These small changes in the chemical composition of the fruit can account for large differences in the potential for bioethanol production using the non-starch polysaccharides found in the oil extraction coproducts.

Pretreatment plays an important role in the production of ethanol from lignocellulosic sources. It is required to make cellulose more accessible to enzymes which convert carbohydrate polymers into fermentable sugars (Mosier et al. 2005). Various types of pretreatments are being studied to be incorporated in the production of bioethanol including physical, chemical and biological treatments as well as combinations (Hendriks and Zeeman 2009). Figure 5 shows the result of effective pretreatment, with the goal of breaking the lignin bonds and disrupting cellulose's crystalline structure.

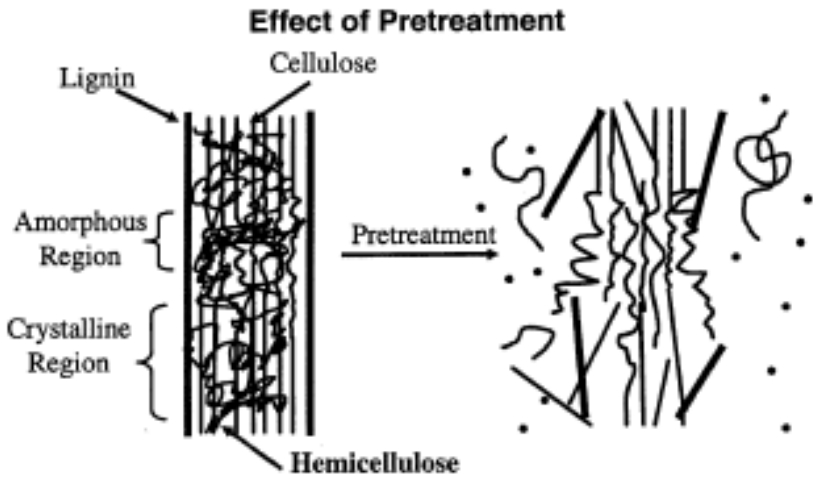

Figure 5. Schematic of pretreatment goals on lignocellulosic biomass (Hsu 1996).

Saccharification of these coproducts is capable of producing ethanol as well as potentially degrading the large polysaccharide chains which are poorly utilized by monogastric animals in animal rations (Huisman, Schols, and Voragen 1998). Cell wall degrading enzymes, such as pectinase, xylanase, and cellulase, increased the digestibility of soybean meal by reducing non-starch polysaccharides into their respective monosaccharides (Karr-Lilienthal et al. 2005). Protein can be concentrated if polysaccharides and lignin are removed during the pretreatment and enzymatic hydrolysis stages, if the high temperatures and acid concentrations have no effect on the protein structures,

In this study, the effectiveness of three different pretreatments on Jatropha curcas coproducts from the oil extraction process with the intent of sugar production via enzymatic hydrolysis was studied, where effectiveness is defined as the concentration of glucan in the pretreated biomass as well as the overall sugar production after enzymatic hydrolysis. The protein and fiber contents were measured initially, after pretreatment and after enzymatic hydrolysis. Sugar production was analyzed using the following three pretreatments: sulfuric acid (Linde, Galbe, and Zacchi 2007), sodium hydroxide (Silverstein et al. 2007), and calcium hydroxide (lime) (Saha and Cotta 2008).

4.2. MATERIAL AND METHODS

4.2.1. Raw Material

Jatropha seeds were acquired from local producers of Viçosa, Minas Gerais, Brazil, with the intent of analyzing biodiesel production. The seeds were cracked and oil was extracted in a soxhlet extraction apparatus using hexane. The remaining biomass was collected, dried and ground to approximately 1 mm particle size. Before further treatment, the solids were diluted with water and placed in a sonicator bath for 10 minutes. The biomass was then rinsed and dried and the water soluble solids quantified.

4.2.2. Pretreatment

Dry biomass samples of 15 grams were treated with either 0.2% H_2SO_4 (v/v), 1.0% NaOH (v/v), 1.5% (w/v) $Ca(OH)_2$ or distilled water in a 500 ml erlenmeyer flask and placed in an autoclave for one hour at 121°C. These values were determined based on the best results obtained from other works using different biomass sources (Linde, Galbe, and Zacchi 2007; Silverstein et al. 2007; Saha and Cotta 2008). Solid concentrations were maintained at 10% (w/v) and all chemical treatments were prepared in duplicate with the control (distilled water) performed with no repetition. After removing the samples from the autoclave, they were washed with distilled water and stored for enzymatic hydrolysis. During no point were the samples dried in an effort to avoid cell wall collapse.

4.2.3. Enzymatic Hydrolysis

Saccharification was performed by mixing 15 grams of the pretreated biomass with a 50 mM sodium citrate buffer (pH 5.0) in a 500 mL flask to reach a solid content of 10% (w/v). Cellulase from *Trichoderma reesei* ATCC 26921

(Sigma-Aldrich Brasil Ltda., Sao Paulo) was applied with a cellulase loading of 30 FPU/gram of substrate and supplemented with an excess of Cellobiase from *Aspergillus niger* (Sigma-Aldrich Brasil Ltda., Sao Paulo) to ensure that the experimental results were not influenced by cellobiose inhibition. Cyclohexamide (40 mg/L) was also added to prevent contamination from outside bacteria.

The glass flasks containing the biomass slurry was placed in a water bath at 42°C and under mechanical agitation at 100 rpm. Samples of 1.5 mL were drawn at 6, 12 and 24 hours using a glass pipette and stored at -20°C until the type of analysis. Figure 6 shows the apparatus used for enzymatic hydrolysis.

Figure 6. Apparatus utilized for enzymatic hydrolysis.

4.2.4. Analysis

Samples were prepared for sugar analyses by centrifuging the samples (2 mL eppendorf tubes) at 13200 rpm and removing the supernatant. Analysis by

HPLC required the dilution of samples to 10% of the initial concentration and the samples were then passed through 0.45 μm filters. The HPLC device was equipped with a refractive index detector (Shimadzu Corporation, Kyoto, Japan). A column (Shim-Pack SCR-101P) was used for the separation of glucose and xylose and was operated at 80 °C with water as eluent, and at a flow rate of 0.4 mL/min.

Reducing sugars analyzed by the dinitrosalicylic acid (DNS) method were measured by spectrophotometry (Beckman DU-65 Spectrophotometer), at a wavelength of 540 nm. As with the HPLC analysis, the supernatant was separated from the solid material by centrifuging. The supernatant was diluted with distilled water in order for absorbance values to be maintained within the reliable measurement interval of the spectrophotometer (0 to 2). Spectrophotometry analyses were performed in duplicate for each of the enzymatic hydrolysis reactions, giving a total of four absorbance readings for each of the treatments. Reducing sugars were measured in order to confirm the results presented by HPLC and also to detect the total reducing sugar content, instead of only glucose and xylose.

Composition data of the Jatropha meal was determined by HPLC carbohydrate analysis. These HPLC analyses were performed in duplicate and the average value was used for analysis. This process was performed at the Paper and Cellulose Laboratory of the Forestry Engineering Department at the Federal University of Viçosa, Brazil. Protein was calculated using the Kjeldahl nitrogen analysis and using nitrogen to protein conversion factor of 6.25, also performed in duplicate.

4.3. RESULTS AND DISCUSSION

4.3.1. Raw Material

The chemical composition of Jatropha varied depending on the growing conditions, harvest period and analysis procedures (Abou Kheira and Atta 2008).

The composition of Jatropha meal after oil extraction by hexane is presented in Table 9. Based on the HPLC carbohydrate analysis, holocellulose (cellulose and hemicellulose) accounted for 22.8% of the total mass, being that glucan (cellulose) accounted for 11.6% of the total mass and xylan, arabinan, galactan and mannan (hemicelluloses) accounted for 11.2% of the total mass. The results shown in Table 9 are average values of two repetitions with the standard deviation in parenthesis.

Martínez-Herrera et al. (2006) reported values of crude protein and neutral detergent fiber (NDF) in processed Jatropha seeds ranging from 60 to 70% and 3 to 11%, respectively. Other sources state that protein content is approximately 40% of the total weight (CETEC 2006). In this work, protein content was measured three months after oil extraction and there exists the possibility of protein degradation (Sadeghi et al. 2006)

Oil concentrations in *Jatropha curcas* were found to be 39% when using the soxhlet apparatus and hexane for solvent extraction. Oliveira et al. (2008) reported an oil content of 31.6% (w/w) for *Jatropha curcas L*. and 23.9% for *Jatropha gossiypiifolia*. Other sources present higher oil concentrations such as CETEC (2006) which found an oil concentration of 38.1% (w/w) in the Jatropha seed.

Table 9. Composition of Jatropha shell biomass expressed as percent of dry matter after oil extraction

Component	Percentage (%)[a]
Glucan	11.6 (0.49)
Xylan	5.6 (0.17)
Arabinan	1.9 (0.06)
Galactan	1.3 (0.02)
Mannan	2.4 (0.07)
Protein	19.0 (0.20)
Lignin[b]	14.2
Ash[b]	7.4

[a]Composition percentages are on a dry-weight basis.
[b]Taken from literature (CETEC 2006).

Water soluble solids accounted for 10.5% the total mass of Jatropha meal, of which nearly no sugars were detected by HPLC, and was therefore discarded. This 10.5% may have consisted of small quantities of water soluble sugars and starch, as well as residual oil which may have been removed with the water.

4.3.2. Pretreatments

The effectiveness of pretreatment is determined both by the chemical composition of the biomass after pretreatment, as well as the hydrolysis efficiency. The initial objective of pretreatment was to investigate its effect on enzymatic hydrolysis efficiency; however, the conservation of protein was also of interest if the material could be detoxified for use as an animal feed. The chemical composition of Jatropha meal water insoluble solids (WIS) after pretreatment can be seen in Table 10. The values in parenthesis represent the standard deviation between the pair of values measured.

The results in Table 10 show, what appears to be, the exceptional NaOH pretreatment results compared with those of H_2SO_4 and Lime since glucan was 84% and 149% greater than that of the H_2SO_4 and lime pretreatments, respectively. Sodium hydroxide proved very effective for the concentration of cellulose and hemicellulose, the desired result when bioethanol production is the only objective. However, sodium hydroxide pretreatment also removed the vast majority of protein from the Jatropha meal biomass according the total Kjeldahl nitrogen test. Figure 7 proves that although the NaOH pretreatment was more effective for the concentration of carbohydrates, little difference was noticed when the different Jatropha meal pretreatments were subjected to enzymatic hydrolysis.

Table 10. Composition of Jatropha meal after pretreatment.

	H_2SO_4 pretreatment	NaOH pretreatment	Lime pretreatment
Content in WIS after pretreatment (% of dry weight)			
Glucan	15.7 (0.28)	28.9 (0.27)	11.6 (0.36)
Xylan	6.5 (0.16)	12.1 (0.32)	4.7 (0.37)
Arabinan	2.5 (0.03)	2.6 (0.06)	1.9 (0.13)
Galactan	1.1 (0.03)	1.0 (0.06)	1.1 (0.04)
Mannan	1.0 (0.03)	1.0 (0.05)	1.9 (0.05)
Protein	16.8 (0.22)	3.2 (0.17)	12.9 (0.15)

4.3.3. Enzymatic Hydrolysis

Each of the pretreatments mentioned above were subjected to enzymatic hydrolysis. In this case, the weight of pretreated biomass submitted to enzymatic hydrolysis was unknown, but instead was determined as a function of the initial weight first submitted to pretreatment. This allowed that the efficiency of the two processes (pretreatment and hydrolysis) be seen in the figures below which show the sugar concentrations during hydrolysis.

Figure 7 shows the concentration of sugar (glucose and xylose) as measured by HPLC as a function of time. It can be seen that the alkaline pretreatments showed greater sugar concentrations after 24 hours of hydrolysis. However, when comparing with hydrolysis of the untreated Jatropha meal sample, the importance of pretreatment for enzymatic hydrolysis is obvious.

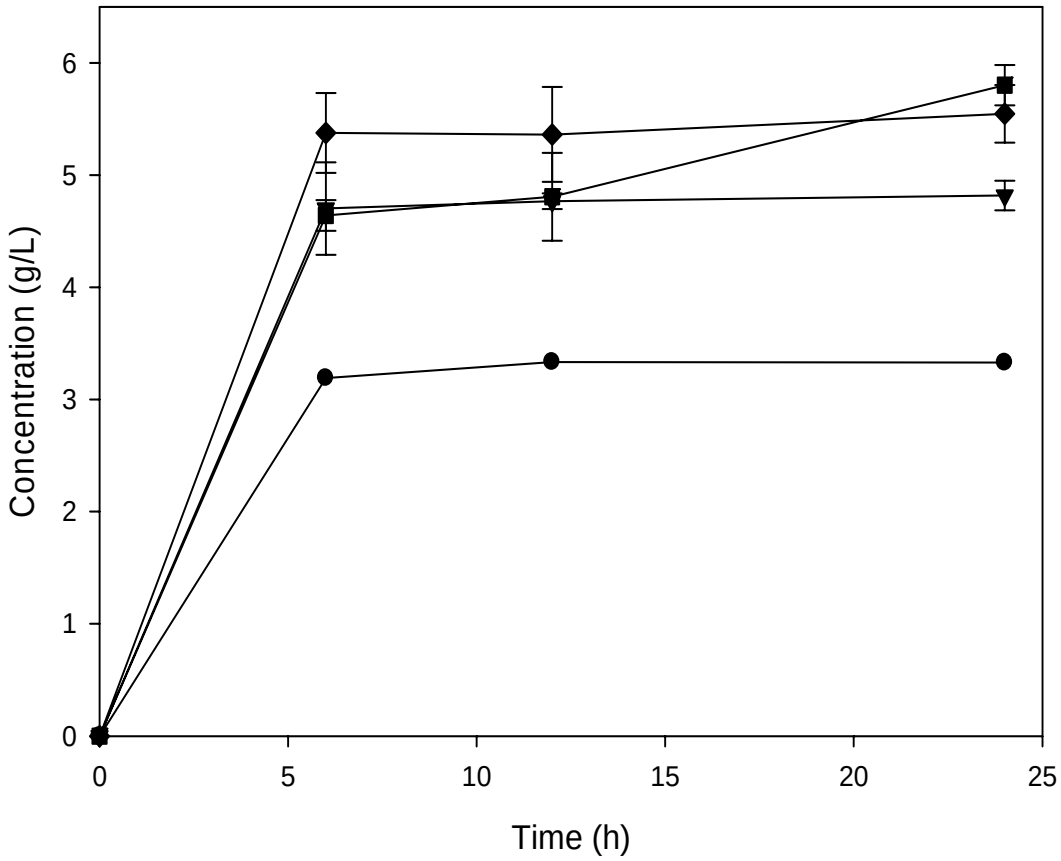

Figure 7. Total concentration of glucose and xylose measured by HPLC after saccharification of the different pretreated biomasses. The untreated (●), sulfuric acid (▼), lime (♦) and sodium hydroxide (■) pretreatment forms are presented as a function of hydrolysis time.

