EVAN MICHAEL VISSER

BIOETHANOL PRODUCTION UTILIZING FUNGAL ENZYME EXTRACTS AND DIFFERENT PROCESSING METHODS

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Agricultural Biochemistry Program, for the attainment of the title *Doctor Scientiae*

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Get all the advice and instruction you can, so you will be wise the rest of your life.

~ Proverbs 19:20

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 December of 2008 he acquired a Master's degree in Agricultural Engineering from the Federal University of Viçosa and in August of 2009 he initiated his Doctorate studies in Biochemistry, obtaining the title of Doctor in July of 2013.

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RESUMO

VISSER, Evan Michael, D.Sc., Universidade Federal de Viçosa, Julho de 2013. **A produção de bioetanol utilizando enzimas fúngicas hidrolíticas e diferentes métodos de processamento.** Orientadora: Valéria Monteze Guimarães. Co-orientadores: Sebastião Tavares de Rezende and Márcio Arêdes Martins.

A produção em escala industrial de bioetanol a partir de fontes lignocelulósicas ainda está em suas fases iniciais, principalmente devido aos altos custos de processamento e, mais especificamente, aos custos elevados de complexos enzimáticos comerciais. No processamento de cana convencional, cerca de 50% do bagaço é queimado para produção de calor, no entanto, quando se considera a conversão de bagaço para etanol, 55% do bagaço pode ser processado para a produção de etanol de segunda geração, enquanto os outros 45% de bagaço fresco e lignina resultantes da hidrólise enzimática são suficientes para alimentar a usina de etanol convencional e também fornecer energia para o processamento/destilação de etanol derivado de lignocelulose. A levedura geneticamente modificada *S. cerevisiae* YRH400 mostrou-se mais eficiente para fermentação de glicose e xilose em etanol do que *K. marxianus* ATCC-8554 e *K. marxianus* UFV-3. A hidrólise enzimática foi realizada em bagaço de cana, inicialmente submetido ao pré-tratamento alcalino, usando um extrato de enzima obtida a partir de *Chrysoporthe cubensis*, produzida pela fermentação em estado sólido. As condições de hidrólise foram temperatura de 50°C e concentração de sólidos de 7.5% (m/v). Este resultado foi comparado a hidrólise e fermentação híbrido (48 e 12 horas de pré-hidrólise), utilizando as leveduras *S. cerevisiae* YRH400 e *K. marxianus* ATCC-8554 sob temperaturas de fermentação de 30 e 40 °C, respectivamente. Hidrólise e fermentação separados pareceu ser mais eficiente, conseguindo converter glicose e

xilose com eficiência de 45 e 97%, respectivamente, no caso de utilização de uma carga enzimática de 10 FPU/g de biomassa pré-tratada, durante um período de 120 horas de hidrólise. Uma combinação de extratos enzimáticos apresentando atividades complementares foi realizada para se obter um complexo enzimático mais completo. Estudos anteriores indicaram que *Chrysoporthe cubensis* é um bom produtor de β-glicosidase, xilanase e outras enzimas acessórias, enquanto que o extrato de *Penicillium pinophilum* é rico em celulases (FPases, endoglucanases e celobiohidrolases). O sinergismo máximo foi observado, entre estes dois extratos, quando misturado na proporção de 50:50, apresentando valores de sinergismo de 76%, 50% e 24% para as atividades de FPase, endoglucanase e xilanase, respectivamente. Este extrato enzimático misturado foi então aplicado na hidrólise do bagaço de cana, submetido a um processo de pré-tratamento alcalino com diferentes cargas enzimáticas e concentrações de biomassa a 45 °C. A conversãoF máxima de glicose e xilose (64% e 93%, respectivamente) foi obtida para o tratamento com a carga enzimática de 20 FPU/g e concentração de sólidos de 8%. Um outro ensaio foi realizado utilizando uma temperatura de reação de 50 °C. À temperatura mais alta, foram obtidos aumentos de 16% e 20% em relação às conversões para glicose e xilose, respectivamente. Além disso, para o tratamento realizado à 50 °C, a taxa de hidrólise foi quase constante após 120 horas, enquanto a taxa de hidrólise dos dois ensaios realizados a 45 °C diminuíram significativamente (0.14 g/l/h at 50°C and 0.10 g/l/h at 45 °C). Neste último experimento, a hidrólise enzimática foi realizada à 50 °C após períodos predeterminados de tempo, a fração sólida foi reciclada na tentativa de reciclar enzimas aderidas à biomassa sólida. Verificou-se que, quando se adiciona 1x (o mesmo valor), 1/2x ou nenhuma enzima durante o segundo período de hidrólise, a mesma quantidade de glicose foi produzida, indicando que as enzimas foram eficientemente recicladas. No entanto, quando adicionado a mesma quantidade de biomassa (8 ou 12% m/v), durante cada período de reciclagem, a concentração de sólidos aumentou e diminuiu significativamente a eficiência de hidrólise. No caso em que a hidrólise foi monitorada continuamente e a concentração de sólidos foi mantida constante (12%), a eficiência da hidrólise de biomassa fresca adicionada a cada ciclo de reciclagem aumentou continuamente, indicando que as celulases e hemicelulases foram eficientemente recicladas e que a lignina não teve nenhum efeito indesejável sobre a hidrólise enzimática.

ABSTRACT

VISSER, Evan Michael, D.Sc., Universidade Federal de Viçosa, July 2013. **Bioethanol production utilizing fungal enzyme extracts and different processing methods.** Adviser: Valéria Monteze Guimarães. Co-advisers: Sebastião Tavares de Rezende and Marcio Arêdes Martins.

Industrial-scale production of bioethanol from lignocellulosic sources is still in its initial phases, mainly due to the high processing costs and more specifically the high costs of commercial enzyme complexes. In conventional sugarcane processing, roughly 50% of bagasse is combusted to power the facility; however when considering the conversion of bagasse to bioethanol, 55% of bagasse can be processed for second generation ethanol production while the other 45% of fresh bagasse and lignin resulting from enzymatic hydrolysis is sufficient to power the conventional ethanol plant and also provide energy for processing/distillation of lignocellulose derived ethanol. The genetically modified yeast strain *S. cerevisiae* YRH400 showed to be more efficient for fermenting of both glucose and xylose to ethanol than *K. marxianus* ATCC-8554 and *K. marxianus* UFV-3. Enzymatic hydrolysis was performed on alkali-pretreated sugarcane bagasse using an enzyme extract obtained from *Chrysoporthe cubensis* produced via solid-state fermentation at the optimal temperature of 50°C with solids loading of 7.5%. This was compared with hybrid hydrolysis and fermentation (48 and 12 hour pre-hydrolysis periods) using the yeasts *S. cerevisiae* YRH400 and *K. marxianus* ATCC-8554 at the fermentation temperatures of 30°C and 40°C, respectively. Separate hydrolysis and fermentation appeared to be the most efficient, achieving glucose and xylose conversion efficiencies of 45 and 97%, respectively, for the enzyme loading of 10 FPU/g pretreated biomass over a 120 hour hydrolysis period. Blending of enzyme extracts with complementing activities was performed to obtain a more complete enzyme complex. Previous studies indicated that *Chrysoporthe cubensis* is a good

producer of β-glucosidase, xylanase and other accessory enzymes, while the extract from *Penicillium pinophilum* is rich in cellulases (FPase, endoglucanases and cellobiohydrolases). Maximum synergy was observed between these two extracts when blended at the concentration of 50:50, presenting synergism values of 76%, 50% and 24% for FPase, endoglucanase and xylanase activities, respectively. This blended enzyme extract was then applied for hydrolysis of alkali-pretreated sugarcane bagasse at different enzyme and biomass loadings at 45°C. A maximum conversion of glucose and xylose (64% and 93%, respectively) was obtained for the treatment with enzyme loading of 20 FPU/g and solids loading of 8%. Another assay was performed utilizing a reaction temperature of 50°C. At the higher temperature increases of 16% and 20% were obtained with respect to the glucose and xylose conversions, respectively. Moreover, for the treatment performed at 50°C the hydrolysis rate was nearly constant after 120 hours while those of the assays performed at 45°C showed to decrease more significantly. In this last experiment enzymatic hydrolysis was performed at 50°C and after predetermined time periods the solid fraction was recycled in an attempt to recycle enzymes adhered to the solid biomass. It was found that when adding the 1x (the same amount), 1/2x or no additional enzyme in the second hydrolysis period the same amount of glucose was produced, indicating the enzymes were efficiently recycled. However when adding the same amount of biomass (8 or 12%) during each recycle period the solids concentration increased and hydrolysis efficiency decreased significantly. In the experiment in which hydrolysis was continuously monitored and the solids concentration maintained constant (12%), hydrolysis efficiency of the fresh biomass added continuously increased, indicating that cellulase and hemicellulase enzymes were efficiently recycled and that lignin had no negative effect on enzymatic hydrolysis.

GENERAL INTRODUCTION

1.1. BACKGROUND

According to the Brazilian National Energy Balance, the Brazilian transportation energy demand was 79.3 million tons of oil equivalent of which 85% is derived from petroleum derivatives, 12.5% from sugarcane products, 2.1% from natural gas and a very small fraction from electricity (Ministério de Minas e Energia 2012). Brazil has a long history of biofuel production dating back to 1975 when the National Alcohol Fuel Program (ProAlcool) was initiated. This program sought to increase production of bioethanol to substitute gasoline which at the time was expensive and extremely scarce (Cortez 2012).

The production of second generation ethanol (bioethanol derived from lignocellulosic biomass) has great potential because cellulose is the most abundant renewable resource in the world. Utilization of agricultural residues presents a sustainable method for biofuels production while not requiring that food crops are converted to biofuels. Rice straw, wheat straw, corn stover and sugarcane bagasse are the major sources of agricultural residues throughout the world (Kim and Dale 2004). Of these, sugarcane bagasse is the most abundant lignocellulosic material in tropical countries. It also presents the advantage of being processed directly at the mill, therefore this residue if found concentrated at a single location. As a general rule, in Brazil one ton of sugarcane generates 280 kg of bagasse and the estimate for the 2012/2013 sugarcane harvest is for 588.37 million tons (UNICA 2013). About 50% of this residue is used in distilleries as a source of energy and the remainder is stockpiled (UNICA 2013). Due to the large quantity of this biomass as an industrial waste, it presents potential for application of the biorefinery concept which permits for the production of fuels and chemicals that offer economic, environmental, and social advantages.

Production of ethanol from lignocellulosic biomass contains three major processes, including pretreatment, hydrolysis, and fermentation. Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic structural and chemical composition to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars (Njoku, Ahring et al. 2012). Hydrolysis refers to the processes that convert the polysaccharides into monomeric sugars. The fermentable sugars obtained from hydrolysis process could be fermented

into ethanol by ethanol producing microorganisms, which can be either naturally obtained or genetically modified (Casey, Mosier et al. 2013).