Figure 8 shows the same sugar concentrations as Figure 7, but includes the separation of glucose and xylose. Here it can be seen that the glucose produced from both the NaOH and Lime pretreated biomass was nearly equal, however, slightly more xylose was produced from the lime pretreated biomass and therefore showing the greater overall sugar (glucose and xylose) content in Figure 7. In all cases, xylose content was roughly 10% of the glucose production, however, from Table 10 it can be seen that xylan consisted of roughly 40% of glucan, signifying much poorer hydrolysis of xylan, likely due to the fact that xylanase activity in the cellulase solution was quite low.

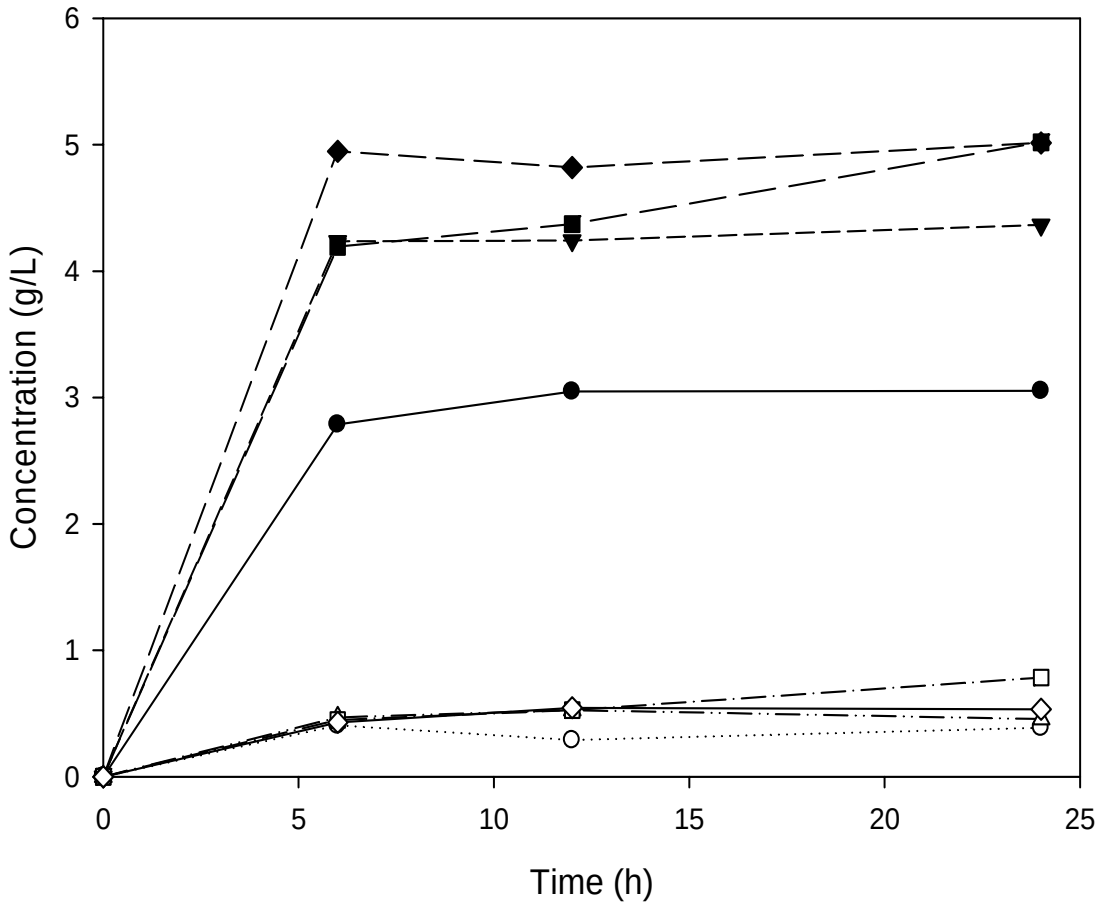

Figure 8. Concentration of glucose and xylose measured by HPLC after saccharification of the different pretreated biomasses. The untreated (●), sulfuric acid (▼), lime (♦) and sodium hydroxide (■) pretreatment forms are presented as a function of hydrolysis time. The hollow symbols represent xylose and the solid symbols glucose.

The chromatograms in Figure 9, Figure 10 and Figure 11 show the peaks of glucose (19.95 min) and xylose (21.48 min). From these figures, it could be noted the difficulty in interpretation of the various substances detected during chromatographic analysis. Many small peaks were recorded and ignored since the objective of this study was to calculate the quantities of glucose and xylose produced by the enzymes, however, the amount of additional peaks shows the complexity of the biomass under study.

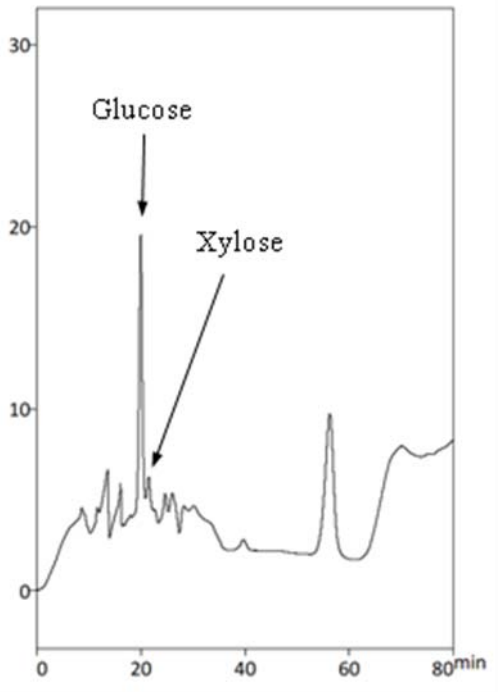

Figure 9. Chromatogram of the hydrolysate after 6 hours of enzymatic hydrolysis using the H_2SO_4 pretreated biomass.

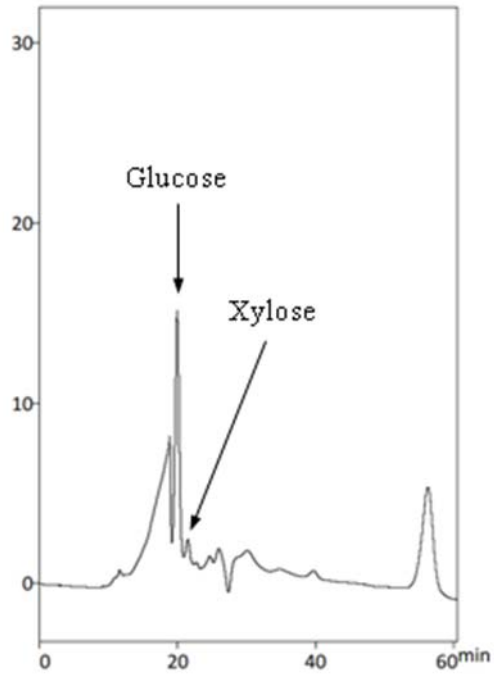

Figure 10. Chromatogram of the hydrolysate after 6 hours of enzymatic hydrolysis using the Lime pretreated biomass.

Each of the hydrolysates analyzed by HPLC show slightly different results based on the type of pretreatment used before enzymatic hydrolysis. It was found that the wide peak found which appeared before that of glucose was the result of organic acids and instead of only increasing the in the vertical direction with concentration, also grew in the horizontal direction, requiring the dilution of the samples in order the organic acids to completely pass through the chromatography column before glucose. The incorporation of a deashing guard column may be adequate for the removal of organic acids by ion/cation exchange in order to better maintain the baseline, preventing the possible interference on the monosaccharide peak areas.

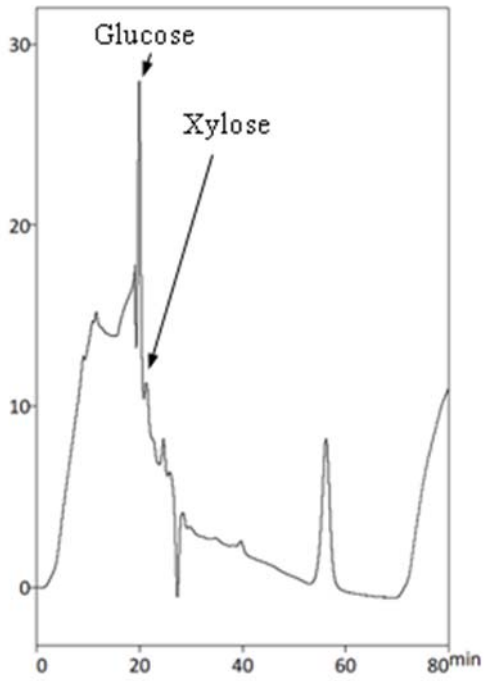

Figure 11. Chromatogram of the hydrolysate after 6 hours of enzymatic hydrolysis using the NaOH pretreated biomass.

Reducing sugars were also determined during the same time period using the DNS (dinitrosalicylic acid) method. When measuring for reducing sugars, concentrations found were nearly double those of glucose and xylose measured by HPLC and also appeared that after 24 hours hydrolysis had not yet stopped. As shown in Figure 12, the treated samples showed greater sugar concentrations after hydrolysis, however, the NaOH pretreatment showed to be more effective than lime and H_2SO_4, as would be expected due to the chemical composition of the pretreated biomass.

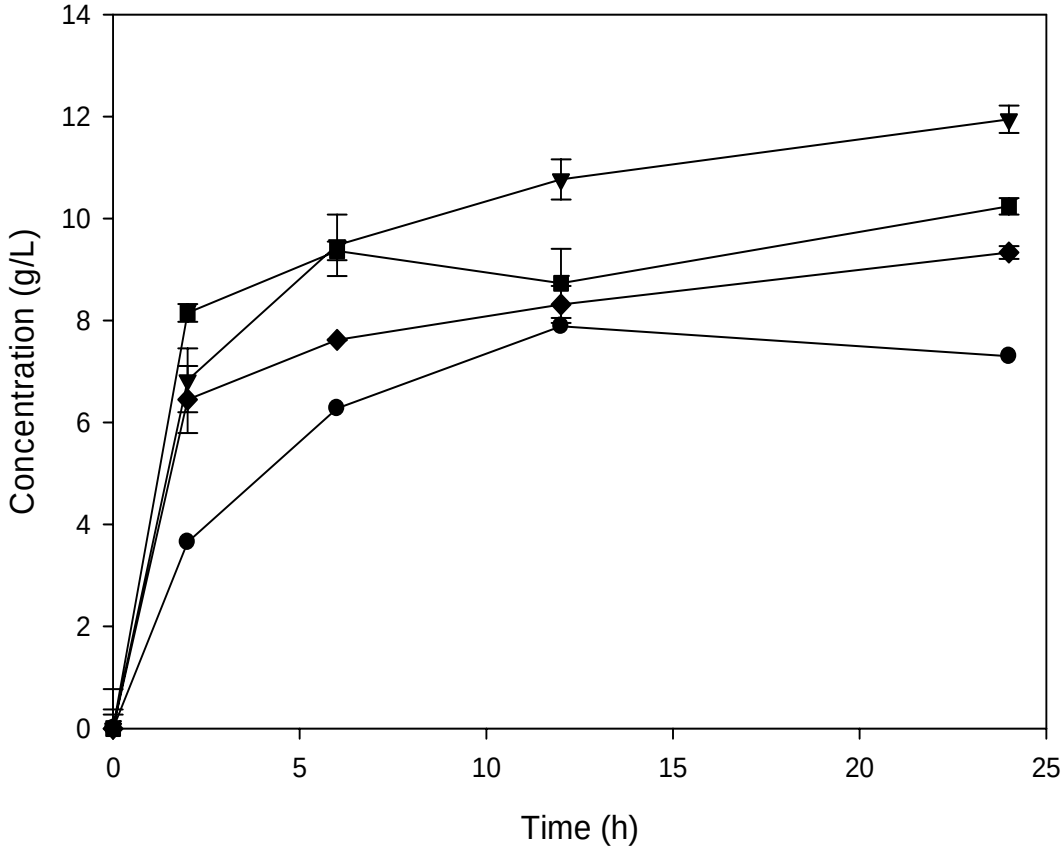

Figure 12. Total concentration of reducing sugars measured by DNS after saccharification of the different pretreated biomasses. The untreated (●), sulfuric acid (▼), lime (♦) and sodium hydroxide (■) pretreatment forms are presented as a function of hydrolysis time.

From the results of the chemical composition of the Jatropha meal after enzymatic hydrolysis shown in Table 11, it was determined which of the pretreatment-hydrolysis combinations was most effective for the production of fermentable sugars. Based on the data from Table 10 and Table 11, 51.0, 63.7 and 62.1% of glucan was hydrolyzed from the H_2SO_4, NaOH and lime pretreatments, respectively. The greatest difference noticed between the acid and alkaline pretreatments was in the hydrolysis of xylan. Only 15.4% of xylan in the H_2SO_4 pretreated biomass was hydrolyzed, while 45.4 and 51.1% of xylan was converted into xylose from the NaOH and lime pretreated biomasses, respectively.

Table 11. Composition of Jatropha meal after hydrolysis

	H_2SO_4 pretreatment	NaOH pretreatment	Lime pretreatment	Untreated
Content in WIS after hydrolysis (%)[a]				
Glucan	7.7 (0.28)	10.5 (0.09)	4.4 (0.25)	9.6 (0.44)
Xylan	5.5 (0.06)	6.6 (0.03)	2.3 (0.14)	7.6 (0.49)
Arabinan	2.4 (0.03)	2.6 (0.02)	1.9 (0.13)	1.9 (0.05)
Galactan	0.4 (0.03)	0.5 (0.01)	0.3 (0.03)	0.6 (0.02)
Mannan	0.5 (0.02)	0.6 (0.01)	0.5 (0.04)	0.8 (0.01)
Protein	12.3 (0.01)	11.0 (0.38)	13.4 (0.07)	11.6 (0.01)

[a]Composition percentages are on a dry-weight basis.

After hydrolysis, all forms of the biomass showed comparable protein concentrations. The increase in protein content from 3.15 to 11.01% in the NaOH pretreated biomass may be due to the hydrolysis of a large portion of the fibers, and thereby concentration the protein fraction. However, in the untreated Jatropha meal sample, 38.9% of protein was lost after hydrolysis. Of the pretreated samples, protein concentrations increased after hydrolysis in both of the alkaline pretreated samples. After hydrolysis of the acid pretreated Jatropha meal, 26.8% of the initial protein content was lost.

4.5. CONCLUSIONS

This work provided evidence that sugar can be produced from Jatropha meal from the enzymatic hydrolysis of non-starch polysaccharides with the intention of fermentation for bioethanol production. There also exists the possibility of the addition of amylase enzymes for the hydrolysis of starch found in the meal, which can reach up to 11% (Martínez-Herrera et al. 2006). The incomplete saccharification of glucan is also of concern. It is possible that as the glucose concentration increased, it inhibited the hydrolysis of the remaining glucan. If this was the case, the addition of fermenting microorganisms for the

simultaneous saccharification and fermentation (SSF) of the Jatropha meal may be a more attractive option.

Although the pretreatment and hydrolysis processes had a negative effect on protein concentrations, a large portion of the non-starch polysaccharides were removed during these processes. Removal of these long polysaccharide chains improves digestibility in monogastric animals. Ávila Filho et al. (2006) reported that for the detoxification of castor bean meal, one hour in an autoclave at 15 psi and 121°C was adequate to remove 100% of the toxins present. If the same is true for Jatropha meal, the pretreatment process is then capable of both preparing the biomass for enzymatic hydrolysis as well as detoxification.

A more complete chemical composition analysis of the Jatropha meal is necessary to determine the starch content as well as the level of toxins. The effect of each of the processes (pretreatment and hydrolysis) was shown in Table 10 and Table 11 but the concentrations of starch and toxins must also be added. The determination of these other nutritional values is also necessary to for calculation of the economic feasibility of this process. It is likely that the sole production of bioethanol from Jatropha meal is not economically feasible, but if it also contributes to the detoxification and improved digestibility for use as an animal ration, the economic feasibility of this process increases dramatically.

4.5. REFERENCES

Abou Kheira, A. A., and N. M. M. Atta. 2008. Response of *Jatropha curcas L.* to water deficit: Yield, water use efficiency and oilseed characteristics. *Biomass and Bioenergy* In Press.

Ávila Filho, S., F. Duarte, and N. M. Ribeiro. 2006. Métodos para Desintoxicação de Tortas de Oleaginosas. Paper presented at I Congresso da Rede Brasileira de Tecnologia do Biodiesel.

CETEC. 2006. Composição Química da Torta de Pinhão-Manso. accessed: 30 de Setembro, 2008

Hendriks, A. T. W. M., and G. Zeeman. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology* 100 (1):10-18.

Hsu, T.A. 1996. Pretreatment of Biomass. In *Handbook on Bioethanol, Production and Utilization*, edited by C. E. Wyman. Washington DC: Taylor & Francis.

Huisman, M. M. H., H. A. Schols, and A. G. J. Voragen. 1998. Cell wall polysaccharides from soybean (Glycine max.) meal. Isolation and characterisation. *Carbohydrate Polymers* 37 (1):87-95.

Karr-Lilienthal, L. K., C. T. Kadzere, C. M. Grieshop, and Jr G. C. Fahey. 2005. Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: A review. *Livestock Production Science* 97 (1):1-12.

Linde, M., M. Galbe, and G. Zacchi. 2007. Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. *Enzyme and Microbial Technology* 40 (5):1100-1107.

Martínez-Herrera, J., P. Siddhuraju, G. Francis, G. Dávila-Ortíz, and K. Becker. 2006. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of Jatropha curcas L. from Mexico. *Food Chemistry* 96 (1):80-89.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96 (6):673-686.

Oliveira, J. S. de , P. M. Leite, L. B. de Souza, V. M. Mello, E. C. Silva, J. C. Rubim, S. M. P. Meneghetti, and P. A. Z. Suarez. 2008. Characteristics and composition of Jatropha gossypiifoliaand *Jatropha curcas L*. oils and application for biodiesel production. *Biomass and Bioenergy* In Press.

Sadeghi, A. A., A. Nikkhah, P. Shawrang, and M. M. Shahrebabak. 2006. Protein degradation kinetics of untreated and treated soybean meal using SDS-PAGE. *Animal Feed Science and Technology* 126 (1-2):121-133.

Saha, B. C., and M. A. Cotta. 2008. Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. *Biomass and Bioenergy* In Press.

Silverstein, R. A., Y. Chen, R. R. Sharma-Shivappa, M. D. Boyette, and J. Osborne. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology* 98 (16):3000-3011.

Sims, R. E. H., A. Hastings, B. Schlamadinger, G. Taylor, and P. Smith. 2006. Energy crops: current status and future prospects. *Global Change Biology* 12 (11):2054-2076.

Chapter 5

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) OF *JATROPHA CURCAS* SHELLS: UTILIZATION OF COPRODUCTS FROM THE BIODIESEL PRODUCTION PROCESS

5.1. INTRODUCTION

The demand for biofuels has increased in recent years due to the rising prices of oil and concern for the environment and carbon dioxide emissions (Sims et al. 2006). This has made countries such as Brazil, with a predominantly tropical climate and large arable region, one of the exporters of biodiesel and ethanol to countries looking to decrease their dependence on foreign oil and meet national targets for biofuel usage. The current installed biodiesel capacity in Brazil is slightly more than 3 billion liters of biodiesel per year (ANP 2008), much of which is produced from soybeans. Ethanol production in 2005 was roughly 16 million cubic meters per year, but is rapidly expanding. Currently, all ethanol produced in Brazil is from sugarcane (MME 2006).

Estimates show that the use of global agricultural residues have the potential to produce up to 442 GL of ethanol from lignocellulosic per year, equivalent to 29% of global gasoline consumption (Kim and Dale 2004). In Brazil alone, large quantities of agricultural residues are produced both from food production as well as when extracting oil for biodiesel production. As new sources of vegetable oil are being applied for the production of biodiesel, with efficiencies and yields much greater than those of soybeans, new sources of biomass will be available for the production of bioethanol.

Various agricultural residues including corn stover (Öhgren, Vehmaanperä et al. 2007), wheat straw (Saha et al. 2005), rice straw and hulls (Karimi, Emtiazi, and Taherzadeh 2006; Saha and Cotta), barley straw (Linde, Galbe, and Zacchi 2007), cotton stalks (Silverstein et al. 2007) and sugarcane bagasse (Adsul et al. 2005) have all been studied for their potential as sources of cellulose for bioethanol production.

Jatropha curcas is one of the oil plants considered to have the greatest potential for biodiesel production in various countries around the world, including Brazil. Its high oil yield (nearly four times that of soybeans) and resistant growing conditions (capable of growing diverse tropical and subtropical climates) make it appealing for biodiesel production. However, the amount of

water available to the plant plays a significant role in the productivity of the shrub (Abou Kheira and Atta).

Jatropha residues can be classified as either outer shells or seed from which the oil was extracted. The outer shell is composed of fibers (53%) lignin (36 %) as well as protein, ash and other extractives (CETEC 2006). In the meal produced from oil extraction from the seed, fiber content is often dependant on the extraction method applied (Evangelista et al. 2004).

The complete process of bioethanol production is dependent on the characteristics of the biomass, however, it is the cost to effectiveness ratio which finally determines the pretreatment form to be used (Eggeman and Elander 2005; Mosier et al. 2005). Pretreatment of lignocellulosic biomass produces a hydrolysate containing large quantities of inhibitors to both saccharification and fermentation (Chandel et al. 2007). These inhibitors which include sugar degradation products, lignin degradation products, acetic acid, and other compounds (Tengborg, Galbe, and Zacchi 2001).

In order to prevent the accumulation of glucose, which is an inhibitor of hydrolysis, simultaneous saccharification and fermentation (SSF) is often applied. Pretreated solids are often washed with hot water to remove inhibitors created during pretreatment; however, at low concentrations these inhibitors may actually have a positive effect on ethanol production due yeast stressing (Öhgren, Bura et al. 2007; Linde, Galbe, and Zacchi 2007). This would therefore eliminate the need for washing of the biomass and allow for direct co-fermentation if the appropriate yeasts are available.

The objective of this study was to evaluate the production of bioethanol from shells of the Jatropha seed via the enzymatic hydrolysis of cellulose found in the shells. The effects of two pretreatment techniques, as well as the use of either washed or unwashed pretreated solids were studied for saccharification and fermentation with cellulase enzymes from *Trichoderma reseei* and *Saccharomyces cerveisiae* yeast for the production of ethanol.

5.2. MATERIAL AND METHODS

5.2.1 Raw Material

Jatropha curcas shells were obtained from a local producer in the region of Viçosa, Minas Gerias – Brazil. The shells were delivered to the Federal University of Viçosa directly after being cracked to obtain the oil seed. Moisture content was determined by placing the samples in a drying oven at 80°C and comparing the initial weight and the weight after 24 hours in the drying oven. The biomass was ground to approximately 1 mm in a mill (Marconi TE 040, Piracicaba, SP - Brazil).

The chemical composition of the raw material was determined at the Cellulose and Paper Laboratory in the Forestry Engineering Department at the Federal University of Viçosa.

5.2.2 Pretreatment

Percent solid recovery determination

Three pretreatment repetitions using both sulfuric acid and sodium hydroxide were performed to calculate the percent solids required. Twenty grams of ground biomass was placed in a 500 mL flask and 0.5% H_2SO_4 or 1.0% NaOH was added to reach a solid:liquid concentration of 10%. The flasks were then heated in an autoclave to 121 °C for one hour before being cooled. The samples were then washed with distilled water, filtered and dried at 80 °C. After drying, the samples were weighed to calculate the percentage of solids recovered after pretreatment.

Pretreatment for SSF

Pretreatment for simultaneous saccharification and fermentation included the same process as was used for the percent solid recovery determination, however, the initial amount of biomass to be pretreated was altered in order to produce exactly 20 grams of pretreated solids. The process was again performed in triplicate using 0.5% H_2SO_4 and 1.0% NaOH at 121 °C for one hour, two of which were prepared for SSF and the other was washed and dried in order to quantify cellulose and hemicellulose contents.

The pretreated biomass slurry was filtered and washed with distilled water or the entire slurry was used for SSF. The pretreated biomass was washed and filtered using a vacuum filter assembly as shown in Figure 13, coupled with a vacuum pump and a glass fiber filter. Samples were then stored in a refrigerator at 4°C until utilized for SSF. It is also important to note that the samples were maintained moist in an effort to avoid cell wall collapse.

Figure 13. Vacuum filter assembly

5.2.3 Enzyme production

Enzymes from *Aspergillus japonicus* were produced in two liters of liquid medium consisting of 6.0 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 0.01 g/L $FeSO_4$, 0.01 g/L $ZnSO_4$ and 10 g/L sugarcane bagasse. The fungi were allowed to grow for 120 hours at 42°C and 200 rpm. After this time, the liquid was filtered through cotton to remove the fungi. Precipitation of the enzymes was done with 80% ammonium sulfate. The solution was then centrifuged for 30 min at 10000 rpm and 2 °C. The supernatant was discarded and the enzymes resuspended in approximately 40 mL of distilled water which was tested for xylanase activity using birchwood substrate.

5.2.4 Yeast cultivation

The *Saccharomyces cerevisiae* strain M70 (Vicente et al. 2006) was produced in order to ferment glucose produced during enzymatic hydrolysis. The yeast was inoculated in an YPD medium of 5 ml and placed in a shaker at 30 °C and 200 rpm for 24 hours. After 24 hours the medium was transferred to 50 ml of YPD medium and after another 24 hours transferred to 500 ml of YPD medium, maintaining the same inoculation conditions. After inoculation, the yeast suspension was centrifuged for 5 min at 3500 rpm and the supernatant was discarded (Öhgren, Bura et al. 2007). The yeast cells were resuspended in approximately 75 ml of distilled water and quickly transferred to the appropriate SSF flask. Lapse time between resuspension and application of the yeast to the SSF was less than one hour. From this solution, 1 ml was injected into a 1.5 ml eppendorf and dried in an oven at 50 °C in order to calculate the concentration of dry cell mass in the suspension.

5.2.5 SSF

The SSF experiments were performed in 500 mL glass Erlenmeyer-flasks with a 275 mL working volume. In the unwashed samples, the slurry was neutralized to a pH of 5.0 and 50 mM sodium citrate buffer with a pH of 5.0 was added to maintain a WIS concentration of 7.5% (w/v). The same sodium citrate buffer was also added to the washed and filtered samples to each the same WIS concentration of 7.5% (w/v).

Cellulase from *Trichoderma reesei* ATCC 26921 and Cellobiase from *Aspergillus niger* were purchased from Sigma-Aldrich Brazil (São Paulo, Brazil) and an enzyme mixture rich in xylanases from *Aspergillus japonicus* was produced in the laboratory. All samples were subjected to a cellulase enzyme loading of 15 FPU/g biomass and supplemented with cellobiase consisting of 125% of the volume of cellulase added. The influence of increasing 4 U/g biomass of the xylanase rich enzyme mixture on the overall efficiency was also studied.