Cellulose in lignocellulosic biomass is usually organized into microfibrils, each measuring about 3 to 6 nm in diameter and containing up to 36 glucan chains having thousands of glucose residues. According to the degree of crystallinity, cellulose is classified into crystalline and paracrystalline (amorphous) cellulose (Kumar and Murthy 2013). Cellulose can be hydrolytically broken down into glucose either enzymatically by cellulytic enzymes or chemically by sulfuric or other acids (Zhang, Liu et al. 2012). Hemicellulose, a branched polymer composed of pentose (5-carbon) and hexose (6-carbon) sugars, can be hydrolyzed by hemicellulases or acids to release its component sugars, including xylose, arabinose, galactose, glucose and/or mannose. Hexoses such as glucose, galactose, and mannose are readily fermented to ethanol by many naturally occurring organisms, but pentoses including xylose and arabinose are fermented to ethanol by few native strains, and usually at relatively low yields. Since xylose and arabinose generally comprises a significant fraction of lignocellulosic biomass, especially hardwoods, agricultural residues and grasses, its utilization makes the economics of biomass to ethanol conversion more feasible. The development of recombinant ethanogenic strains resulted in bacteria and yeasts capable of co-fermenting pentoses and hexoses into ethanol and other value-added products at high yields (Ota, Sakuragi et al. 2013).

Various pretreatment technologies have been extensively studied to process different biomass for cellulosic ethanol production. However, none can be declared the "best" because each pretreatment has its intrinsic advantages and disadvantages. Effective pretreatment is characterized by several criteria: size reduction, preserving hemicellulose fractions, limiting formation of inhibitors due to degradation products, minimizing energy input, and cost-effectiveness (Saddler and Kumar 2013). Other than these criteria, several other factors must also be considered, including recovery of high value-added co-products (e.g., lignin and protein), pretreatment catalyst, catalyst recycling, and waste treatment. When comparing these different pretreatment options, all the mentioned criteria should be comprehensively considered as a basis. Moreover, pretreatment results must be weighed against their impact on the ease of operation and cost of the downstream processes and the trade-off between several costs, including operating costs, capital costs, and biomass cost.

Saccharification is the critical step for bioethanol production where complex carbohydrates are converted to simple monomers. Compared to acid hydrolysis, enzymatic hydrolysis requires less energy and mild environment conditions (El-Zawawy, Ibrahim et al. 2011). Another advantage of enzyme hydrolysis over acid or alkali hydrolysis is the fact that no fermentation inhibitor products are generated.

Optimal conditions for cellulases have been reported as temperature of 40–50°C and pH 4–5, while optimal assay conditions for xylanase are often time the same. For complete cellulose degradation the synergistic action of the four cellulase enzymes is necessary: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.176), exoglucohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21). The hemicellulose fraction hydrolysis requires a more complex group of enzymes, referred to as hemicellulases. Complete enzymatic hydrolysis of xylan, the major polymer founded in hemicelluloses, requires endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and several accessory enzymes including α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (Yeh, Huang et al. 2010; Van Dyk and Pletschke 2012; Kumar and Murthy 2013). Figure 1 shows the action of these enzymes on the glucose chains making up cellulose. CBH1 and CBH2 represent the cellobiohydrolase enzymes that attack from the reducing and non-reducing ends of the glucose chain, respectively.

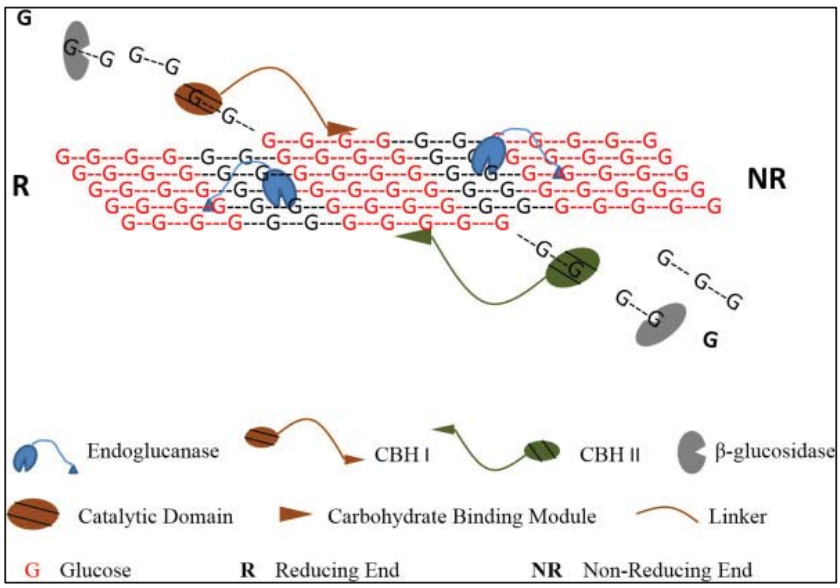

Figure 1. Action of various cellulases enzymes on surface layer of cellulose (Adopted from Kumar and Murthy (2013))

Among the various cellulolytic microbial strains, *Trichoderma* has been reported to show the greatest potential for synthesis of cellulase and hemicellulase enzymes, and for this reason it has been one of the most studied to date. However, *Trichoderma* generally lacks β-glucosidase activity which is necessary to break the β(1→4) bond to liberate the two glucose molecules. On the other hand, *Aspergillus* is one of the most studied producers of β-glucosidase (Sarkar, Ghosh et al. 2012). Many studies have reported blending cellulases from these two microorganisms as a method to maximize conversion of lignocellulose to monosaccharide sugars. However, these enzymes are often used as purified enzyme extracts marketed by enzyme distributers which significantly elevates costs. A more cost-effective option may therefore be the application of crude enzyme extracts obtained from enzyme producing fungi, in which case an array of enzyme activities is maintained and enzyme concentration costs are minimized.

Fermentation of glucose to ethanol is a well-known process, however the mixture of pentose and hexose sugars resulting from enzymatic hydrolysis of cellulose and hemicellulose is not readily fermented by a single microorganism. The yeast *S. cerevisiae* is the most studied and is known for its inherent resistance to low pH, high temperature, and various inhibitors (Hasunuma and Kondo 2012). Other wild-type microorganisms used in the fermentation process include *Escherichia coli*, *Zymomonas mobilis*, *Kluyveromyces marxianus*, *Pichia stipitis*, and *Candida brassicae*, where some are capable of fermenting pentose sugars, but often at rates significantly lower than that of *S. cerevisiae* (Lin, Huang et al. 2012; Wirawan, Cheng et al. 2012; Moreno, Ibarra et al. 2013). Temperature resistance of fermentation microorganisms is of great importance due to the high temperature of enzymatic hydrolysis, therefore in order for effective application of SSF, the fermentation temperature should be as close to the optimal enzyme hydrolysis temperature as possible.

Multiple process models have been developed for lignocellulose hydrolysis, seeking to maximize efficiency while reducing capital and operational costs and simplifying the overall process. Examples of these processes include: Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Hybrid Hydrolysis and Fermentation (HHF), Separator or Simultaneous Co-Fermentation (SSCF) and Consolidated Bioprocessing (CPB), and these processes can also be broken down into batch, fed-batch and continuous processes. Integration of the

different processes (enzyme production, saccharification and fermentation) reduces costs, but also complicates the process since optimal operating conditions are typically different (Hasunuma and Kondo 2012; Elliston, Collins et al. 2013). This is further complicated in consolidated bioprocessing where is single microorganism is utilized for enzyme synthesis as well as monosaccharide fermentation.

Commercial enzyme extracts have evolved from mixtures of cellulases only to include β-glucosidases and more recently hemicellulases to ensure the most cost-efficient conversion of pretreated lignocellulosic materials to fermentable sugars (Novozymes 2013). It is possible to reduce enzyme costs if they are produced on-site (Delabona, Farinas et al. 2012). This may be due to the fact that enzymes do not need to be highly concentrated and furthermore no accessory enzyme activity is lost in intense concentration/purification processes. Blending of complementing crude enzyme extracts is therefore a strategy that shows promise, i.e., one enzyme extract rich in cellullase enzymes and another rich in hemicellulase enzymes. Blending of enzyme extracts cultivated via solid state fermentation may present even greater advantages since highly concentrated enzyme extracts can be obtained.

A few studies have been performed on recycling of cellulases (Tu, Zhang et al. 2009; Qi, Chen et al. 2011; Weiss, Borjesson et al. 2013); where most focus on separating the enzyme from the solid or liquid phase or recycling of the solid and/or liquid phase directly. Membrane filtration and surfactants have been utilized in an attempt to recovery enzymes from hydrolysis products, however a more straightforward approach to enzyme recycling is direct recycle of the solid fraction to which most of the cellulase and hemicellulase enzymes are likely bound. Studies have shown that enzyme productivity (product yield per quantity of enzyme applied) can be significantly increased in this type of recycle process. Moreover, when utilized in combination with an alkali pretreatment the recycle process can be repeated multiple times since lignin accumulation is slowed.

Although improved reducing sugar or ethanol yields have been the objective of multiple previous studies focusing on the conversion of lignocellulosic biomass, the present thesis sought to reduce processing costs by simplifying the enzyme production process instead of improving efficiency when utilizing commercial enzyme extracts. This was performed by utilizing different processing techniques and application of

crude enzyme extracts not submitted to the various concentration and purification steps which increase enzyme costs.

1.2. THESIS STRUCTURE

This work was divided into the following chapters:

Chapter 1 presents the review paper from my Qualification Exam. This paper provides an overview of the lignocellulose to ethanol conversion process, focusing on sugarcane bagasse, as well as the conventional ethanol production process from sugarcane juice. Calculations were performed to indicate: the amount of bagasse which can be processed for second generation ethanol production, ethanol yields from second generation ethanol production and the amount of bagasse which must be destined for power generation.

Chapter 2 introduces the first experimental studies that were performed as part of the investigation on bioethanol processing techniques. Different yeasts were evaluated with respect to their ability to ferment both glucose and xylose and an enzyme extract produced from the fungi *Chrysoporthe cubensis* was applied for analysis of separate hydrolysis and fermentation and simultaneous saccharification and fermentation.

Chapter 3 presents more advanced experimental results in which an enzyme blend was produced by blending the crude extracts from *Chrysoporthe cubensis* and *Penicillium pinophilum*. Herein the enzyme blend was characterized with regards to enzyme synergy between enzyme activities of both extracts, and saccharification experiments were then performed to assess the hydrolytic potential of the enzyme blend.

Chapter 4 indicated the potential for recycling of cellulase and hemicellulase enzymes. Here it was shown that by recycling the solid fraction after a predetermined hydrolysis period permitted that enzymes were recycled as part of the insoluble solids. This study further showed that the presence of increasing lignin concentrations did not hinder enzymatic hydrolysis, but instead hydrolysis efficiency increase with the increasing lignin concentration.

References

Casey, Elizabeth, NathanS Mosier, Jiri Adamec, Zachary Stockdale, Nancy Ho, and Miroslav Sedlak. 2013. Effect of salts on the Co-fermentation of glucose and xylose by a genetically engineered strain of Saccharomyces cerevisiae. *Biotechnology for Biofuels* 6 (1):1-10.