Directly after addition of the enzyme mixtures, the produced *S. cerevisiae* cell suspension was added to the concentration of 0.8 g/L (dry yeast cells). This yeast ferments glucose but not xylose. The SSF experiments were performed in a shaker at 35°C and 150 rpm. Samples of 1.5 mL were removed at 0, 2, 4, 8, 12, 24, 48, 72, 96 and 120 h using sterilized equipment to avoid contamination.

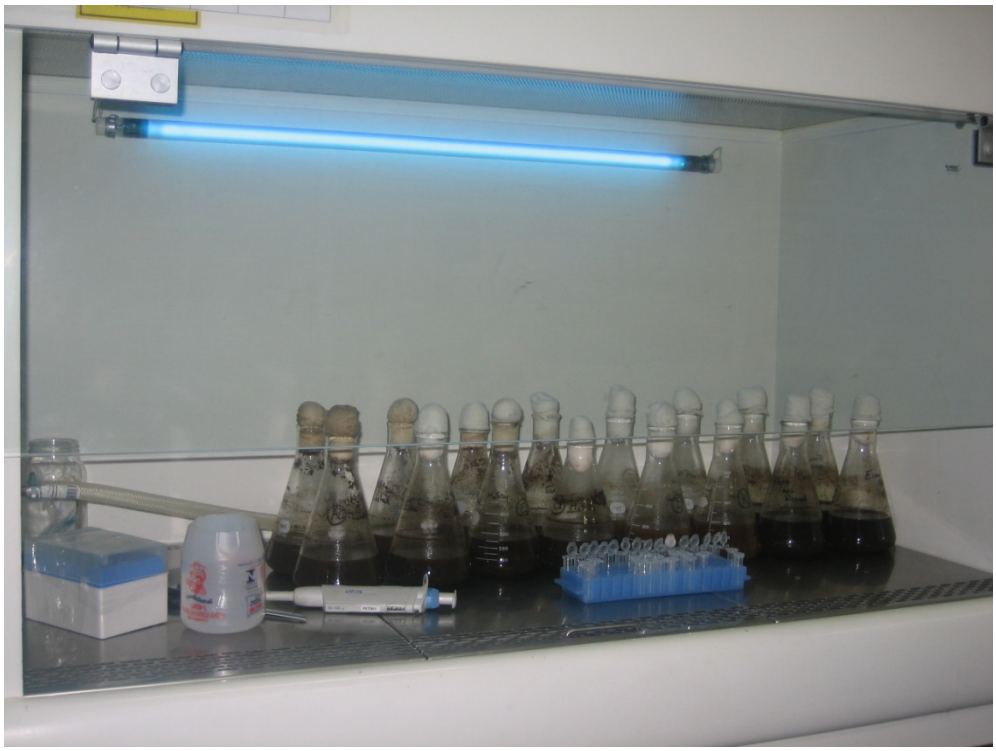

Figure 14. Preparation for sample taking in a sterilized environment.

5.2.6 Analysis

The amounts of ethanol, glucose, xylose, furfural and HMF were determined by a high performance liquid chromatography system. Ethanol, glucose, xylose, furfural and HMF concentrations were determined by HPLC with a Shim-Pack SCR-101P column (Shimadzu Corporation, Kyoto, Japan) at 80 °C and 0.5 ml/min with water as the eluent. Prior to being injected for chromatographic analysis, samples were filtered through a 0.45 filter (MilliQ) and properly diluted with water.

Cellulase and xylanase activities were determined by measuring the reducing sugars using a DNS (dinitrosalicylic acid) reagent, conforming to the specific methodology of each enzyme activity measurement. Whatmans number one filter paper was used as the cellulose source for cellulase activity and expressed as FPU (filter paper units) (Ghose 1987). Xylanase activity was determined using Birchwood substrate as the carbohydrate source and expressed as U (enzyme unit).

Efficiency of the cellulose conversion process was determined using the theoretical values for glucan conversion to glucose (1.11 g/g) and fermentation of glucose to ethanol (0.51 g/g) (US Department of Energy 2006).

Composition data of the Jatropha shells was determined by HPLC carbohydrate analysis. These HPLC analyses were performed in duplicate and the average value was used for analysis. This process was performed at the Paper and Cellulose Laboratory of the Forestry Engineering Department at the Federal University of Viçosa, Brazil. Protein was calculated using the Kjeldahl nitrogen analysis and using nitrogen to protein conversion factor of 6.25, also performed in duplicate.

5.3. RESULTS

5.3.1 Raw material

The composition of the raw material can be found in Table 12. Little information is found regarding the chemical composition of Jatropha meal and even less on the outer shells used in this study; however, published data on the chemical composition shows large variations depending on the region and rainfall (Martínez-Herrera et al. 2006; Abou Kheira and Atta; Openshaw 2000; Abou Kheira and Atta 2008). Oil extraction techniques also play a role in the resulting chemical composition of the meal (Evangelista et al. 2004). Since only the outer shell, which isn't subjected to the oil extraction processes, was evaluated during this study, the extraction method was disregarded. Table 12 shows the average value of the two repetitions with the standard deviation in parenthesis.

Table 12. Composition of Jatropha shell biomass expressed as percentage of dry matter

Component	Percentage (%)[a]
Glucan	26.5 (0.21)
Xylan	6.1 (0.21)
Arabinan	3.5 (0.21)
Galactan	2.7 (0.01)
Mannan	1.2 (0.03)
Protein	6.8 (0.05)
Lignin	22.1 (0.42)
Ash[b]	8.4

[a]Composition percentages are on a dry-weight basis.
[b]Taken from literature (CETEC 2006)

The determined chemical composition of Jatropha shells was very similar to the data presented by Cetec (2006) which found fiber, lignin and protein contents of 53.52, 36.72 and 7.80 %, respectively. Results obtained from Jatropha grown in the region of Viçosa, Minas Gerais – Brazil, shown in Table 12 are quite similar, except that the lignin concentration is significantly lower, concentration of fibers, if only considering glucan, xylan, arabinan, galactan and mannan total 40% of the total dry weight.

5.3.2 Pretreatments

Two different pretreatment methods (0.5% H_2SO_4 and 1.0% NaOH) were considered for the pretreatment of the Jatropha shells. The polysaccharides recovered after pretreatment of the Jatropha shells is shown in Table 13. The values presented in parenthesis represent the standard deviation of the samples measured.

Table 13. Composition of Jatropha shell biomass after pretreatment

	Acid pretreatment	Alkaline pretreatment
WIS in slurry (%)	72.9 (0.23)	50.4 (0.23)
Content in WIS (% of dry weight)		
Glucan	33.9 (0.26)	50.0 (0.31)
Xylan	8.1 (0.22)	12.0 (0.22)
Arabinan	4.2 (0.14)	1.6 (0.03)
Galactan	2.1 (0.10)	1.3 (0.03)
Mannan	0.5 (0.02)	0.4 (0.01)
Protein	7.8 (0.20)	1.4 (0.40)
Lignin	27.1 (0.14)	16.2 (0.07)

The effect of pretreatment on the Jatropha shells was much as expected. The acid pretreatment was more effective for the hydrolysis of hemicelluloses while the alkaline pretreatment was able to solubilize a greater portion of lignin. In both cases, more than 92% of glucan was maintained. It is also interesting to note that more than 96% of xylan was maintained after acid pretreatment and slightly more than 99% after alkaline pretreatment. The greatest difference was found in arabinan where 87.5% was recovered after acid pretreatment and only 23% recovered after alkaline pretreatment. When comparing with results from the pretreatment of cotton stalks with both sulfuric acid and sodium hydroxide, Silverstein et al. (2007) showed that sulfuric acid had a greater effect on xylan, completely hydrolyzing all xylan in the biomass. However, the authors used 2% H_2SO_4 instead of the 0.5% used for this study. Silverstein et al. (2007) also showed that 59.07% of solids were recovered after pretreatment with 1.0% NaOH at 121°C for 1 h. For the 0.5% H_2SO_4 pretreatment at 121°C for 1 h, 73.7% of the solids were recovered, nearly identical to the results obtained in this study. The pretreatment results are in accordance with Silverstein, Chen et al. (2007) who reported that alkaline pretreatments are more effective on agricultural residues than wood materials.

Pretreatment's effect on the shell's protein content was of little interest since the initial protein content was relatively low. However, in the case of Jatropha meal (cracked and defatted kernel), the correct pretreatment may not

only be used as a preparatory process for enzymatic hydrolysis, but also for the detoxification of the meal (Martínez-Herrera et al. 2006).

Ethanol yield from SSF after 48 hours was 6.07 g/L and 7.68 g/L in the washed samples treated with the simple cellulase loading for the acid and alkaline pretreatments respectively. For all SSF forms tested (washed or unwashed biomass and addition of xylanase enzymes), the samples treated with NaOH presented higher ethanol concentrations than those treated with H_2SO_4. The effects of the different pretreatment methods can be seen in Table 13.

Figure 15. SSF of the various treatments in a controlled environment (35°C and 150 rpm).

The SSF experiments were performed using 20 grams of pretreated material, both from the acid and alkaline pretreatments (Figure 15). It is important to note that the alkaline pretreatment was much more effective for the concentration of cellulose, as could be seen in Table 13. Comparison of the SSF results for the acid and alkaline pretreatments are shown in Figure 16 and Figure 17 where the WIS are washed and unwashed, respectively.

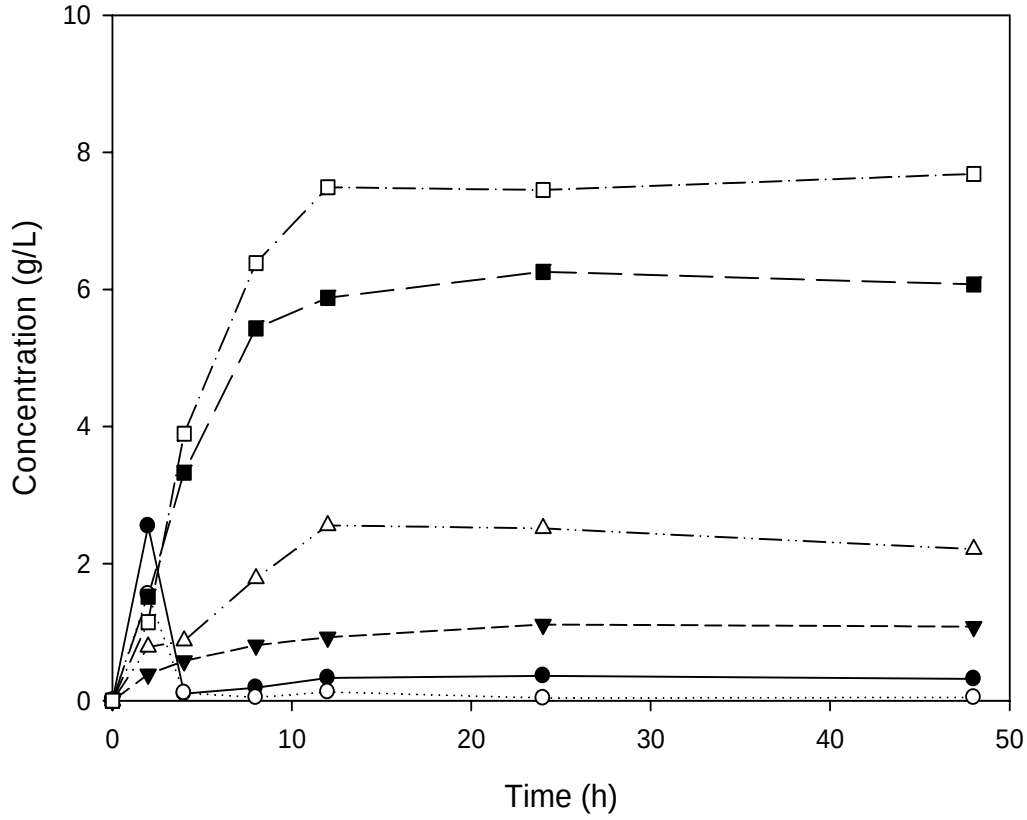

Figure 16. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the washed WIS. The solid symbols represent the dilute sulfuric acid pretreatment and the hollow symbols the dilute alkali pretreatment.

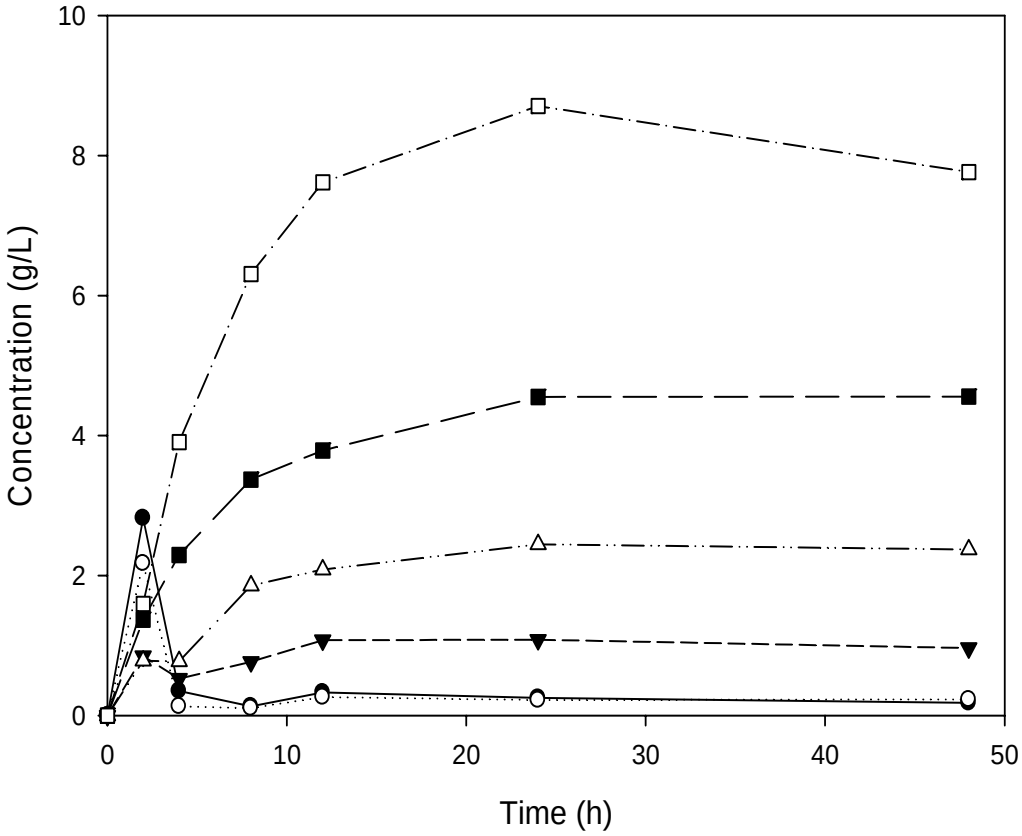

Figure 17. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the unwashed slurry with the addition of supplemental xylanase enzymes. The solid symbols represent the dilute sulfuric acid pretreatment and the hollow symbols the dilute alkali pretreatment.

The sodium hydroxide pretreatment proved to be of greater efficiency than sulfuric acid when the composition of the pretreated biomasses was not considered. The higher concentration of ethanol from the alkaline pretreatment is most likely due to the fact the alkaline pretreated biomass contained greater quantities of cellulose than the acid pretreated biomass.

When comparing the two pretreatment methods, the highest ethanol concentration (7.76 g/L) was reached when the alkaline pretreated was used, the WIS were not washed and supplemental xylanase enzymes were added. The lowest value (4.55 g/L) was encountered when using the same conditions, but acid pretreated biomass instead of alkaline. Efficiencies for cellulose (glucan)

conversion to ethanol were 36.55% and 31.6% for the acid and alkaline pretreated biomasses, respectively.

In only one case was the ethanol concentration greater when acid pretreatment was utilized. Figure 31 shows ethanol, xylose and glucose concentrations for the conditions of alkaline pretreatment and unwashed WIS. In this case, the ethanol concentration declined dramatically after 24 h of SSF. The results were consistent in both repetitions, but this phenomenon could not be explained.

5.3.3 Simultaneous saccharification and fermentation (SSF)

Two types of biomass slurries and two different enzyme loadings for SSF were considered in this study being: whole slurry, washed slurry, whole slurry with additional enzymes from *A. japonicus* and washed slurry with additional enzymes from *A. japonicus*. The DNS reducing sugar method with birchwood extract showed that the concentrated enzyme mixture from *A. japonicus* had a xylanase activity of 21.6 U/ml. Cellulase activity was disregarded since xylanase enzymes are much more prevalent from *A. japonicus* produced using sugarcane bagasse as a carbohydrate source. To the slurries containing the additional xylanase enzyes, 75 U were added (3.75 U/g of WIS).

Although lower than concentrations published in other works which used 2 to 5 g/L of dry yeast cells (Linde, Galbe, and Zacchi 2007; Linde et al. 2008), the yeast concentration of 0.8 g/L was sufficient to ferment all glucose present in the sample, proven by the minimal concentration of glucose.

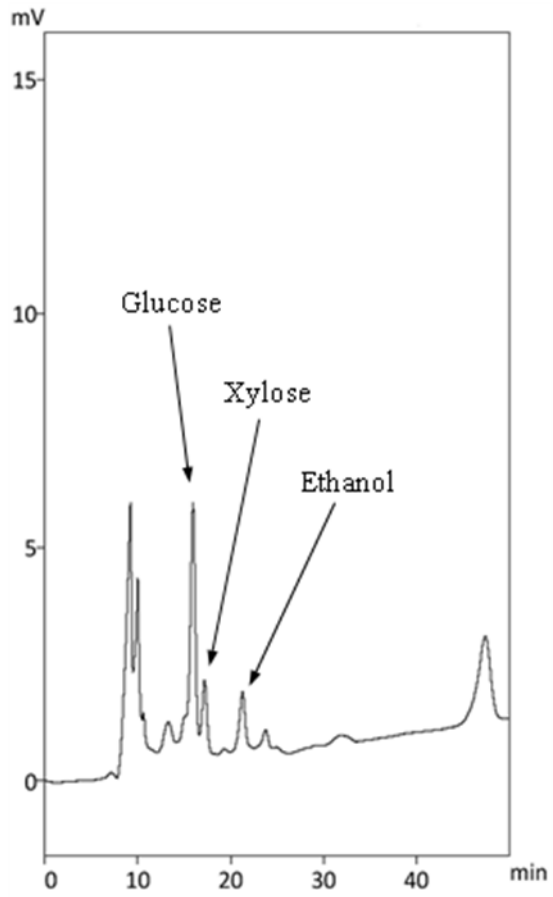

Figure 18. Chromatogram of the SSF solution containing NaOH pretreated and washed solids after 2 h

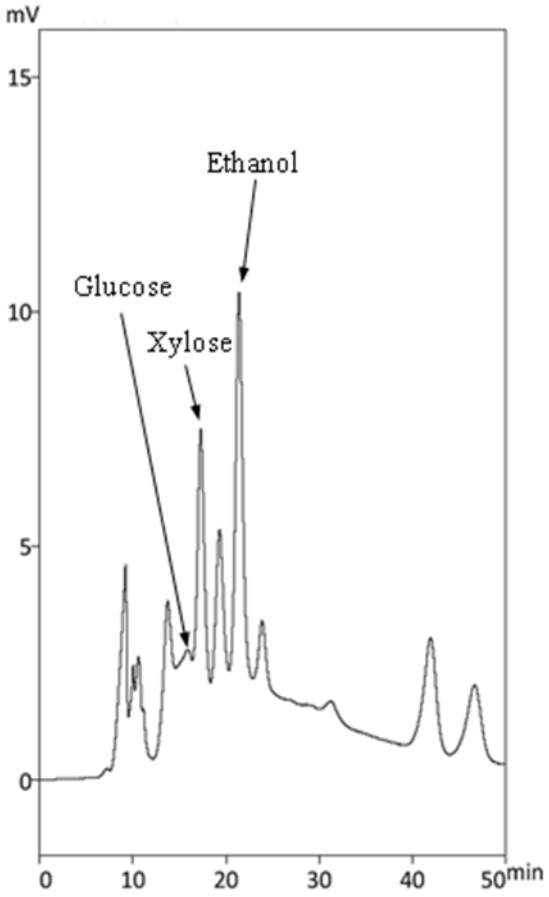

Figure 19. Chromatogram of the SSF solution containing NaOH pretreated and washed solids after 48 h

The chromatograms in Figure 18 and Figure 19 show the changes in sugar, ethanol and organic acids from 2 to 48 hours after beginning the SSF process. The retention times for glucose, xylose and ethanol were 15.8, 17.1 and 21.3 min, respectively. An increase was observed in ethanol and xylose concentrations while a drastic decrease of glucose was noticed. This was expected since *S. cerevisiae* is not capable of fermenting pentose sugars (xylose). Other compounds found with retention times of 19.4, 23.9 and 42.0 min were observed, but not specified.

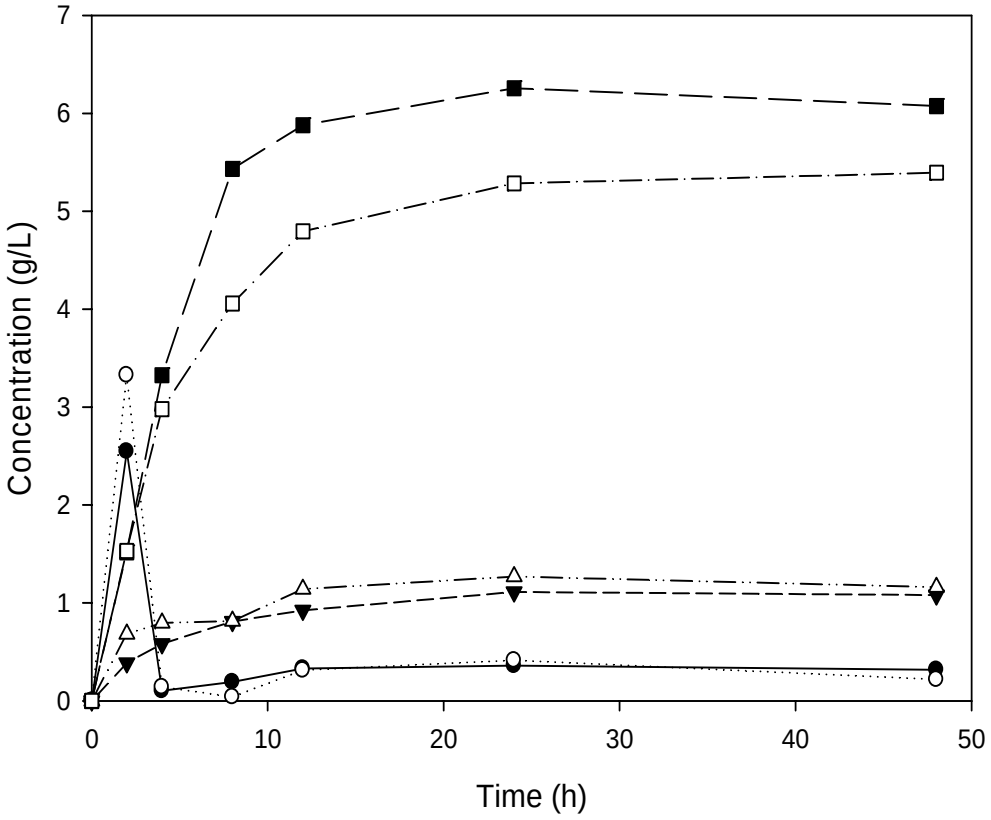

Figure 20. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF with the acid pretreated WIS. The solid symbols represent the washed WIS and the hollow symbols the unwashed slurry.

Figure 20 shows the difference between use of washed WIS and the original slurry containing the inhibitors produced during acid pretreatment. As expected, the washed WIS showed greater potential for ethanol production, but requires greater amounts of energy to wash the biomass. A complete economic analysis of the washing process is necessary to determine if the increases in SSF potential are worth the costs of washing the WIS.

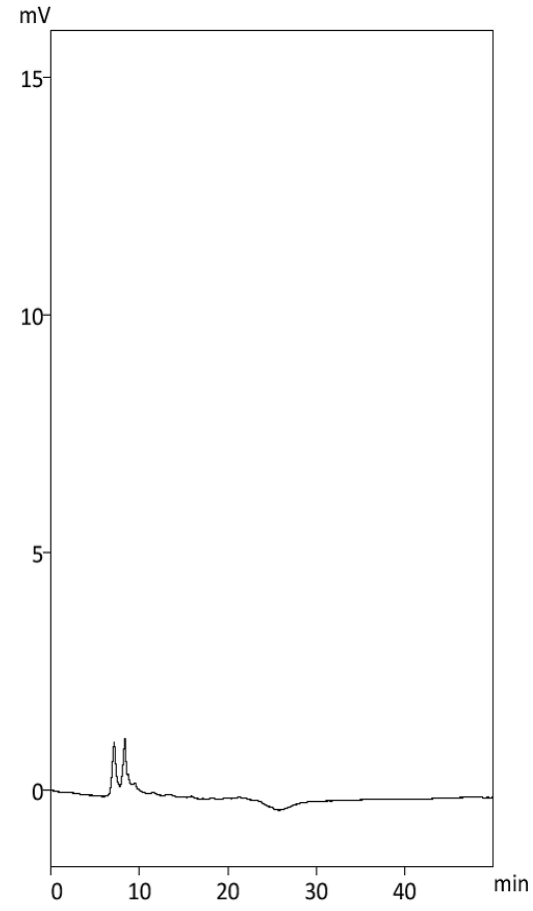

Figure 21. Chromatogram of the SSF solution containing H_2SO_4 pretreated and washed solids after 0 h.

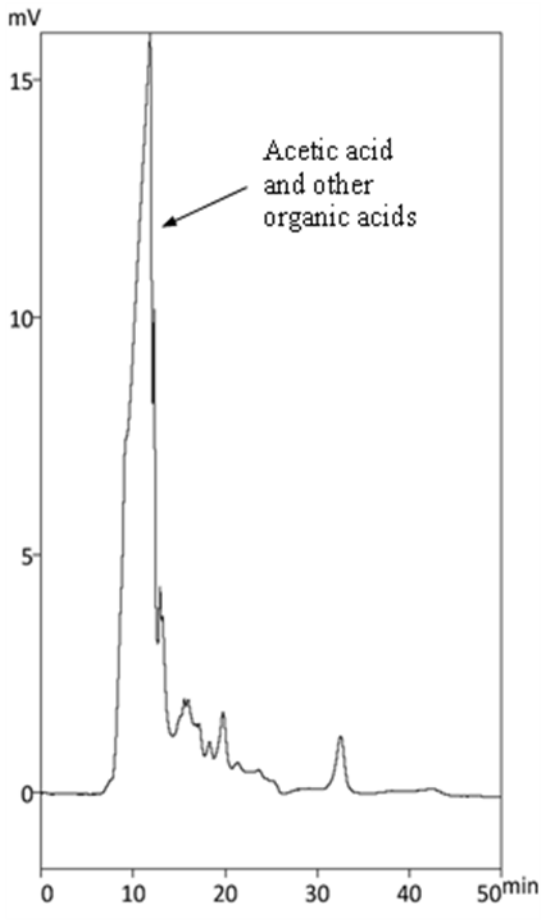

Figure 22. Chromatogram of the SSF solution containing the unwashed slurry of H_2SO_4 pretreated solids after 0 h.

The influence of washing the pretreated solids can be not only from the graphs in Figure 20 and Figure 23, but the presence of inhibitors formed can be seen when comparing Figure 21 to Figure 22. Tests for acetic acid were determined, but the chromatography column used is not suitable for the quantification of organic acids. Pure acetic acid produced a peak much like that found at 11.8 min in Figure 22. It was determined that organic acids were the main inhibitor to this process since no furfural was detected during this time. The fact that no furfural (byproduct of hemicellulose acid hydrolysis) was found signified that the sulfuric acid concentration was quite low or that the pretreatment time was short. Although the objective is was to pretreat the biomass without generating large quantities of inhibitors, the fact that no furfural

was encountered may suggest that higher acid concentrations or longer pretreatment retention times are necessary to improve process efficiency.