Cortez, Luís. 2012. Building up sugarcane ethanol sustainability. *Biofuels, Bioproducts and Biorefining* 6 (6):599-600.

Delabona, Priscila da Silva, Cristiane Sanchez Farinas, Mateus Ribeiro da Silva, Sindelia Freitas Azzoni, and José Geraldo da Cruz Pradella. 2012. Use of a new Trichoderma harzianum strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. *Bioresource Technology* 107 (0):517-521.

El-Zawawy, Waleed K., Maha M. Ibrahim, Yasser R. Abdel-Fattah, Nadia A. Soliman, and Morsi M. Mahmoud. 2011. Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production. *Carbohydrate Polymers* 84 (3):865-871.

Elliston, Adam, Samuel R. A. Collins, David R. Wilson, Ian N. Roberts, and Keith W. Waldron. 2013. High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. *Bioresource Technology* 134 (0):117-126.

Hasunuma, Tomohisa, and Akihiko Kondo. 2012. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. *Process Biochemistry* 47 (9):1287-1294.

Hasunuma, Tomohisa, and Akihiko Kondo. 2012. Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. *Biotechnology Advances* 30 (6):1207-1218.

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

Kumar, Deepak, and GantiS Murthy. 2013. Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. *Biotechnology for Biofuels* 6 (1):1-20.

Lin, Ting-Hsiang, Chiung-Fang Huang, Gia-Luen Guo, Wen-Song Hwang, and Shir-Ly Huang. 2012. Pilot-scale ethanol production from rice straw hydrolysates using xylose-fermenting Pichia stipitis. *Bioresource Technology* 116 (0):314-319.

Ministério de Minas e Energia. 2012. Balanço Energetico Nacional (Brazilian National Energy Balance).

Moreno, Antonio D., David Ibarra, Ignacio Ballesteros, Alberto González, and Mercedes Ballesteros. 2013. Comparing cell viability and ethanol fermentation of the thermotolerant yeast Kluyveromyces marxianus and Saccharomyces cerevisiae on steam-exploded biomass treated with laccase. *Bioresource Technology* 135 (0):239-245.

Njoku, S. I., B. K. Ahring, and H. Uellendahl. 2012. Pretreatment as the crucial step for a cellulosic ethanol biorefinery: Testing the efficiency of wet explosion on different types of biomass. *Bioresource Technology* 124 (0):105-110.

Novozymes. *Novozymes Cellic® CTec3 Application Sheet* 2013 [cited June 2013. Available from http://bioenergy.novozymes.com/en/cellulosic-ethanol/CellicCTec3/Documents/AS_2012-01394-01.pdf.

Ota, Miki, Hiroshi Sakuragi, Hironobu Morisaka, Kouichi Kuroda, Hideo Miyake, Yutaka Tamaru, and Mitsuyoshi Ueda. 2013. Display of Clostridium cellulovorans xylose isomerase on the cell surface of Saccharomyces cerevisiae and its direct application to xylose fermentation. *Biotechnology Progress* 29 (2):346-351.

Qi, Benkun, Xiangrong Chen, Yi Su, and Yinhua Wan. 2011. Enzyme adsorption and recycling during hydrolysis of wheat straw lignocellulose. *Bioresource Technology* 102 (3):2881-2889.

Saddler, Jack, and Linoj Kumar. 2013. Special Issue from the NSERC Bioconversion network workshop: pretreatment and fractionation of biomass for biorefinery/biofuels. *Biotechnology for Biofuels* 6 (1):17.

Sarkar, Nibedita, Sumanta Kumar Ghosh, Satarupa Bannerjee, and Kaustav Aikat. 2012. Bioethanol production from agricultural wastes: An overview. *Renewable Energy* 37 (1):19-27.

Tu, Maobing, Xiao Zhang, Mike Paice, Paul MacFarlane, and Jack N. Saddler. 2009. The potential of enzyme recycling during the hydrolysis of a mixed softwood feedstock. *Bioresource Technology* 100 (24):6407-6415.

UNICA. 2013. Brazilian Sugarcane Industry Association - Sugarcane Production and Processing.

Van Dyk, J. S., and B. I. Pletschke. 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnology Advances* 30 (6):1458-1480.

Weiss, Noah, Johan Borjesson, Lars Saaby Pedersen, and Anne Meyer. 2013. Enzymatic lignocellulose hydrolysis: Improved cellulase productivity by insoluble solids recycling. *Biotechnology for Biofuels* 6 (1):5.

Wirawan, Ferdian, Chieh-Lun Cheng, Wei-Chen Kao, Duu-Jong Lee, and Jo-Shu Chang. 2012. Cellulosic ethanol production performance with SSF and SHF processes using immobilized Zymomonas mobilis. *Applied Energy* 100 (0):19-26.

Yeh, An- I., Yi-Ching Huang, and Shih Hsin Chen. 2010. Effect of particle size on the rate of enzymatic hydrolysis of cellulose. *Carbohydrate Polymers* 79 (1):192-199.

Zhang, Zehui, Bing Liu, and Zongbao Zhao. 2012. Efficient acid-catalyzed hydrolysis of cellulose in organic electrolyte solutions. *Polymer Degradation and Stability* 97 (4):573-577.

Chapter 2.

PROCESS ANALYSIS OF INTEGRATED SECOND GENERATION ETHANOL PRODUCTION

Submitted as part of the Qualification Exam

Abstract

Ethanol from lignocellulose presents great potential for production of renewable fuels due to the wide availability of cellulose biomass across the globe, where this potential is even greater in countries with tropical climates, as in the case of Brazil. Biorefineries integrated with conventional sugarcane mills for processing the bagasse co-product to ethanol present the opportunity to significantly increase ethanol production while still maintaining the facility free of external energy demands. The present paper reviewed second generation ethanol production from sugarcane bagasse from a process standpoint, as it may be integrated with existing conventional ethanol production. Ethanol production from sugarcane bagasse was evaluated and it was determined that slightly more than half (55%) of sugarcane bagasse can be used to increase total ethanol production by 18%, while utilizing the remaining bagasse (45%) and waste products to maintain the facility independent of external energy sources. Analysis was based on process efficiencies reported for lignocellulosic biomass conversion and the heating values of both raw bagasse and the by-products of cellulosic ethanol production. The amount of water used for processing (solids loading in pretreatment and saccharification) also plays an important role on process efficiency as well as energy demands for heating and separation.

Keywords: Ethanol, Pretreatment, Saccharification and fermentation, Mass and energy balance.

1. Introduction

The concept of a biorefinery has been gaining popularity around the world in recent years, especially when referring to utilization of lignocellulosic biomass for the production of fuels, power and chemicals (Naik, Goud et al. 2010). Brazil has been the focus of many studies and investments in this field, especially due to its tropical climate and extensive area of agricultural production (Visser, Filho et al. 2012). Sugarcane is the base agricultural product used for the production of ethanol in Brazil and national production is the largest in the world where roughly 7.5 million hectares are planted (De Figueiredo and La Scala 2011). The fibrous residue produced after sugarcane milling is referred to as bagasse and is commonly used for cogeneration of process steam and

electricity; however there is also the potential to use this biomass for production of more valuable products.

It was estimated that in 2009 milled Brazilian sugarcane production was 575 million tons (CONAB 2011), where this results in an accumulation of bagasse estimated to be roughly one third of the dry weight of the harvested cane (Seabra, Tao et al. 2010). In the old sugarcane mills commonly found throughout Brazil, the use of bagasse for cogeneration of process steam and production of electricity to meet on-site plants demands requires that up to 90% of bagasse be combusted, however large gains in efficiency are achieved if low-pressure boilers are exchanged for those of medium or high-pressure (Coelho, Goldemberg et al. 2006; da Silva, Inoue et al. 2010). When using medium pressure boilers it has been estimated that only 50% of the bagasse available is needed to meet the plant demands (Botha and von Blottnitz 2006), thus leaving a large surplus to potentially be utilized in envisioned biorefineries.

Cellulosic ethanol production typically involves three distinct stages: pretreatment, saccharification and fermentation. Pretreatment is an essential processing step in the production of bioethanol from lignocellulosic biomass such as bagasse, being both technically and economically important. It is required to breakdown the crystalline structure of lignocellulose and allows that enzymatic hydrolysis proceeds at an acceptable rate and suitable conversion efficiency is obtained. Pretreatment is typically one of the most energy intensive steps in the process and thus is a substantial cost factor; and as a result, a wide variety of pretreatments methods have recently been studied; however pretreatment methods are typically specific to the biomass employed as well as the enzyme cocktail to be used for saccharification (Shi, Sharma-Shivappa et al. 2009; Alvira, Tomás-Pejó et al. 2010; Carrasco, Baudel et al. 2010; da Silva, Inoue et al. 2010).

Enzymatic hydrolysis continues to be bottleneck for economic feasibility of the second generation ethanol production process, however enzymatic saccharification is preferred over acid hydrolysis for both technical and environmental reasons (El-Zawawy, Ibrahim et al. 2011). Development in the production of more advanced enzyme cocktails and also fermenting microorganisms has also resulted in greater process efficiencies and the potential for process integration which can effectively

reduce the number of bottlenecks in the overall process and decrease energy demands (Cardona, Quintero et al. 2010).

The majority of biomass pretreatment methods encountered in literature are based on the use of dilute acids, typically in combination with mechanical and thermal processing, specifically aiming to remove the hemicellulose portion of the lignocellulosic structure by chemical hydrolysis. Significantly less attention is given to alkali-based pretreatment methods used specifically for lignin solubilization. Because many raw enzyme substrates, especially those obtained from filamentous fungi, are rich in hemicellulose hydrolyzing enzymes (xylanases, arabinases, etc.) (Meyer, Rosgaard et al. 2009; Buaban, Inoue et al. 2010; de Almeida, Guimaraes et al. 2011), utilization of alkali pretreatment to preserve the hemicellulose fraction in its respective polysaccharides may be advantageous to increase ethanol yields and also minimize production of fermentation inhibitors.

The present review evaluates the specific unit operations involved in conventional ethanol production as well as second generation ethanol production from sugarcane bagasse. Specific attention is given to the alkaline pretreatment method followed by saccharification and fermentation of both cellulose and hemicellulose fractions. A mass and energy balance was performed to evaluate the energy available in recovered lignin and unconverted cellulose and hemicellulose fractions to determine the potential for cogeneration utilizing these products to meet energy demands of the process.

2. Ethanol production process from sugarcane

The first step of the ethanol production process occurs in the field when sugarcane is harvested. The sugarcane plant itself can be divided into three parts: roots, stalk and leaves. Mechanical harvesting separates the stalk from the leaves, in which the stalk is collected for transportation to the processing plant and the leaves are left in the field to protect the soil and act as a natural fertilizer. Studies have indicated that it is also possible to convert sugarcane leaves to ethanol via saccharification and fermentation (Dawson and Boopathy 2007; Jutakanoke, Leepipatpiboon et al. 2012), but there are conflicting reports on the quantity of leaves which must be left in the field to prevent soil degradation.