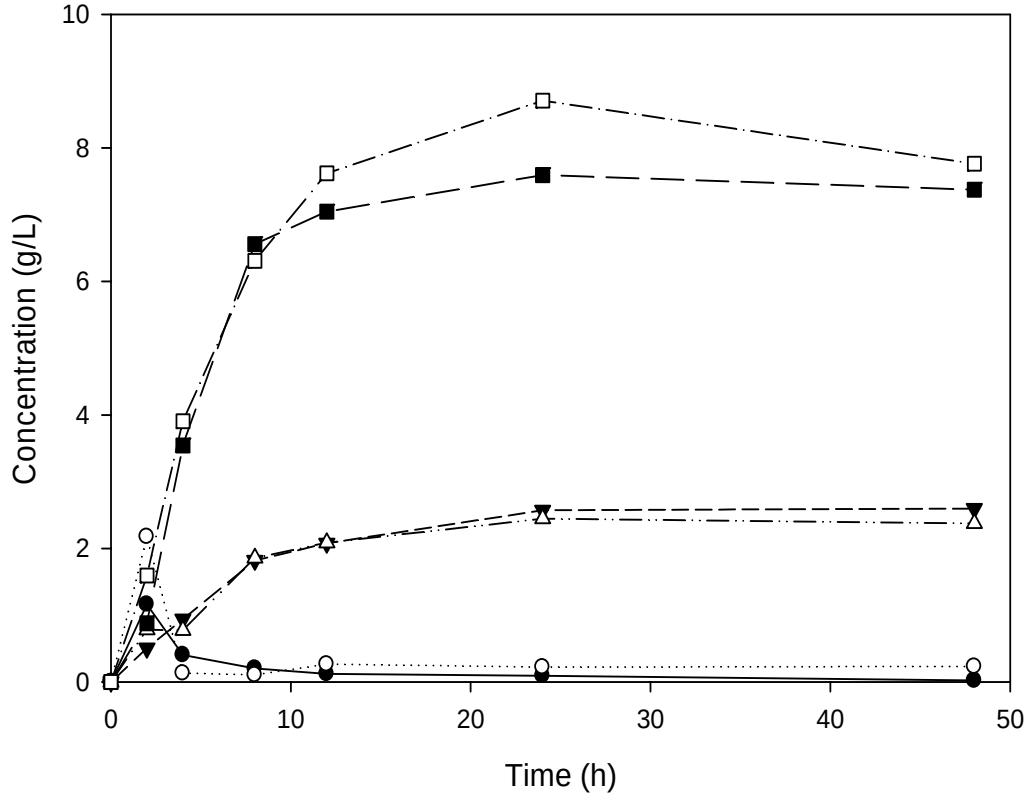

Figure 23. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF with the alkaline pretreated WIS and supplemented with xylanase enzymes. The solid symbols represent the washed WIS and the hollow symbols the unwashed slurry.

From Figure 23 it can be seen that there was very little difference in xylose production when comparing the washed and unwashed WIS. However, in contradiction to the results shown in Figure 20, ethanol yield was slightly higher when the unwashed slurry was used for SSF. It can be assumed that there were fewer inhibitors to hydrolysis and fermentation in the NaOH pretreatment in comparison with that of the acid pretreatment. The small quantity of sugars produced during pretreatment was also maintained when the solids were not washed. After 48 h, the ethanol concentrations for the washed and unwashed

WIS were 7.37 and 7.76 g/L, respectively. These values corresponded to 34.72 and 36.55% conversion of glucan to ethanol.

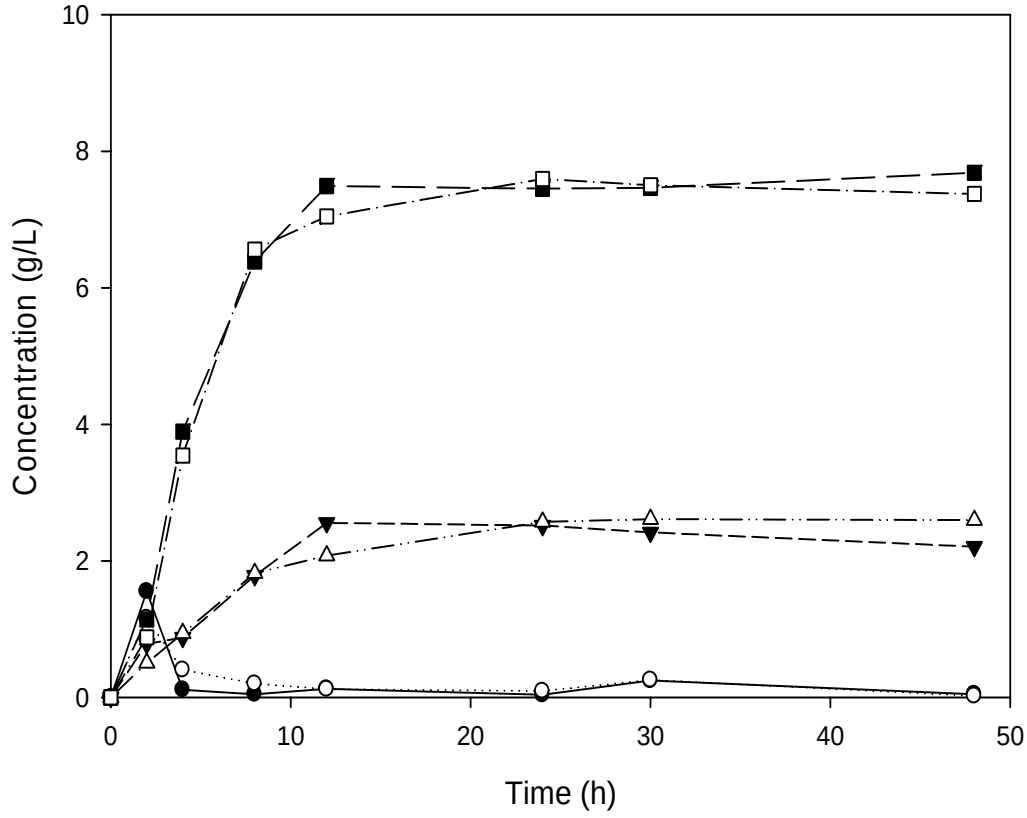

Figure 24. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF in the unwashed slurry with alkaline pretreated WIS. The solid symbols represent pure cellulase loading and the hollow symbols represent the mixture of cellulase and xylanase enzymes.

The supplementation with xylanase enzymes precipitated from *A. japonicus* had no effect on the production of ethanol or xylose, as can be seen in Figure 24. No increase in ethanol concentration was expected since *S. cerevisiae* is not capable of fermenting pentose sugars, however, the fact that no difference was seen in the xylose concentration indicates that the xylanase activity in the purchased cellulase solution was significant.

The highest efficiency for glucan conversion to ethanol was from the acid pretreatment with washed WIS. A yield of 6.07 g/L was obtained, corresponding to an efficiency of 42.17%. In another study, treatment of wheat straw with

concentrated gave only 49% conversion of total carbohydrates to sugars (Saha et al. 2005). Xylan hydrolysis showed even poorer efficiencies of approximately 20% and other sugars produced were not measured.

Repeatability of the SSF process is shown in Figure 25 to Figure 32, where the standard deviation is represented by the error bars on the graph. All results showed relatively good repeatability considering the difficulty in maintaining the content of WIS equal in all treatments without drying the samples.

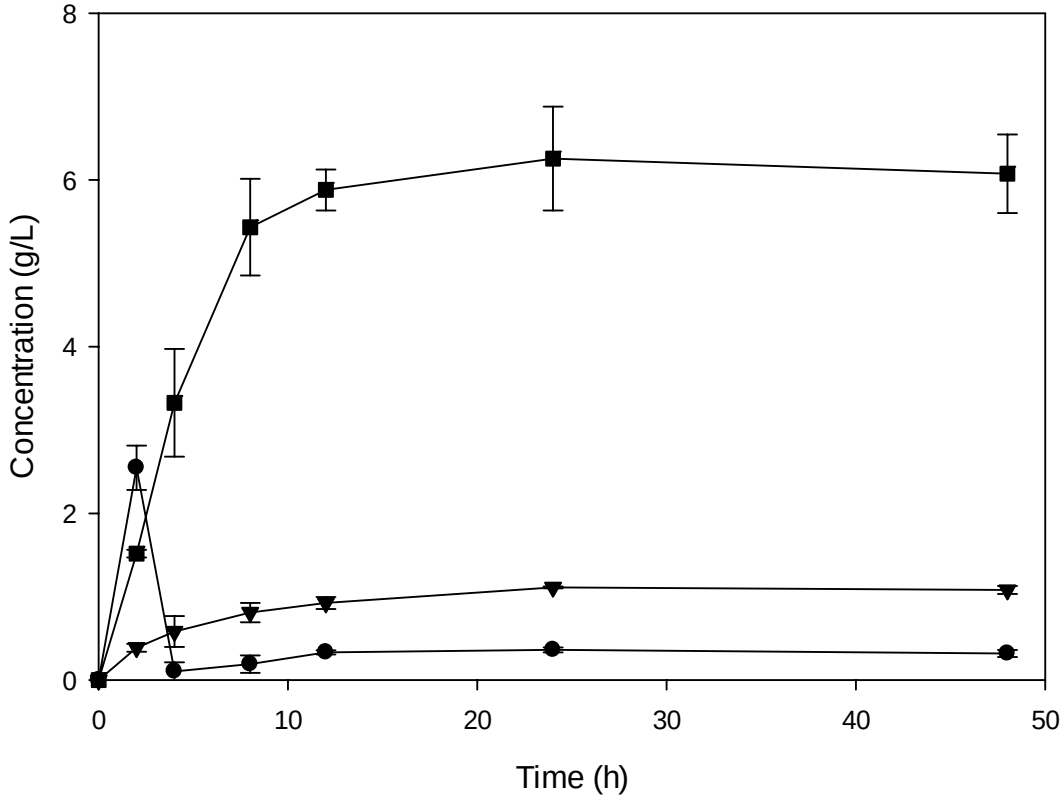

Figure 25. Glucose (●), xylose (▼) and ethanol (■)concentrations during SSF of the washed WIS, pretreated with dilute acid.

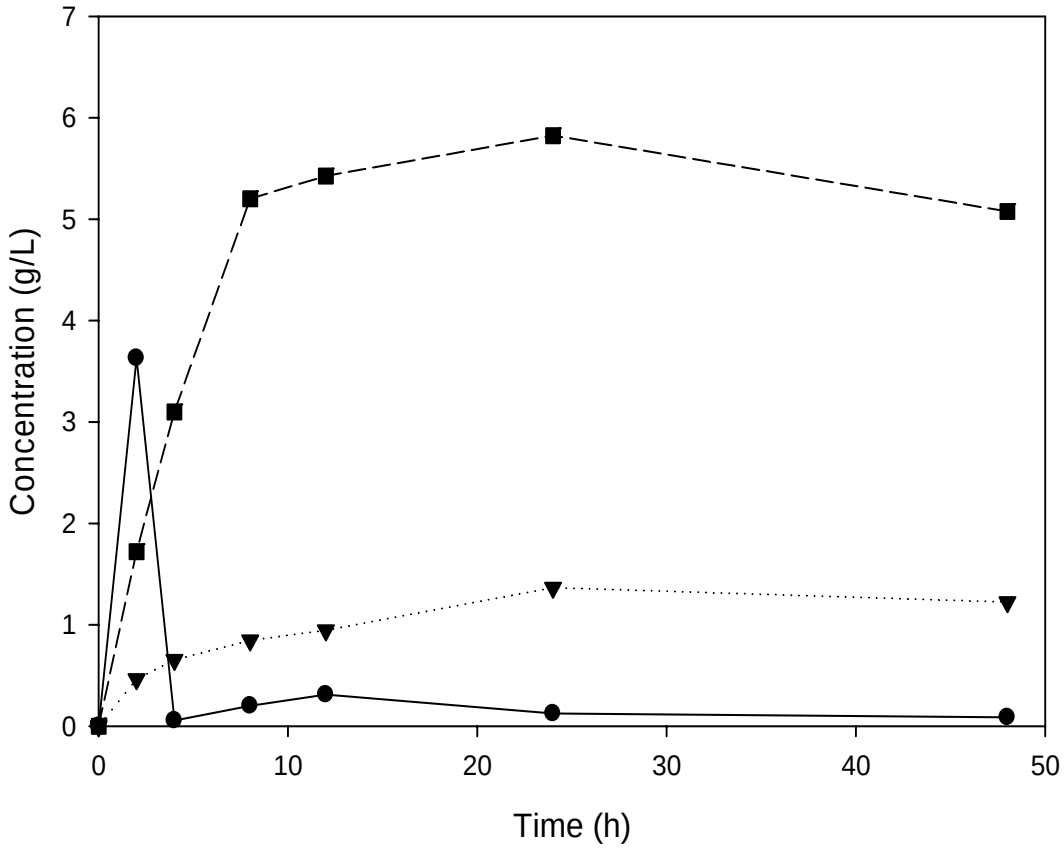

Figure 26. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the washed WIS and addition of supplemental xylanase, pretreated with dilute acid.