Production of bioethanol from sugarcane is performed in the following main steps: reception and cleaning of sugarcane, extraction of sugars, juice treatment, concentration and sterilization, fermentation and distillation (Luo, van der Voet et al. 2009). Many of these steps are unaltered when integrating the lignocellulose ethanol production process, but the entire process must be analyzed to evaluate all potential possibilities of interaction. A brief summary of these steps in the bioethanol production process are presented in the following subsections.

2.1. Sugarcane reception and cleaning, extraction of sugars and juice treatment

Sugarcane stalks are initially cleaned for removal of gross impurities (dirt, sand and other large impurities) before being subjected to crushing and extraction of the juice. In order to maximize recovery of sugars, water is added in an imbibition process and the insoluble bagasse is separated from the liquid sugar slurry. Physical treatments such as screens and hydrocyclones are then used to remove sand and fiber from the juice (Chen and Chou 1993). This slurry of dissolved sugars receives chemical treatments typically consisting of the addition of phosphoric acid to enhance decantation during settlement, followed by heating of the mixture. The pre-heated mixture is then supplemented with lime and further heated to the boiling point of the liquid. Air bubbles are removed from the hot juice and a flocculant is added before feeding the juice into a settler.

Two streams are obtained from the settler: one composed of clarified juice and the other of mud containing impurities. A filter is typically used to further recover sugars contained in the mud and this filtrate is recycled back to the point before the second heating operation.

2.2. Juice concentration and sterilization

Clarified juice contains roughly 15 wt% sugars and therefore is concentrated prior to fermentation so that energy demands are minimized for separation of ethanol from water in the proceeding purification steps. Multi-effect evaporators are typically found in industry to concentrate a portion of the juice to concentrations up to 65 wt% sucrose. This concentrated fraction is then mixed with the diluted fraction (15 wt%) to obtain a final sucrose concentration in the juice of approximately 22 wt%. The juice is

sterilized to avoid contamination during the fermentation process and then cooled to the desired fermentation temperature.

2.3. Conventional Fermentation

In conventional fermentation, sucrose encountered in the sugarcane juice is consumed by yeast to produce ethanol and carbon dioxide, as well as the minor components of higher alcohols, organic acids, glycerol and yeast cells. Gases exiting the fermenter are scrubbed for recovery of ethanol vapors and the wine exiting the fermenter is centrifuged for yeast recovery. Yeasts are recovered to a concentration of up to 70% (volume basis) and then treated with sulfuric acid and water to avoid bacterial contamination and diluted to roughly 28% (volume basis). It is desired that maximal ethanol concentrations are obtained during fermentation since energy demands for distillation increase significantly as a function of decreasing ethanol concentrations. Prior to distillation ethanol concentrations are typically in the range of 10-12% (v/v)

2.4. Distillation

Ethanol purification is typically performed in subsequent distillation columns, in sections of distillation and refraction (Sánchez and Cardona 2012). The unit operation of distillation is energy intensive which makes acquisition of a maximal ethanol concentration prior to distillation of great importance. Because ethanol and water form an azeotrope at the concentration of 95.6 wt% at atmospheric pressure, further processing is required to produce anhydrous bioethanol. This is typically done by adsorption with molecular sieves or extractive distillation in the presence of a compound such as ethylene glycol.

Distillation column bottoms, also known as vinasse, is a by-product of ethanol processing generated at the rate of roughly 12 to 1 per volume of ethanol in this step, and is often recycled back to the fields as a fertilizer (Goldemberg, Coelho et al. 2008). Although disposal of vinasse is often viewed as a problem, some studies have been performed on using this by-product to generate biogas on site.

3. Production of ethanol from sugarcane bagasse

Sugarcane bagasse is one of the main by-products generated during conventional bioethanol production from sugarcane. Bagasse is readily available at the plant site, and

production of bioethanol from sugarcane bagasse may be integrated with existing infrastructure where conventional bioethanol is produced, including the unit operations of evaporation, fermentation and distillation.

Production of ethanol from lignocellulosic sources such as sugarcane bagasse using biological catalysts is typically performed in three steps: pre-treatment, enzymatic saccharification and fermentation. Optimal treatments vary as a function of biomass composition and the desired methods for integration with existing bioethanol production sites. These different processes stages are evaluated more in-depth in the following subsections.

3.1. Pretreatment

During pretreatment, the lignocellulosic matrix of biomass is broken down in order to reduce the degree of crystallinity and increase the portion of amorphous cellulose most suitable for enzymatic attack. Diverse forms of pretreatment have been studied, and those most commonly used are combinations of physical, chemical and thermal treatments. Physical treatment involves increasing surface area for enzyme attack and often times dilute acids or bases under high temperatures are used to further prepare biomass for hydrolysis. Selection of acid, alkaline or other pretreatment solutions is dependent on biomass composition and enzymes available for hydrolysis, since acids are generally more effective for hydrolyzing hemicelluloses from the substrate and bases are more effective for solubilizing lignin. Despite the predominance of studies evaluating dilute-acid pretreatments, it has also been shown that pretreatments focused on removing lignin have more effectively improved enzymatic digestibility than those with the objective of removing hemicelluloses (Zhao, Peng et al. 2009). Alkali pretreated biomass is composed principally of cellulose and hemicellulose while the liquid fraction contains dissolved lignin; fewer fermentation inhibitors are also produced in this process (Alvira, Tomás-Pejó et al. 2010).

When using crude enzyme extracts for enzymatic hydrolysis, permanence of the xylan fraction is of great interest since many of the filamentous fungi known for production of cellulase enzymes also produce significant quantities of xylanase enzymes (de Almeida, Guimaraes et al. 2011). Selection of alkali pretreatment therefore takes into consideration the assumption that the enzyme cocktail to be used not only contains the necessary cellulose degrading enzymes, but also significant quantities of

hemicellulase enzymes. It has been proven that supplementation of these enzymes to the cocktail not only increases the total soluble sugar yield, but also increases glucose yield when cellulase loading remained constant (Kumar and Wyman 2009); the absence of large concentrations of fermentation inhibitors (especially furfural and hydroxymethylfurfural but also including lignin derivatives) is another advantage of the alkali pretreatment which facilitates subsequent fermentation steps.

Energy consumption during the pretreatment phase may be significant, especially for physical treatment (grinding) of biomass. Although most pretreatment techniques are a combination of mechanical techniques with chemical, thermal or biological, studies have been performed in which sugarcane bagasse obtained from the existing process plant is not further milled (Carrasco, Baudel et al. 2010). Diverse studies have been performed on the energy necessary for milling of corn stover, switchgrass and hardwoods to facilitate enzyme digestibility (Alzate and Toro 2006; Lavigne and Powers 2007; Piccolo and Bezzo 2009), as well as the degree of mechanical pretreatments for economic feasibility (da Silva, Inoue et al. 2010).

Pretreatments at diverse conditions are encountered in literature. Results from different pretreatment studies with sugarcane bagasse are presented in Table 1. Correlations are typically observed between pretreatment intensity and lignin recovery when using alkali-based pretreatments, and in the case of acid-based pretreatments more significant variations of pentose recovery are observed. This is highlighted in the study performed by Mesa, Gonzáles et al. (2011) using acid compared to the others in Table 1.

Table 1. Results of different pretreatments encountered in published works.

Particle size	Chemical/ Concentration	Temperature	Time	Hexose recovery	Pentose recovery	Lignin recovery	Citation
0.3 mm	3.1% NH_4OH-H_2O_2 (w/w)	120°C	1 h	85%	72%	50%	(Zhu, Zhu et al. 2012)
0.45-0.9 mm	1% NaOH (w/v)	30°C	20 h	97.5%	72%	45%	(Cheng, Zhang et al. 2008)
0.45-0.9 mm	0.6% H_2O_2 (w/v)	30°C	20 h	99.7%	62%	87%	(Cheng, Zhang et al. 2008)
0.45-0.9 mm	1% NaOH+ 0.3% H_2O_2 (w/v)	30°C	20 h	96.5%	74.6%	35.5%	(Cheng, Zhang et al. 2008)
0.45-0.9 mm	1% NaOH+ 0.6% H_2O_2 (w/v)	30°C	20 h	95.8	78.5%	30%	(Cheng, Zhang et al. 2008)
>1 cm	0.2 M H_2SO_4	120°C	40 min	99.6%	26.9%	99.2%	(Mesa, González et al. 2011)
In natura	Steam explosion + 1% NaOH	190°C/ 100°C	15 min/ 1 h	92% 92%	18% 8%	98% 14%	(Rocha, Gonçalves et al. 2012)

3.2. Saccharification and Fermentation

During hydrolysis, cellulose and hemicellulose polymers are broken down in order to produce fermentable sugars. This process can be done with the use of acids or enzymes. The current tendency is to use the enzymatic process due to environmental concerns and also because enzymatic hydrolysis results in improved fermentation since fewer inhibitors are generated from the acid hydrolysis of the cellulose/hemicellulose fractions, although the process is slower. However, the cost of enzymes is one of the most important factors contributing to the total costs of ethanol production from biomass, accounting for up to 15% (Novozymes 2009). Cellulase enzymes are inhibited by glucose, therefore high enzyme loadings are required or saccharification must be consolidated with fermentation to permit that glucose is converted to ethanol and this

inhibitory effect is diminished (as indicated in Figure 1). When using an enzyme cocktail consisting of both cellulase and hemicellulase enzymes, both fractions can be simultaneously hydrolyzed to produce sugars for further fermentation, as well as increase overall saccharification yields (Kumar and Wyman 2009).

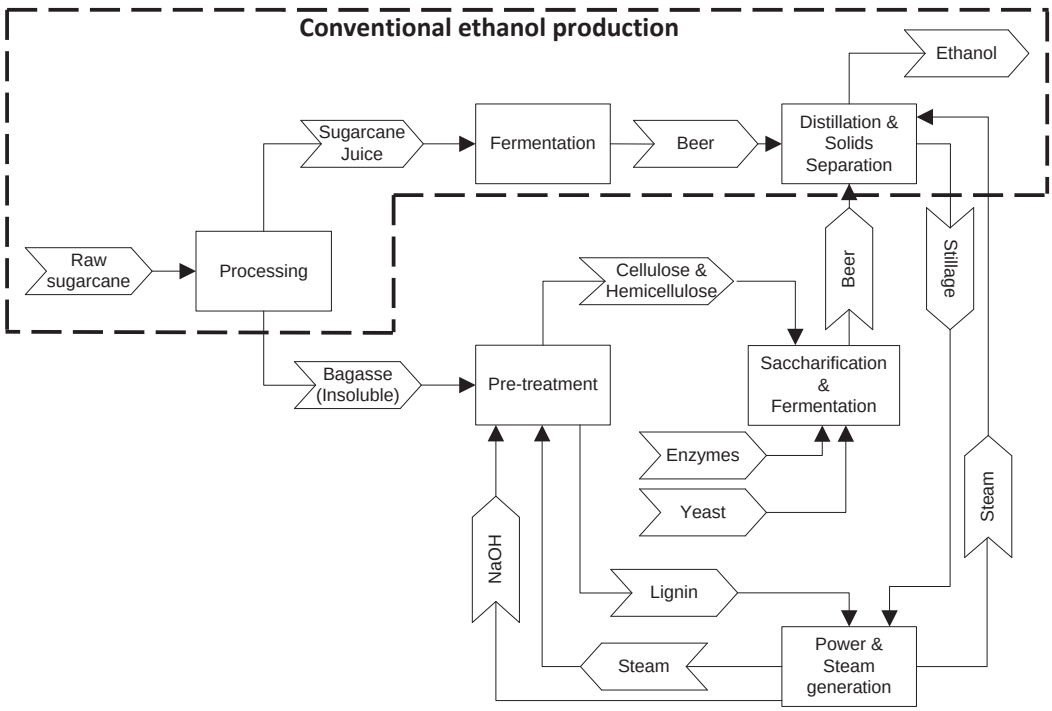

Figure 1. Block diagram of ethanol production from lignocellulosic biomass with integrated ethanol distillation.

Separate hydrolysis and fermentation permits for greater process integration, where the sugars produced from hydrolysis may be combined with those from the cane juice. Both steams may then be concentrated and fermented together (Simultaneous Saccharification and Fermentation – SSF) (Macrelli, Mogensen et al. 2012), however this may present additional technical difficulties due to the presence of pentose sugars together with hexoses. Additional methods such as Cascade Simultaneous Saccharification and Fermentation (CSSF) seek to further improve efficiencies and reduce costs by recycling of enzymes (Li and Kim 2012).

Just as pretreatment methods must be tailored to the specific biomass to be processed, enzyme cocktails also need to be specific to the individual pretreated biomass compositions. Numerous studies have been performed for analyzing the effect

of different enzyme cocktails on saccharification, utilizing purified enzymes purchased on the market (Saha and Cotta 2008; Engel, Krull et al. 2012), or the enzyme cocktail produced by a specific microorganism (Buaban, Inoue et al. 2010; Maeda, Serpa et al. 2011).

For fermentation, the yeast *Saccharomyces cerevisiae* is that most commonly used in the fermentation of glucose to ethanol, however the natural strain is incapable of metabolizing pentose sugars. Co-fermentation of both pentose and hexose sugars has been the focus of diverse studies using microorganisms including *Kluyveromyces marxianus* (Lane and Morrissey 2010; Kang, Kim et al. 2012) and *Pichia stipitis* (Watanabe, Watanabe et al. 2011), as well as genetically modified strains of *Saccharomyces cerevisiae* (Hector, Dien et al. 2010), *Zymomonas mobilis* (Zhang, Eddy et al. 1995), *Escherichia coli* (Qureshi, Dien et al. 2006) and others. Commercial utilization of xylose-fermenting microorganisms is often limited due to slow fermentation rates, carefully regulated oxygen requirements, sensitivity to inhibitors and low ethanol tolerance.

Few fermentation inhibitors are produced when utilizing alkaline pretreatment (Alvira, Tomás-Pejó et al. 2010), and when combined with the fact that various cellulolytic microorganisms are known to produce cellulases and hemicellulases capable of converting cellulose and hemicellulose respectively into soluble monomeric or oligomeric sugars, the demand for microorganisms for co-fermentation of hexose and pentose fractions is continuously increasing (Kuhad, Gupta et al. 2011).

Published yields for the conversion of polysaccharides to monosaccharides and their subsequent fermentation vary significantly. Increased enzyme loading obviously increases hydrolysis efficiency, but solids loading also has a significant effect on monosaccharide yield (Kristensen, Felby et al. 2009). This is shown graphically in Figure 3 of the following modeling section. Data presented in the present paper consider optimistic conversions which may potentially be obtained on the commercial scale, as encountered in different published studies (Table 2).

Table 2. Percentage of theoretical conversion during hydrolysis and fermentation

	Hydrolysis[a]		Fermentation[a,b]	
Glucan	90%	(Rivera, Rabelo et al. 2010)	90%	(Humbird, Davis et al. 2011)
Xylan	95%	(Saha, Nichols et al. 2011)	80%	(Humbird, Davis et al. 2011)
Arabinan	95%	(Saha, Nichols et al. 2011)	80%	(Humbird, Davis et al. 2011)

[a]Percentage of theoretical conversion

[b]Fermentation considers the corresponding monosaccharide sugar

3.3. Ethanol concentration

After fermentation, conventional distillation methods are typically used for separation of liquid fractions for concentration of ethanol. Although distillation is energetically intensive, it is widely used across the world. With respect to energy necessary for separation, distillation is ranked as one of the largest energy consumers worldwide, accounting for nearly 3% of the world energy consumption (Enweremadu, Waheed et al. 2009).

In light of the high energy requirements for distillation, the ethanol industry currently utilizes evaporators for concentration of sugars so that microorganisms may produce the greatest ethanol concentration possible, utilizing microorganisms to increase ethanol concentration instead of distillation. As indicated in section 3.2, integration of the second generation ethanol process with conventional ethanol production may initiate at the evaporation stage.

Pervaporation and membrane separation are alternative techniques being increasingly studied but have received limited acceptance in industry (Lewandowicz, Białas et al. 2011). Because these methods do not required elevated temperatures such as conventional distillation, yeasts and enzymes can potentially be separated from the ethanol fraction and reused for continuous fermentation (Gryta, Morawski et al. 2000).

3.4. Lignin recovery, alkali recycle and co-generation

Despite its negative effect on enzymatic hydrolysis of cellulose and hemicellulose, lignin is energy dense and can be effectively used as a fuel in combustion for production of process steam. Because alkaline pretreatment is much the same as the Kraft process in the pulp and paper sector, many of the methods used for black liquor treatment can be applied to the herein pretreatment step to not only

recovery lignin but also allow for recycle of the alkaline solution (Amidon 2006; Minu, Jiby et al. 2012).

Lignin can be precipitated from an alkali solution by decreasing the pH. At pH 3 nearly all lignin in solution is precipitated as a solid which is removed by simple filtration or use of a hydrocyclone (Minu, Jiby et al. 2012). Another option for treatment of black liquor is gasification, in which the lignin fraction is oxidized for production of a high caloric syngas composed predominantly of hydrogen and carbon monoxide, which is one of the intensely studied options for treatment of black liquor and recycle of alkali components (Carlsson, Wiinikka et al. 2010). Studies have also been performed indicating that ultrafiltration processes may be suitable for this purpose, in which case there would be no need for further consumption of chemicals or cooling of the pretreatment liquid fraction (Wallberg and Jonsson 2006). Ultimately, lignin is utilized for the energy generation.

The production of ethanol from biomass requires significant quantities of process water which have a direct effect on energy demands. Decreases in the solid to liquid ratio both in the pretreatment and saccharification steps aids in process efficiency, but also requires greater energy demands for both heating and separation. This generated wastewater must then be processed for separation of organic material from both pretreatment and distillation stillage as well as recovery of the alkali pretreatment solution. Recovery of these fractions allows for co-generation to meet the energy demands of production as well as potentially produce electricity to be marketed to the local grid.

Anaerobic digestion of organic material resulting from the ethanol process for production of biogas/energy is already employed in some ethanol plants for treatment of vinasse. The process models proposed by Macrelli et al. (2012) consider anaerobic digestion of pentose sugars resulting from acid pretreatment for production of biogas. Xylose and fermentation byproducts are dissolved molecules, and thus can be rapidly digested (Shafiei, Karimi et al. 2011).

Co-generation of process steam and electricity to meet the power demands of the plant allows that sugarcane processing be self-sufficient. In the past inefficient low pressure boilers were used for power generation and required that nearly all bagasse be combusted to meet energy demands, however these are being replaced by more efficient

medium pressure boilers capable of producing significantly more electricity per unit of biomass. This means that more bagasse is left over for lignocellulosic ethanol production after meeting power demands of the plant (Goldemberg, Coelho et al. 2008).

4. Process integration analysis

Process integration requires that one have sufficient knowledge of the individual processes involved so that equipment installations and costs may be minimized and efficiencies optimized. Luo et al. (2009) presented a life cycle assessment for conventional ethanol production as well as second generation ethanol from "field-to-wheel", detailing the system boundaries for each with all inputs and outputs.

Conversion of sugarcane bagasse to ethanol presents the logistical advantage of encountering the raw material available on-site without having to deal with issues such as transportation, production, harvest, etc., since these are all part of the existing sugarcane processing process. Integrated production of cellulosic ethanol with conventional ethanol permits the use of existing infrastructure to significantly minimize capital expenditures. Simulations have previously been performed confirming the economic benefits of process integrations, especially when considering advanced hydrolysis technologies and fermentation of pentose sugars (Dias, Junqueira et al. 2012). There are varying levels of process integration which may be proposed. These may be limited to integration of distillation equipment only (as shown in Figure 1), or include integrated evaporation, fermentation and distillation (Macrelli, Mogensen et al. 2012).

The publication of Macrelli et al. (2012) presents multiple different scenarios for integration of cellulosic with conventional ethanol production. However, in all cases pentose sugars are destined for anaerobic digestion/biogas production instead of fermentation to ethanol. Modeling performed by the National Renewable Energies Laboratory of the United States suggested that fermentation of both pentose and hexose sugars can be performed by genetically modified microorganisms (Table 2) (Humbird, Davis et al. 2011). Because hemicellulose, especially xylans, often makes up nearly 25% of raw biomass, its conversion to ethanol can greatly improve products yields.

4.1. Process modeling

In order to analyze both the mass and energy balance of an integrated cellulosic ethanol production facility the process was modeled as shown in Figure 1. Different from what is typically observed in other models published, this assumes alkali

pretreatment for lignin removal followed by enzymatic hydrolysis of both cellulose and hemicellulose fractions to their respective monosaccharide sugars.

Composition of sugarcane bagasse varies among species, however for process analysis the databank of the US Department of Energy Biomass Program was accessed to provide bagasse composition (Table 3). The higher heating value (HHV) of the sugarcane bagasse as well as the fractions recovered for power generation was based on the relationship provided by Sheng and Azevedo (2005).

Table 3. Sugarcane bagasse composition

Composition (wt%, dry basis)[a]	
Glucan	43.4%
Xylan	23.1%
Lignin	24.1%
Arabinan	1.6%
Galactan	0.5%
Mannan	0.3%
Ash	2.8%
Uronic Acids	1.3%
Ultimate analysis (wt%, dry basis)	
C	49.0%
H	5.5%
N	0.2%
O	39.2%
S	0.0%
HHV (MJ/kg)[b]	21.085

[a]Values reported by the US Department of Energy – Biomass Program (US Department of Energy 2004)

[b]Calculated according to the relationship prosed by Sheng and Azevedo (2005)

Model parameters were based on published data presented in section 3 (Tables 1-2), and the specific conversions/recoveries utilized are shown in Table 4. The monosaccharide fractions considered were limited to glucose, xylose and arabinose, along with their respective polymers, to maintain simplicity and also because little is encountered in literature regarding fermentation of additional sugars. Another very important factor to be included in the model is the solids loading in both the pretreatment and hydrolysis steps, which were considered as 12.5% and 20% (w/v), respectively. Solids loading of 20% is considered high, but can easily be obtained via fed-batch processes.