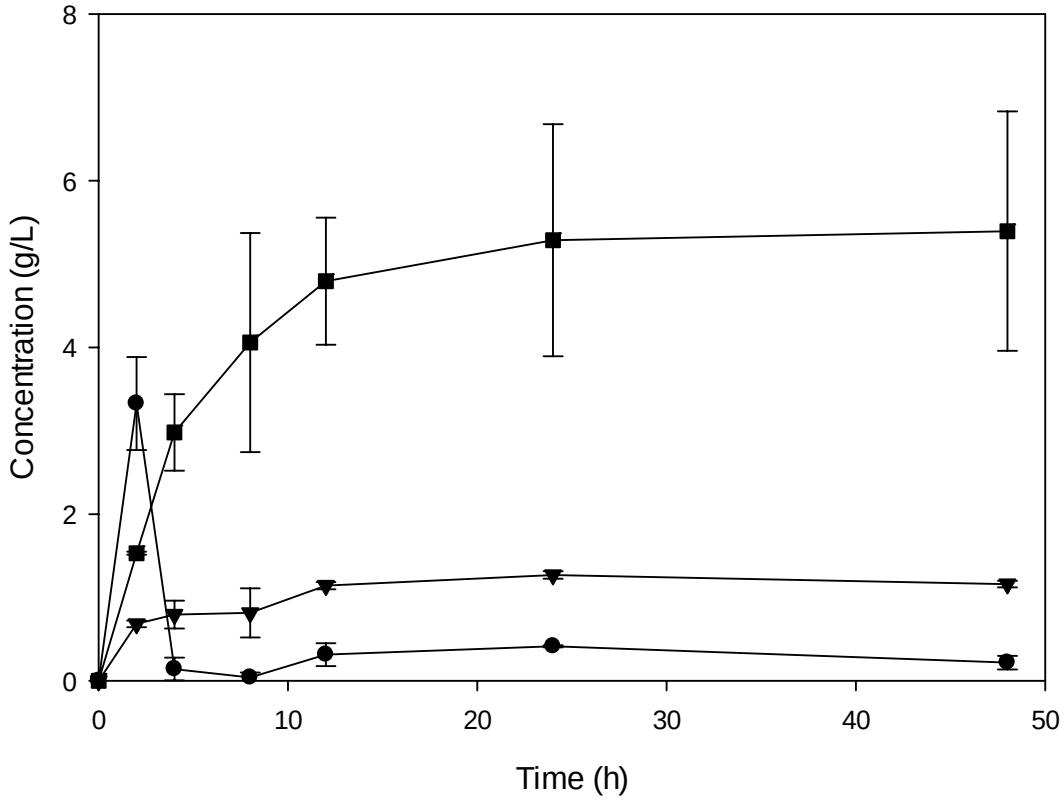

Figure 27. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the unwashed slurry, pretreated with dilute acid.

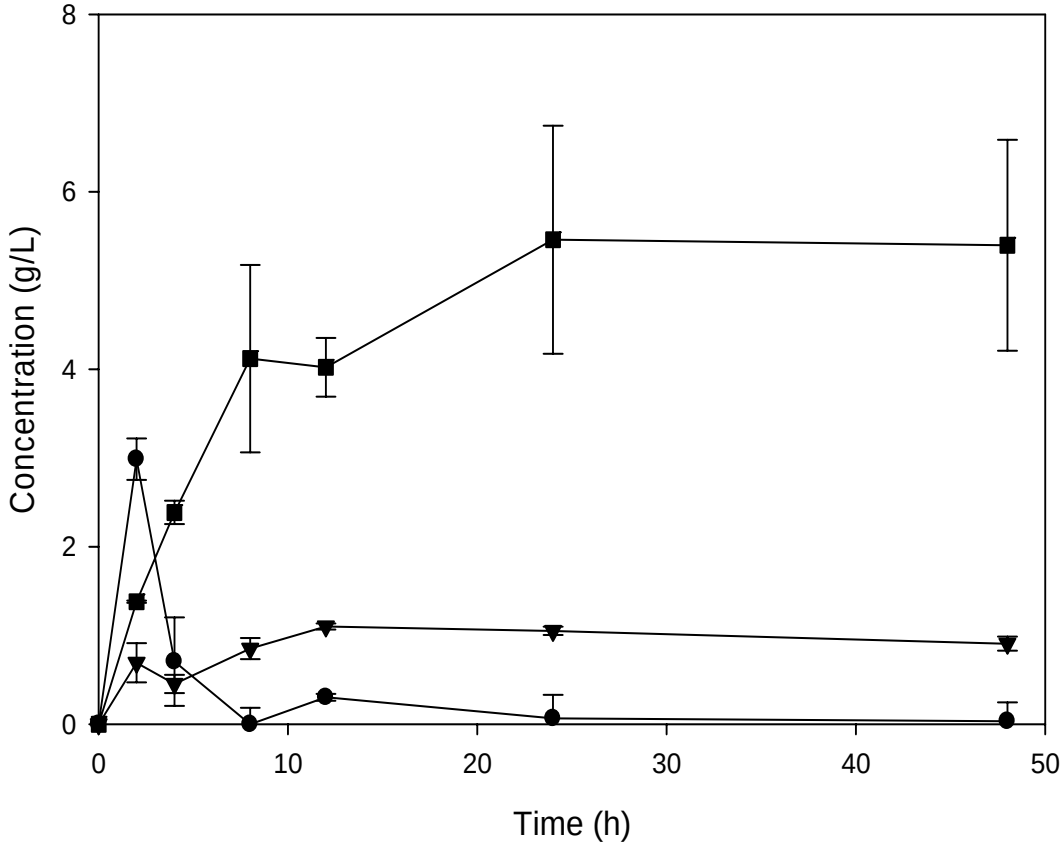

Figure 28. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the unwashed slurry and supplemented with additional xylanases, pretreated with dilute acid.

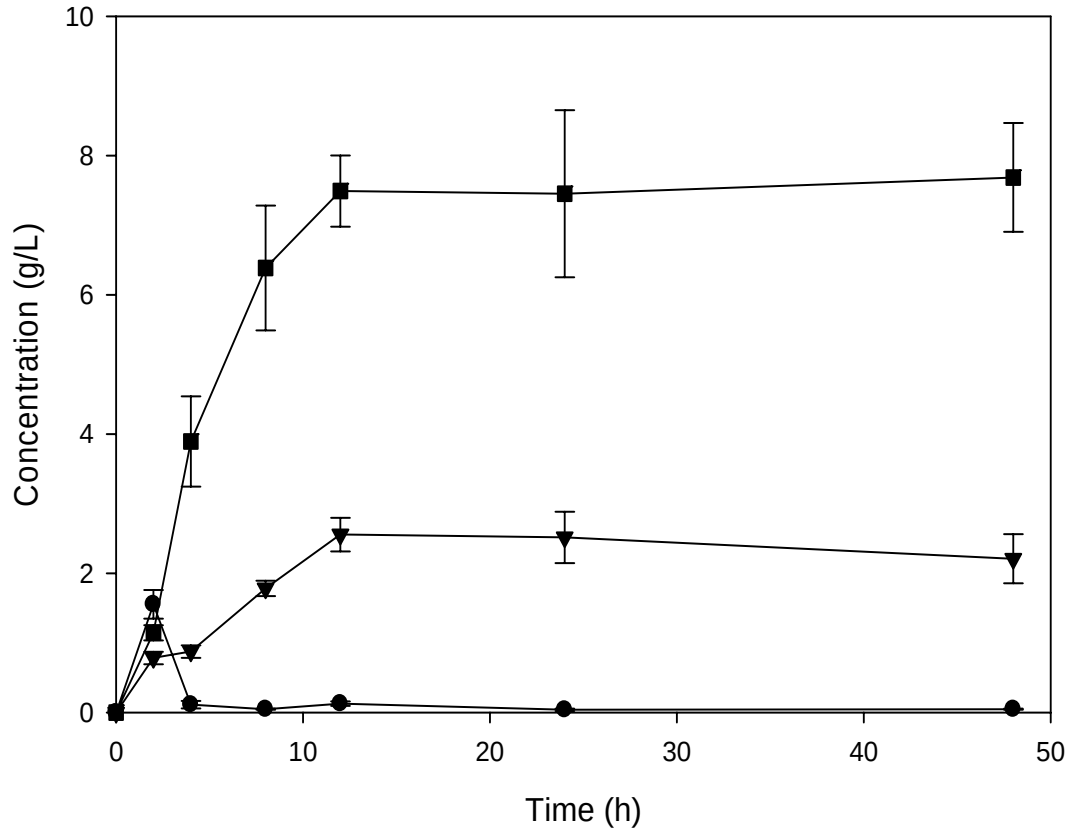

Figure 29. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the washed WIS, pretreated with NaOH.

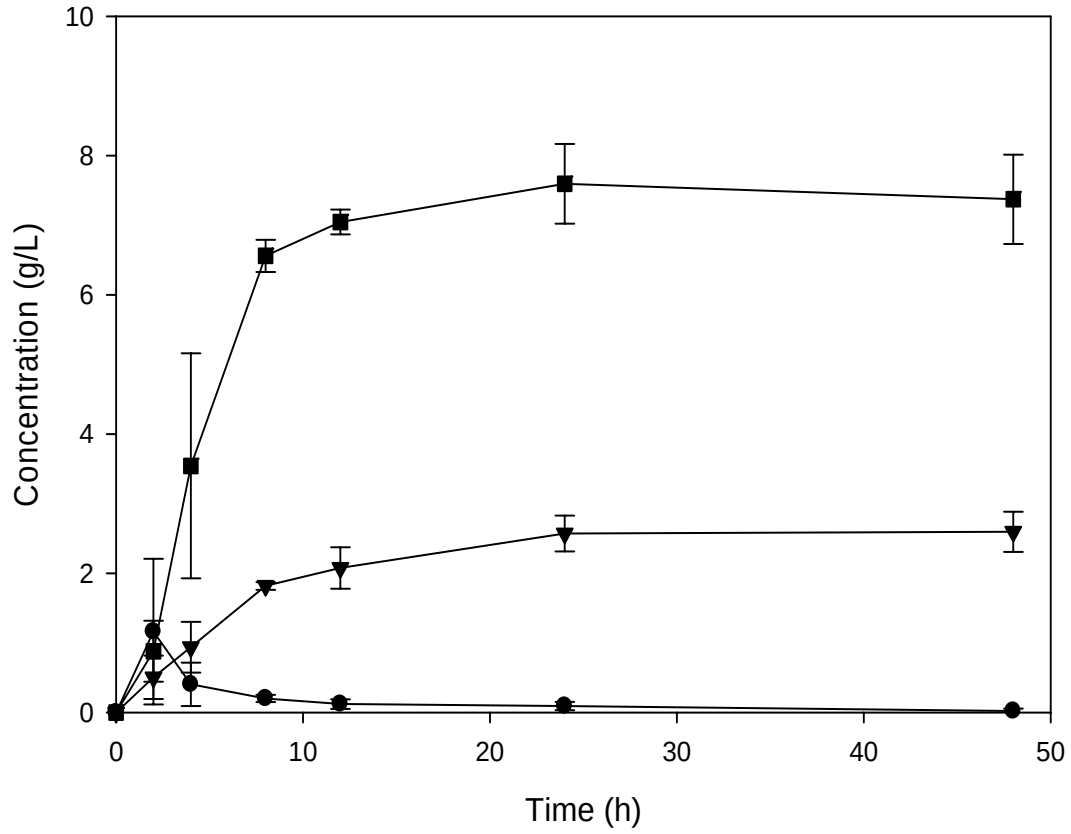

Figure 30. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the washed WIS and supplemented with additional xylanases, pretreated with NaOH.

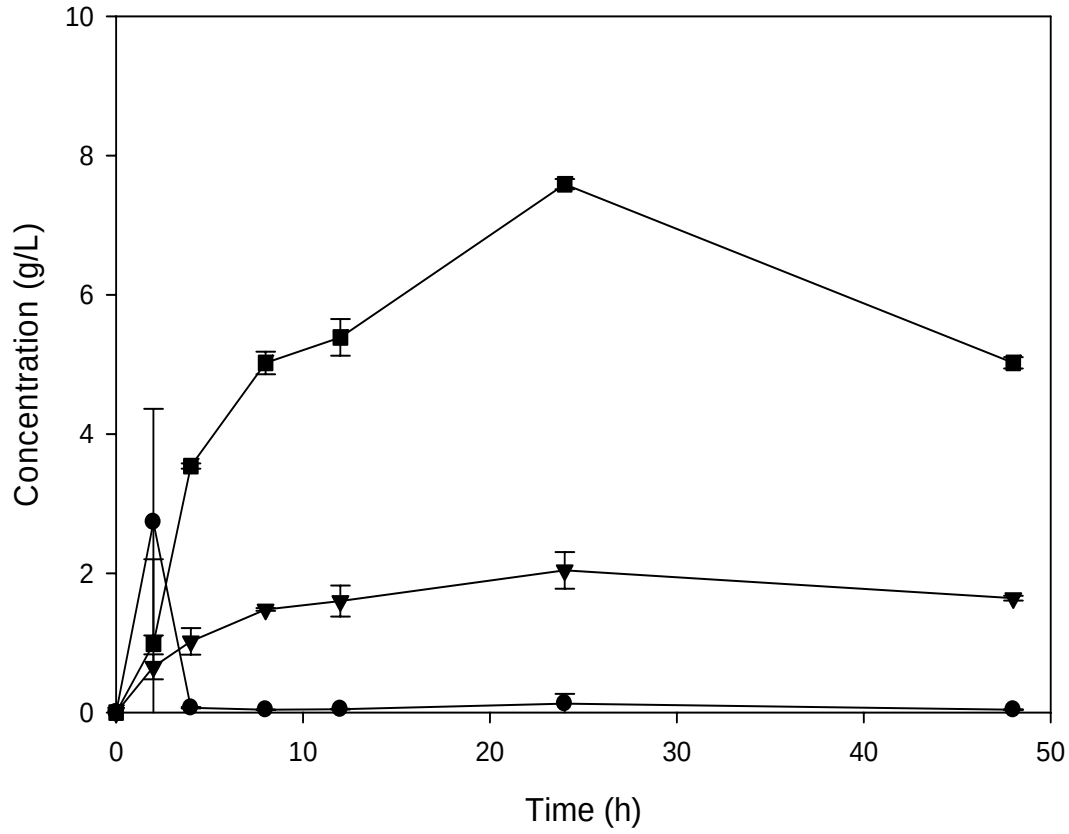

Figure 31. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the unwashed slurry, pretreated with NaOH.