Complete conversion of polysaccharides to fermentable sugars considers the theoretical factors of 1.11 (w/w) for C6 sugars and 1.136 (w/w) for C5 sugars; however losses are assumed and the conversion factors utilized in the present model are slightly lower. Fermentation is also based on the theoretical conversion of sugar to ethanol of 0.51 (w/w), where the actual percentages used in the present study take into consideration the fact that pentose sugars are less readily fermented than hexose sugars (Katahira, Ito et al. 2008).

Table 4. Efficiencies of recovery/conversion used in the integrated plant model

Pretreatment component recoveries (wt %)		Hydrolysis percent of theoretical conversion		Fermentation percent of theoretical conversion	
Glucan	90%	Glucan	90%	Glucose	90%
Xylan	70%	Xylan	90%	Xylose	70%
Arabinan	70%	Arabinan	90%	Arabinose	70%
Lignin	25%	Lignin	-	Lignin	-

The distillation process was modeled considering the production of hydrous ethanol (94 %wt), since a large portion of the Brazilian ethanol market is based on hydrous ethanol sales (MME - Brazilian Federal Government Ministry of Mines and Energy 2011). Also assumed was the fact that 99% (w/w) of ethanol was recovered in the distillate stream. Therefore, a distillation system was modeled to estimate process heat requirements for the column reboiler based on conventional distillation technology (Green and Perry 2007).

4.2. Modeling results

Utilization of sugarcane bagasse as a raw material for lignocellulose ethanol production represents one of the most viable biorefinery concepts since conventional mills already produce both ethanol and energy on-site, and these sites are also home to excess quantities of bagasse. Annually, a 3 Mt y^{-1} sugarcane mill generates approximately 0.375 Mt of dry bagasse. This total quantity represents the potential for ethanol production of 180 ML, i.e., roughly 66% of the ethanol conventionally produced via sugarcane juice (273 ML y^{-1}).

4.2.1. Mass and energy balance

It has been estimated that traditional ethanol produced from sugarcane sugars has a net energy balance of more than 8 times (energy output divided by energy input), however the vast majority of energy inputs are in the form of agricultural production (Coelho, Goldemberg et al. 2006). In the case of ethanol produced from bagasse there are no additional agricultural energetic production requirements because it is a co-product, however processing energy inputs are increased due to the difficulty of accessing fermentable sugars from biomass.

Roughly 50% of the bagasse resultant of sugarcane milling is needed to meet the electricity and process steam demands for conventional ethanol production (Botha and von Blottnitz 2006). Ethanol plants in Brazil typically use the bagasse co-product as a fuel for CHP generation (combined heat and power) and often export electricity due to the abundance of bagasse available. Although the percentage of bagasse required to meet energy demands varies significantly as a function of boiler efficiency (steam pressure), for modeling purposes it was assumed that the energy necessary for conventional ethanol production was equivalent to the energy content of 50% of all bagasse produced (1,190 MJ t^{-1} of cane processed).

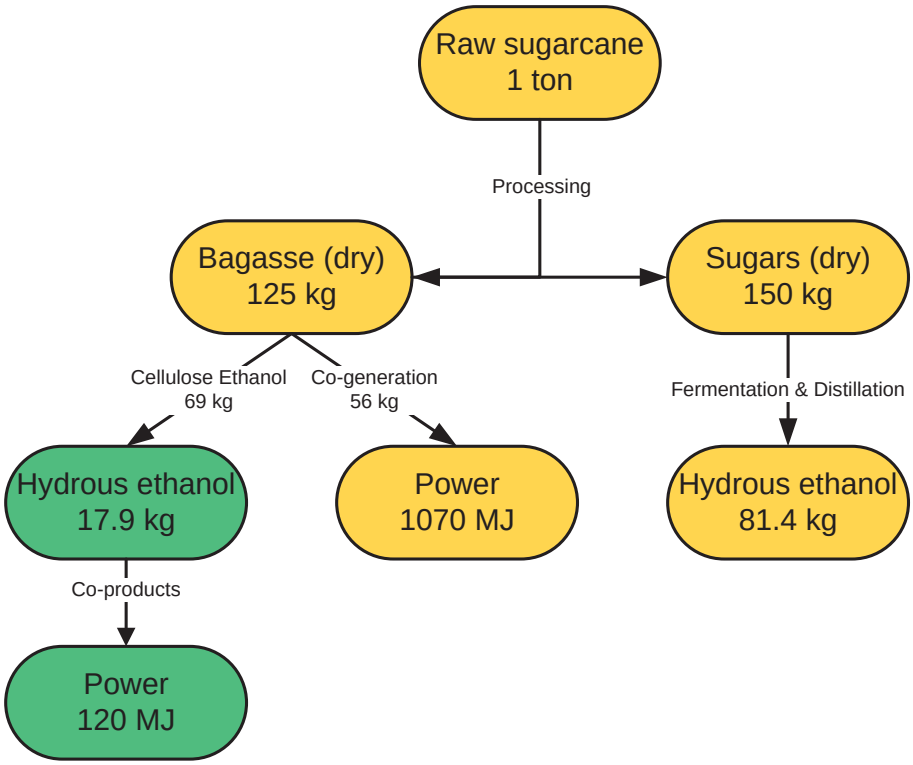

Figure 2. Mass flow of ethanol and bagasse. The conventional process is shown in light-gray and lignocellulosic ethanol conversion in the darker color.

Energy demands for bagasse-based ethanol production can be met by the combustion of co-products from the saccharification and fermentation processes (unconverted biomass and recovered lignin), and even generate a small excess. Because excess energy is available, 55% of the bagasse can be submitted to the cellulose ethanol process and still meet all energy demands of the integrated plant. Processing of 55% of the bagasse, up from 50%, results in almost a 10% increase in the volume of ethanol to be produced from sugarcane bagasse (Figure 2). In the case of a 3 Mt y^{-1} sugarcane processing plant, which is approximately the average size in Brazil (UNICA - União da Indústria de Cana-de-Açúcar), production of ethanol from bagasse as shown in Figure 2 may result in annual production of 68 ML y^{-1}.

Changes in process efficiency, lignin recovery and heat integration result in modifications in the amounts of biomass necessary/available for generation of electricity and process steam. If assuming that all bagasse were to be converted to ethanol, 32 kg of hydrous ethanol per ton of sugarcane processed could be produced,

however, this would result in a lack of roughly 1,000 MJ per ton of sugarcane processed to meet all energy demands for the process to be completely self-sufficient.

4.2.2. Comparison of different solid loadings

Increasing the liquid to solid ratio significantly improves enzymatic hydrolysis while maintaining enzyme loading constant (enzyme activity per biomass). Numerous studies have been published using different substrates, solid loadings and enzyme loadings, making comparison analyses extremely difficult. Studies in which saccharification and fermentation are preformed separately often cite product (glucose) inhibition as one of the factors limiting hydrolysis efficiency (Dien, Ximenes et al. 2008), however, the same can be observed in simultaneous saccharification and fermentation systems (SSF), suggesting that mass transfer limitations or other effects related to the increased content of insoluble solids, such as non-productive adsorption of enzymes may explain this limitation (Kristensen, Felby et al. 2009).

From data obtained in previously published works, it was possible to make a general relationship between the percent solids loading in the SSF reactor and conversion efficiency. It was demonstrated that even during consolidated processing (SSF), an increased solids loading resulted in a decrease in conversion percentage (Kristensen, Felby et al. 2009). The effect of solids loading on the energy necessary for processing was evaluated and as expected, as the percent solids loading increased and efficiency decreased, the energy needs also decreased due to the extra energy required for distillation of a more diluted is greater (Figure 3). Decreased solids loading percentage also requires greater quantities of water which must then be treated from the distillation column bottoms.

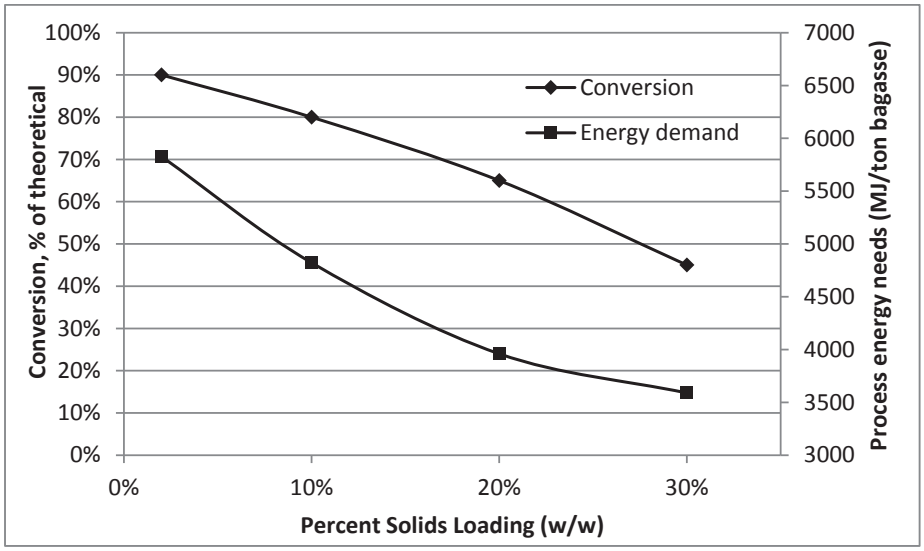

Figure 3. Effect of solids loading on conversion efficiency and process energy needs.

4.2.3 Power generation

Co-generation of process steam and electricity to meet the power demands of the plant allows that sugarcane processing be self-sufficient, when disregarding energy demands of agricultural production. Inefficient low pressure boilers were previously for power generation and required that nearly all bagasse be combusted to meet energy demands, however these are being replaced by efficient medium pressure boilers and thus a greater portion of the bagasse is used for generation of electricity, or potentially lignocellulosic ethanol (Goldemberg, Coelho et al. 2008).

The bottoms stream from distillation is composed principally of water but also contains un-hydrolyzed biomass and sugars which were not fermented. Solids can be easily separated from this stream via filtration of centrifugation and used together with lignin for co-generation. In the generated model, it was calculated that for each ton of bagasse subjected to the ethanol conversion process, recovered lignin and distillation bottoms accounted for 5,600 MJ.

5. Conclusions

Sugarcane bagasse possesses the potential to significantly improve ethanol production while allowing that sugarcane mills remain independent of external energy sources for processing, utilizing only the biomass co-product. In the case of sugarcane bagasse and utilization of a complex enzyme cocktail, it was considered that a delignification pretreatment would be more adequate than an acid-based method for hemicellulose hydrolysis. It was then concluded that of the bagasse generated during conventional sugarcane milling, slightly more than half (55%) could be converted to ethanol while the other 45% summed with the by-products of the cellulosic ethanol process were sufficient to meet all energy demands. Process inefficiencies in the conversion of long-chain polysaccharides to monosaccharide sugars, as well as poor pentose fermenting ability of yeasts, results in overall process efficiencies which will likely be improved with advances in biochemical process optimization; however, these inefficiencies allow that the unconverted biomass fraction be sufficient to meet the energy demands of the process. Process integration opportunities are numerous, but should not be generalized due to the complexity of each individual scenario.