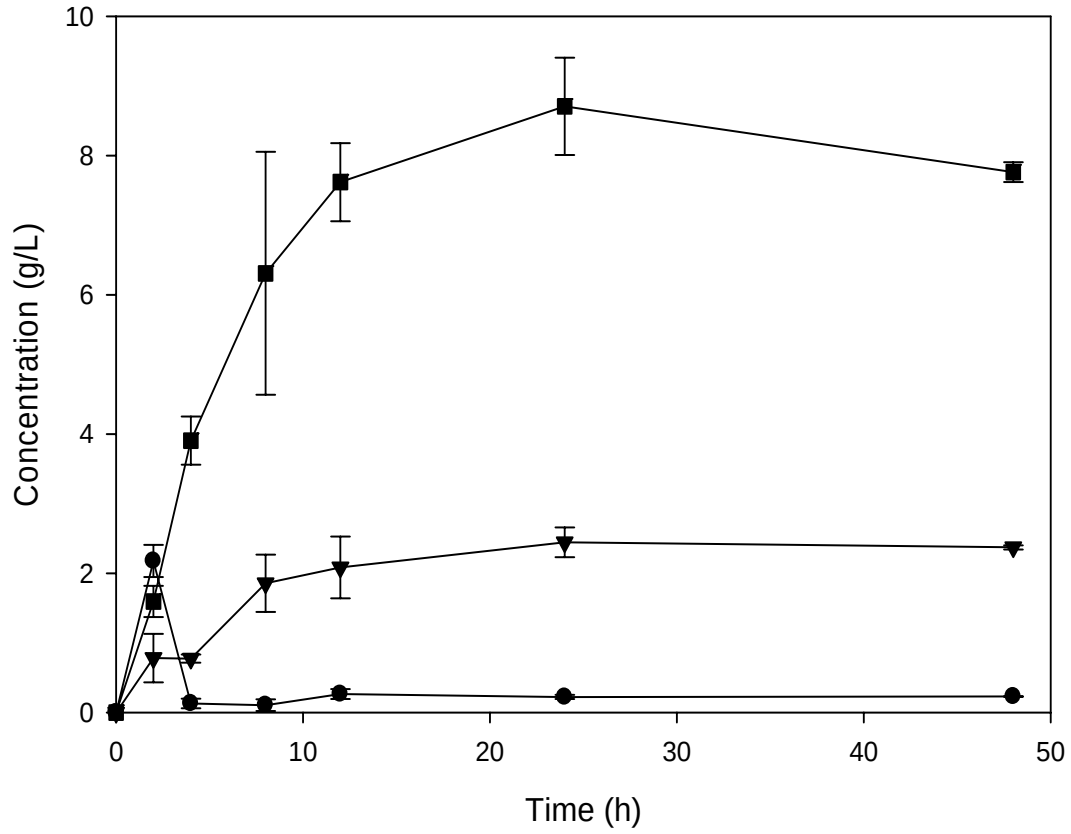

Figure 32. Glucose (●), xylose (▼) and ethanol (■) concentrations during SSF of the unwashed slurry and supplemented with additional xylanases, pretreated with NaOH.

5.4. CONCLUSIONS

This work showed the technical feasibility of ethanol production from Jatropha shells. No other works related to this have been published so this article can be used as a starting point for the improvement of this process. In order to improve this process, each of the individual steps needs to be reevaluated (pretreatment, hydrolysis and fermentation). Although the chemical pretreatment proved to significantly increase the glucan content in the biomass, the enzymatic hydrolysis results show that the use of highly diluted H_2SO_4 and NaOH may not have been sufficient to adequately expose the biomass for enzymatic activities. It is possible that the composition of Jatropha shells require concentrated acid

pretreated, higher temperatures or longer exposure to both acid and elevated temperatures.

The low efficiencies were the effect of poor enzymatic hydrolysis since all glucose produces was consumed by the yeast. This may be due to the use of inadequate enzymes, inadequate pretreatment techniques or another of the multiple variables which could be modified. It is also possible that another compost inhibited enzymatic activity. Since no other studies are encountered related to the use of vegetable oil extraction coproducts as biomass sources for the production of bioethanol via enzymatic hydrolysis, inhibitors which are not found in traditional biomasses and agricultural residues may have been the cause of the poor efficiency in the studied process.

In this experiment, if the chemical composition of the biomass was measured after saccharification and fermentation, the amount of cellulose successfully converted to glucose could be determined by the difference in glucan concentrations. This would in turn also allow for calculation of the yeast´s efficiency to ferment the produced glucose and determine if ethanol respiration was responsible for diminishing ethanol yields.

Many other works show ethanol concentrations between 10 and to 20 g/L (Kuo and Lee 2008). The majority of these studies look to optimize the conversion of cellulose and hemicellulose to ethanol and often the final ethanol concentration is not the main objective. These low ethanol concentrations, however, reduce the economic viability of the process due to the large amount of water which must be evaporated during distillation, greatly increasing energy demands of the process.

5.5. REFERENCES

Abou Kheira, A. A., and N. M. M. Atta. 2008. Response of Jatropha curcas L. to water deficit: Yield, water use efficiency and oilseed characteristics. *Biomass and Bioenergy* In Press.

Adsul, M. G., J. E. Ghule, H. Shaikh, R. Singh, K. B. Bastawde, D. V. Gokhale, and A. J. Varma. 2005. Enzymatic hydrolysis of delignified bagasse polysaccharides. *Carbohydrate Polymers* 62 (1):6-10.

Agência Nacional de Petroleo. 2007. Authorized capacity of biodiesel production plants http://www.anp.gov.br/biocombustiveis/capacidade_plantas.asp. accessed: September 28, 2008

CETEC. 2006. Composição Química da Torta de Pinhão-Manso. www.PinhaoManso.com.br. accessed: September 30, 2008

Chandel, A. K., R. K. Kapoor, A. Singh, and R. C. Kuhad. 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. *Bioresource Technology* 98 (10):1947-1950.

Eggeman, T., and R. T. Elander. 2005. Process and economic analysis of pretreatment technologies. *Bioresource Technology* 96 (18):2019-2025.

Evangelista, A. R., J. Gonçalves de Abreu, A. J. Peron, A. C. Fraga, and P. C. Neto. 2004. Avaliação da composição química de tortas de mamona e amendoim obtidas por diferentes métodos de extração de óleo. Paper read at I Congresso Brasileiro de Mamona, at Campina Grande - PB.

Ghose, T.K. 1987. Measurement of cellulase activities. *Pure and applied chemistry* 59 (2):257-268.

Karimi, K., G. Emtiazi, and M. J. Taherzadeh. 2006. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae. *Enzyme and Microbial Technology* 40 (1):138-144.

Kim, S., and B. E. Dale. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy* 26 (4):361-375.

Kuo, C., and C. Lee. 2008. Enhanced enzymatic hydrolysis of sugarcane bagasse by N-methylmorpholine-N-oxide pretreatment. *Bioresource Technology* In Press.

Linde, M., M. Galbe, and G. Zacchi. 2007. Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. *Enzyme and Microbial Technology* 40 (5):1100-1107.

Linde, M., E. Jakobsson, M. Galbe, and G. Zacchi. 2008. Steam pretreatment of dilute H_2SO_4 impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. *Biomass and Bioenergy* 32 (4):326-332.

Martínez-Herrera, J., P. Siddhuraju, G. Francis, G. Dávila-Ortíz, and K. Becker. 2006. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of Jatropha curcas L. from Mexico. *Food Chemistry* 96 (1):80-89.

Ministério de Minas e Energia. 2006. Balanço Energético Nacional (Brazilian National Energy Balance).

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96 (6):673-686.

Öhgren, K., R. Bura, G. Lesnicki, J. Saddler, and G. Zacchi. 2007. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry* 42 (5):834-839.

Öhgren, K., J. Vehmaanperä, M. Siika-Aho, M. Galbe, L. Viikari, and G. Zacchi. 2007. High temperature enzymatic prehydrolysis prior to simultaneous saccharification and fermentation of steam pretreated corn stover for ethanol production. *Enzyme and Microbial Technology* 40 (4):607-613.

Openshaw, K.. 2000. A review of Jatropha curcas: an oil plant of unfulfilled promise. *Biomass and Bioenergy* 19 (1):1-15.

Saha, B. C., and M. A. Cotta. 2008. Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. *Biomass and Bioenergy* In Press, Corrected Proof.

Saha, B. C., L. B. Iten, M. A. Cotta, and Y. V. Wu. 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochemistry* 40 (12):3693-3700.

Silverstein, R. A., Y. Chen, R. R. Sharma-Shivappa, M. D. Boyette, and J. Osborne. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology* 98 (16):3000-3011.

Sims, R. E. H., A. Hastings, B. Schlamadinger, G. Taylor, and P. Smith. 2006. Energy crops: current status and future prospects. *Global Change Biology* 12 (11):2054-2076.

Tengborg, C., M. Galbe, and G. Zacchi. 2001. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. *Enzyme and Microbial Technology* 28 (9-10):835-844.

US Department of Energy. 2006. Theoretical Ethanol Yield Calculator 2006. http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html. accessed: September 15, 2008

Vicente, M. .A. , L. G. Fietto, I. .M. Castro, A. N. Gonçalves dos Santos, M. X. Coutrim, and R. L. Brandão. 2006. Isolation of Saccharomyces cerevisiae strains producing higher levels of flavoring compounds for production of "cachaça" the Brazilian sugarcane spirit. *International Journal of Food Microbiology* 108 (1):51-59.

Chapter 6

GENERAL CONCLUSION AND SUGGESTIONS FOR FUTURE STUDIES

6.1. INTRODUCTION

This study was developed with the goal of producing ethanol from sources of lignocellulosic biomass, in particular, coproducts from the oil extraction of *Jatropha curcas*. Initially, various oil crops were studied in order to determine their potential as both sources of vegetable oil for biodiesel and non-starch polysaccharides for the production of bioethanol from lignocellulosic material. The African palm proved to be the greatest producer of biodiesel per hectare as well as have the greatest potential for bioethanol production due to the large amount of biomass produced per area and its high fiber content. Jatropha was the second most productive both in terms of biodiesel and bioethanol production, but it was decided that Jatropha would be the material studied due to its growing acceptance as a vegetable oil producer in the region of Minas Gerais.

Cellulase enzymes were used for the experimental study of cellulose enzymatic hydrolysis. Different ethanol production techniques were studied and compared, being:

i. separate hydrolysis of Jatropha meal, comparing H_2SO_4, NaOH and Lime pretreatments with and untreated sample (Chapter 4)
ii. simultaneous saccharification and fermentation of Jatropha shells, comparing H_2SO_4 and NaOH pretreatments (Chapter 5)
iii. simultaneous saccharification and fermentation of Jatropha shells, comparing the influence of inhibitors produced during pretreatment (Chapter 5)
iv. simultaneous saccharification and fermentation of Jatropha shells, different enzyme loadings (addition of xylanase) (Chapter 5)

The chemical composition of both the Jatropha meal (leftover biomass after oil extraction) and the Jatropha shells (removed before oil extraction) were determined with the objective of quantifying structural carbohydrates.

In chapter 4, where Jatropha meal was evaluated as a carbohydrate source for bioethanol production, glucan was found to consist of only 11.6% of the total mass, an extremely low percentage if considering that only ethanol is produced.

However, if digestibility improves significantly after hydrolysis of the long polysaccharide chains, the economic feasibility improves significantly. When comparing the three pretreatments, NaOH was the most effective for the concentration of glucan and xylan, but had a negative effect on the protein content. After hydrolysis of the pretreated Jatropha meal, the two alkaline pretreatments showed to be more effective than the acid pretreatment when comparing the concentration of produced glucose and xylose by HPLC. When DNS was used for the comparison of reducing sugars, the acid pretreatment showed greater sugar concentrations. Knowing that acids are more effective for the hydrolysis of hemicelluloses, it was likely that a greater portion of hemicelluloses was hydrolyzed into various pentose sugars (xylose, arabinose, ect.), causing the higher reducing sugar concentration for acid pretreated solids in comparison with alkaline treated biomass. After 24 hours, sugar concentrations averaged roughly 5 g/L for the three pretreatments. If theoretical fermentation of 0.51 g ethanol/g monomeric sugar is assumed, ethanol concentrations will not surpass 2.5 g/L (3.15%).

Simultaneous saccharification and fermentation of Jatropha shells, evaluated in chapter 5, was capable of producing 7 to 8 g/L of ethanol even when using pretreated solids loading of 7.5% (w/v). The Jatropha shell has a much higher content of glucan (26.5%) and xylan (6.1%), much more suitable for bioethanol production. Once again, the NaOH proved to be much more suitable for concentrating cellulose during pretreatment, increasing the percentage of glucan to nearly 200% its previous value. When comparing the overall SSF process, glucan to ethanol efficiency was greater when the material was pretreated with acid than with the alkaline (42.17% to 34.72%). All glucose was converted into ethanol, as could be seen in the graphs relating ethanol, glucose and xylose production to SSF time.

In all cases, it was shown that Jatropha biomass can be used for the production of bioethanol from cellulose. In the case of the Jatropha meal, some nutritional gains or the desintoxication of the meal during this process is necessary to make the process economically feasible. Starch should also be

quantified to determine if amylase enzymes could further improve ethanol yields. Jatropha shells showed the greatest potential for bioethanol production, but further studies must be done to improve the hydrolysis efficiencies. An economic analysis is also necessary to determine if this process can be applied today.

6.2. SUGGESTIONS FOR FUTURE STUDIES

The following suggestions are proposed for further studies in this field:

i. Prepare a more detailed analysis of the chemical composition of the Jatropha meal and shell, being sure to include values for starch and toxins. Starch can also be used for enzymatic hydrolysis, increasing ethanol yields;
ii. Utilize a deashing guard column to be coupled with the adequate monosaccharide detection column. This will help to maintain the baseline straight, therefore making HPLC readings more accurate and eliminating the masking of detected substances;
iii. Prepare an economic analysis of the overall process;
iv. Improve the hydrolysis efficiency by testing different enzymatic hydrolysis conditions. Small changes in temperature, pH and solid concentration make significant differences in the efficiency of the overall process;
v. Test enzymatic hydrolysis efficiencies using a commercial enzyme cocktail. A commercial enzyme cocktail often contains various enzymes capable of hydrolyzing hemicelluloses. A microorganism capable of fermenting these pentose created is then needed for their fermentation into ethanol;
vi. Determine the chemical composition of the Jatropha shells after enzymatic hydrolysis, allowing for the calculation of the cellulose conversion percentage.