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7. References

Alvira, P., E. Tomás-Pejó, et al. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review." Bioresource Technology **101**(13): 4851-4861.

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

Amidon, T. E. (2006). "The biorefinery in New York: woody biomass into commercial ethanol." Pulp & Paper-Canada **107**(6): 47-50.

Botha, T. and H. von Blottnitz (2006). "A comparison of the environmental benefits of bagasse-derived electricity and fuel ethanol on a life-cycle basis." Energy Policy **34**(17): 2654-2661.

Buaban, B., H. Inoue, et al. (2010). "Bioethanol production from ball milled bagasse using an on-site produced fungal enzyme cocktail and xylose-fermenting Pichia stipitis." Journal of Bioscience and Bioengineering **110**(1): 18-25.

Cardona, C. A., J. A. Quintero, et al. (2010). "Production of bioethanol from sugarcane bagasse: Status and perspectives." Bioresource Technology **101**(13): 4754-4766.

Carlsson, P., H. Wiinikka, et al. (2010). "Experimental investigation of an industrial scale black liquor gasifier. 1. The effect of reactor operation parameters on product gas composition." Fuel **89**(12): 4025-4034.

Carrasco, C., H. M. Baudel, et al. (2010). "SO2-catalyzed steam pretreatment and fermentation of enzymatically hydrolyzed sugarcane bagasse." Enzyme and Microbial Technology **46**(2): 64-73.

Chen, J. C. P. and C. C. Chou (1993). Cane sugar handbook: a manual for cane sugar manufacturers and their chemists, John Wiley and Sons.

Cheng, K.-K., J.-A. Zhang, et al. (2008). "Sugarcane Bagasse Mild Alkaline/Oxidative Pretreatment for Ethanol Production by Alkaline Recycle Process." Applied Biochemistry and Biotechnology **151**(1): 43-50.

Coelho, S. T., J. Goldemberg, et al. (2006). "Brazilian sugarcane ethanol: lessons learned." Energy for Sustainable Development **10**(2): 26-39.

CONAB, C. N. d. A. (2011). "3º Levantamento cana-de-açúcar Safra - 2011/2012." Retrieved July 20, 2012, from http://www.conab.gov.br/conteudos.php?a=1253&t.

da Silva, A. S., H. Inoue, et al. (2010). "Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation." Bioresource Technology **101**(19): 7402-7409.

da Silva, A. S. A., H. Inoue, et al. (2010). "Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation." Bioresource Technology **101**(19): 7402-7409.

Dawson, L. and R. Boopathy (2007). "Use of post-harvest sugarcane residue for ethanol production." Bioresource Technology **98**(9): 1695-1699.

de Almeida, M. N., V. M. Guimaraes, et al. (2011). "Cellulases and hemicellulases from endophytic Acremonium species and its application on sugarcane bagasse hydrolysis." Applied Biochemistry and Biotechnology **165**(2): 594-610.

De Figueiredo, E. B. and N. La Scala (2011). "Greenhouse gas balance due to the conversion of sugarcane areas from burned to green harvest in Brazil." Agriculture Ecosystems & Environment **141**(1-2): 77-85.

Dias, M. O. S., T. L. Junqueira, et al. (2012). "Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash." Bioresource Technology **103**(1): 152-161.

Dien, B. S., E. A. Ximenes, et al. (2008). "Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol." Bioresource Technology **99**(12): 5216-5225.

El-Zawawy, W. K., M. M. Ibrahim, et al. (2011). "Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production." Carbohydrate Polymers **84**(3): 865-871.

Engel, P., S. Krull, et al. (2012). "Rational approach to optimize cellulase mixtures for hydrolysis of regenerated cellulose containing residual ionic liquid." Bioresource Technology **115**(0): 27-34.

Enweremadu, C., A. Waheed, et al. (2009). "Parametric study of an ethanol-water distillation column with direct vapour recompression heat pump." Energy for Sustainable Development **13**(2): 96-105.

Goldemberg, J., S. T. Coelho, et al. (2008). "The sustainability of ethanol production from sugarcane." Energy Policy **36**(6): 2086-2097.

Green, D. and R. Perry (2007). Perry's Chemical Engineers' Handbook, Eighth Edition, McGraw-Hill Professional.

Gryta, M., A. W. Morawski, et al. (2000). "Ethanol production in membrane distillation bioreactor." Catalysis Today **56**(1-3): 159-165.

Hector, R. E., B. S. Dien, et al. (2010). "Engineering industrial Saccharomyces cerevisiae strains for xylose fermentation and comparison for switchgrass conversion." J Ind Microbiol Biotechnol.

Humbird, D., R. Davis, et al. (2011). "Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover." NREL Report No. TP-5100-47764.

Jutakanoke, R., N. Leepipatpiboon, et al. (2012). "Sugarcane leaves: Pretreatment and ethanol fermentation by Saccharomyces cerevisiae." Biomass and Bioenergy **39**(0): 283-289.

Kang, H.-W., Y. Kim, et al. (2012). "Cellulosic ethanol production on temperature-shift simultaneous saccharification and fermentation using the thermostable yeast Kluyveromyces marxianus CHY1612." Bioprocess and Biosystems Engineering **35**(1): 115-122.

Katahira, S., M. Ito, et al. (2008). "Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating S. cerevisiae via expression of glucose transporter Sut1." Enzyme and Microbial Technology **43**(2): 115-119.

Kristensen, J. B., C. Felby, et al. (2009). "Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose." Biotechnology for Biofuels **2**: 11.

Kuhad, R. C., R. Gupta, et al. (2011). "Bioethanol production from pentose sugars: Current status and future prospects." Renewable and Sustainable Energy Reviews **15**(9): 4950-4962.

Kumar, R. and C. E. Wyman (2009). "Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies." Bioresource Technology **100**(18): 4203-4213.

Lane, M. M. and J. P. Morrissey (2010). "Kluyveromyces marxianus: A yeast emerging from its sister's shadow." Fungal Biology Reviews **24**(1-2): 17-26.

Lavigne, A. and S. E. Powers (2007). "Evaluating fuel ethanol feedstocks from energy policy perspectives: A comparative energy assessment of corn and corn stover." Energy Policy **35**(11): 5918-5930.

Lewandowicz, G., W. Białas, et al. (2011). "Application of membrane distillation for ethanol recovery during fuel ethanol production." Journal of Membrane Science **375**(1–2): 212-219.

Li, X. and T. Kim (2012). "Bioconversion of corn stover derived pentose and hexose to ethanol using cascade simultaneous saccharification and fermentation (CSSF)." Bioprocess and Biosystems Engineering **35**(1): 99-104.

Luo, L., E. van der Voet, et al. (2009). "Life cycle assessment and life cycle costing of bioethanol from sugarcane in Brazil." Renewable and Sustainable Energy Reviews **13**(6–7): 1613-1619.

Macrelli, S., J. Mogensen, et al. (2012). "Techno-economic evaluation of 2nd generation bioethanol production from sugar cane bagasse and leaves integrated with the sugar-based ethanol process." Biotechnology for Biofuels **5**(1): 22.

Maeda, R. N., V. I. Serpa, et al. (2011). "Enzymatic hydrolysis of pretreated sugar cane bagasse using Penicillium funiculosum and Trichoderma harzianum cellulases." Process Biochemistry **46**(5): 1196-1201.

Mesa, L., E. González, et al. (2011). "The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse." Chemical Engineering Journal **168**(3): 1157-1162.

Meyer, A. S., L. Rosgaard, et al. (2009). "The minimal enzyme cocktail concept for biomass processing." Journal of Cereal Science **50**(3): 337-344.

Minu, K., K. K. Jiby, et al. (2012). "Isolation and purification of lignin and silica from the black liquor generated during the production of bioethanol from rice straw." Biomass and Bioenergy(0).

Minu, K., K. K. Jiby, et al. (2012). "Isolation and purification of lignin and silica from the black liquor generated during the production of bioethanol from rice straw." Biomass and Bioenergy **39**(0): 210-217.

MME - Brazilian Federal Government Ministry of Mines and Energy. (2011). "Brazilian Energy Balance." Retrieved July 20, 2012, from https://ben.epe.gov.br/.

Naik, S. N., V. V. Goud, et al. (2010). "Production of first and second generation biofuels: A comprehensive review." Renewable and Sustainable Energy Reviews **14**(2): 578-597.

Novozymes (2009). The Novozymes Report 2009.

Piccolo, C. and F. Bezzo (2009). "A techno-economic comparison between two technologies for bioethanol production from lignocellulose." Biomass and Bioenergy **33**(3): 478-491.

Qureshi, N., B. S. Dien, et al. (2006). "Genetically Engineered Escherichia Coli for Ethanol Production from Xylose: Substrate and Product Inhibition and Kinetic Parameters." Food and Bioproducts Processing **84**(2): 114-122.

Rivera, E. C., S. C. Rabelo, et al. (2010). "Enzymatic hydrolysis of sugarcane bagasse for bioethanol production: determining optimal enzyme loading using neural networks." Journal of Chemical Technology & Biotechnology **85**(7): 983-992.

Rocha, G. J. M., A. R. Gonçalves, et al. (2012). "Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production." Industrial Crops and Products **35**(1): 274-279.

Saha, B. C. and M. A. Cotta (2008). "Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol." Biomass and Bioenergy **32**(10): 971-977.

Saha, B. C., N. N. Nichols, et al. (2011). "Ethanol production from wheat straw by recombinant Escherichia coli strain FBR5 at high solid loading." Bioresource Technology **102**(23): 10892-10897.

Sánchez, Ó. J. and C. A. Cardona (2012). "Conceptual design of cost-effective and environmentally-friendly configurations for fuel ethanol production from sugarcane by knowledge-based process synthesis." Bioresource Technology **104**(0): 305-314.

Seabra, J. E. A., L. Tao, et al. (2010). "A techno-economic evaluation of the effects of centralized cellulosic ethanol and co-products refinery options with sugarcane mill clustering." Biomass and Bioenergy **34**(8): 1065-1078.

Shafiei, M., K. Karimi, et al. (2011). "Techno-economical study of ethanol and biogas from spruce wood by NMMO-pretreatment and rapid fermentation and digestion." Bioresource Technology **102**(17): 7879-7886.

Sheng, C. and J. L. T. Azevedo (2005). "Estimating the higher heating value of biomass fuels from basic analysis data." Biomass and Bioenergy **28**(5): 499-507.

Shi, J., R. R. Sharma-Shivappa, et al. (2009). "Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production." Biomass and Bioenergy **33**(1): 88-96.

UNICA - União da Indústria de Cana-de-Açúcar. (May 1, 2011). Retrieved July 20, 2012, from www.unica.com.br.

US Department of Energy. (2004, May 2011). "Biomass Feedstock Composition and Property Database." Retrieved July 20, 2012, from http://www.afdc.energy.gov/biomass/progs/search1.cgi.

Visser, E., D. Filho, et al. (2012). "Simultaneous saccharification and fermentation (SSF) of Jatropha curcas shells: utilization of co-products from the biodiesel production process." Bioprocess and Biosystems Engineering **35**(5): 801-807.

Wallberg, O. and A. S. Jonsson (2006). "Separation of lignin in kraft cooking liquor from a continuous digester by ultrafiltration at temperatures above 100 degrees C." Desalination **195**(1-3): 187-200.

Watanabe, T., I. Watanabe, et al. (2011). "A UV-induced mutant of Pichia stipitis with increased ethanol production from xylose and selection of a spontaneous mutant with increased ethanol tolerance." Bioresource Technology **102**(2): 1844-1848.

Zhang, M., C. Eddy, et al. (1995). "Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic Zymomonas mobilis." Science **267**(5195): 240-243.

Zhao, X., F. Peng, et al. (2009). "Enhancement of the enzymatic digestibility of sugarcane bagasse by alkali-peracetic acid pretreatment." Enzyme and Microbial Technology **44**(1): 17-23.

Zhu, Z., M. Zhu, et al. (2012). "Pretreatment of sugarcane bagasse with NH4OH–H2O2 and ionic liquid for efficient hydrolysis and bioethanol production." Bioresource Technology **119**(0): 199-207.

Chapter 3

Analysis of three yeast strains and their application in hydrolysis and fermentation of sugarcane bagasse using the enzyme cocktail produced by *Chrysoporthe cubensis*

Abstract

Hybrid saccharification and fermentation (HSF) has been proposed as a method to increase conversion of lignocellulosic biomass to bioethanol. Optimally xylose would be simultaneously fermented so that neither glucose nor xylose inhibit the enzymatic hydrolysis process. In the present study three yeasts (*Saccharomyces cerevisiae* YRH-400, *Kluyveromyces marxianus* ATCC 8554 and *Kluyveromyces marxianus* UFV-3) were evaluated with respect to their ability to ferment both glucose and xylose. The enzyme extract from *Chrysoporthe cubensis* was also evaluated with respect to its ability to hydrolyze sugarcane bagasse alone and when integrated with the analyzed yeasts in HSF. It was found that the yeast strain *Saccharomyces cerevisiae* YRH-400 most efficiently fermented both glucose and xylose to ethanol, acquiring ethanol yields of 0.477 g/g glucose and 0.24 g/g xylose with additional xylitol yield of 0.49 g/g xylose. Separate hydrolysis and fermentation was more efficient that HSF. Similarly, pre-hydrolysis for 48 hours followed by fermentation at 30°C resulted in greater ethanol yields than the 12 hour pre-hydrolysis time with HSF at 40°C. The enzyme extract from *C. cubensis* was very efficient for hydrolyzing hemicellulose and converted roughly 50% of cellulose. Lower efficiencies were obtained for HSF. As the pre-hydrolysis time was reduced the efficiencies decreased accordingly, resultant of lowering the temperature further from the optimal temperature for enzyme hydrolysis.

1. Introduction

Bioethanol is considered a carbon neutral, sustainable and renewable fuel which can be produced from a number of different agricultural residues to replace fossil fuels. Native biomass presents limited enzyme accessibility due to its compact and rigid structure, also known as biomass recalcitrance. To overcome this natural limitation, pretreatment steps are included in the biochemical process for biomass conversion to more valuable products. Application of enzyme extracts rich in hemicellulases as well as cellulases not only allows for the production of pentose sugars, but facilitates access of cellulase enzymes resulting in greater hexose sugar yields which may be due to increased fiber swelling and fiber porosity (Gao, Uppugundla et al. 2011; Hu, Arantes et al. 2011; Sills and Gossett 2011).

The filamentous fungi *Chrysoporthe cubensis* was previously assessed with respect to its enzyme production ability, and it was found that this fungi produces an extract exceptionally rich in hemicellulase enzymes (Falkoski, Guimaraes et al. 2013). The authors compared the extract produced by this fungus with a commercial enzyme product and found that the produced extract was more efficient for hydrolyzing alkali-pretreated sugarcane bagasse per FPU of enzyme applied. Because the extract produced by *C. cubensis* is rich in xylanase enzymes, application of an alkali pretreatment allows for removal of lignin in order to make the cellulose and hemicellulose fractions more enzyme accessible. Many published studies have sought to obtain an optimal enzyme mixtures, but often by blending different commercial extracts (Suwannarangsee, Bunterngsook et al. 2012). Utilization of a single organism capable of generating this blend of enzymes therefore presents the potential to reduce costs of enzyme purification and may potentially facilitate the overall enzyme production process.

Conversion of C6 sugars liberated via enzymatic hydrolysis is a well understood and documented process, typically performed with the yeast *Saccharomyces cerevisiae*. Conversion of C5 sugars to ethanol is more complicated and numerous studies have been performed utilizing native strains of bacteria, yeasts and filamentous fungi capable of fermenting xylose to ethanol (Skoog and Hahn-Hägerdal 1988), however often times these strains do not display desired industrial characteristics, such as resistance to inhibitors commonly found in hydrolyzates (Yuan, Rao et al. 2011). Hybrid saccharaification and fermentation (HSF) has been extensively studied since it permits for integration of saccharification and fermentation processes, while minimizing the effect of the difference in optimal temperature of enzymes and fermenting microorganisms. The term “hybrid saccharification and fermentation” refers to a process wherein biomass is saccharified to a limited extent in a pre-hydrolysis period (incomplete or partial saccharification), followed by continued saccharification and fermentation occurring simultaneously. One of the major advantages of HSF is the fact that the released monosaccharide sugars are rapidly consumed in fermentation, preventing that they inhibit enzymes in the breakdown of cellulose and hemicellulose. A major disadvantage of HSF is due to the fact that yeast are difficult to recover after fermentation, since in centrifugation or filtration unit operations the yeast biomass is found together with the remaining lignocellulosic biomass (Watanabe, Miyata et al.

2012). Due to the advantages and disadvantages of both processes, no general consensus has been reached but is instead dependent on other processing parameters.

The objective of the present study was to evaluate the capacities of three different yeast species (*Saccharomyces cerevisiae* YRH 400, *Kluyveromyces marxianus* ATCC-8554 and *Kluyveromyces marxianus* UFV-3) for fermenting of glucose and xylose to ethanol. The same yeasts were then assessed with regards to their applicability in HSF processes and compared with yields obtained from separate hydrolysis and fermentation.

2. Material and Methods

2.1. Raw materials and pretreatment

Sugarcane bagasse was obtained from the Center for Sugarcane Research and Breeding of the Federal University of Viçosa, Brazil. In the laboratory this was again washed and dried in an oven at 70°C until reaching a constant mass, after which it was further milled in a knife mill (Marconi, Piracicaba, SP, Brazil) to obtain a particle size less than 1 mm with appropriate sieve and submitted to alkaline pretreatment. Sodium hydroxide 1.5% (w/v) was used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v); and treatments were performed in an autoclave at 120°C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with Whatman no. 1 filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at -20°C.

2.2. Microorganisms and cultivation

2.2.1. *Saccharomyces cerevisiae* YRH-400, *Kluyveromyces marxianus* ATCC 8554 and *Kluyveromyces marxianus* UFV-3

The yeast strain *S. cerevisiae* YRH-400 was kindly provided by the National Center for Agricultural Utilization Research, USDA; *K. marxianus* ATCC-8554 was purchased from the ARS Culture Collection (NRRL) with collection number Y-1109; and *K. marxianus* UFV-3 was kindly provided by the Department of Microbiology at the Federal University of Viçosa, Brazil. All yeasts were individually cultured in YPD

medium (yeast extract 1%, peptone 1% and glucose 1%) in a rotary shaker at 28°C and 180 rpm for 24 h, followed by preservation in 20% glycerol in a freezer at -80°C.

For cell production from *S. cerevisiae* YRH-400, *K. marxianus* ATCC-8554 and *K. marxianus* UFV-3, the same YPD medium was employed and cells (20 µL) were transferred directly from the freezer stock to the YPD medium in a 250 mL Erlenmeyer flask with working volume of 100 mL. Flasks were maintained for 24 hours in a rotary shaker at 28°C and 180 rpm. After this period the medium was centrifuged (3,000 x *g*), the supernatant was discarded, and the cells were resuspended in fresh YPD medium. Optical density of the resuspended cells was then measured at 600 nm.

2.2.2. *Chrysoporthe cubensis*

The fungus *C. cubensis* LPF-1 used in this study was obtained from the mycological collection of the Forest Pathology Laboratory, Federal University of Viçosa, MG, Brazil (Falkoski, Guimaraes et al. 2013). The fungus was maintained on PDA plates at 28 °C and subcultured periodically. The inoculum was prepared by growing the fungus under submerged fermentation in 250 mL Erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Each flask was inoculated with 10 agar plugs cut out of a 5 day-old colony of *C. cubensis* grown on PDA plates and maintained in a rotary shaker for 5 days, at 150 rpm and 28 °C. The culture obtained was aseptically homogenized with Polytron® and immediately used to inoculate the solid culture media.

For enzyme production via solid state fermentation, 250 mL Erlenmeyer contained 12.5 g of wheat bran and 18.75 mL of the culture media (final moisture of 60%) consisting of, in g/L, NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2. Furthermore, $MnCl_2$ (0.1 mg/L), H_3BO_3 (0.075 mg/L), Na_2MoO_4 (0.02 mg/L), $FeCl_3$ (1.0 mg/L) and $ZnSO_4$ (3.5 mg/L) also were added to the medium as trace elements. The flasks were autoclaved at 120°C for 20 min and then supplemented with 5 mL (containing 1.5 X 10^7 spores/mL) of inoculum obtained as aforementioned. The flasks were maintained at 28 °C in a controlled temperature chamber and the enzymatic extraction was performed after 7 days of fermentation. Enzymes secreted during HSF were extracted with sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate), under agitation of 150 rpm for 60 min at room temperature. Solids were

separated by filtration through cotton wool followed by centrifugation at 15000*g* for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis and application.

2.3. Batch Fermentation

YP cultures were prepared in 50 mL Erlenmeyer flasks with working volume of 25 mL, and supplemented with 40 g/L xylose or 20 g/L xylose + 20 g/L glucose. Flasks were sealed with rubber stoppers and fitted with one sampling needle and one needle for CO_2 venting. After sterilization, these were inoculated with yeast cells of the three evaluated strains to an OD_{600} of 2.0. Nitrogen was bubbled through the medium of all flasks via the sampling needle for five minutes and the flasks were then transferred to a rotary shaker at 30°C and 150 rpm where the experiment was conducted. Samples were taken from the fermentation flasks at predetermined time intervals and analyzed by HPLC for determination of remaining sugar and fermentation products.

2.4. Enzyme production

The crude enzyme extract produced by *C. cubensis* was partially concentrated using a Micron ultrafiltration unit (Millipore Corporation, Bedford, MA) for later application in sugarcane hydrolysis assays. FPase, endoglucanase, β-glucosidase and xylanase activities were measured according to previously published methods, as reported in (Falkoski, Guimaraes et al. 2013).

2.5. Biomass pretreatment and composition analysis

Sugarcane bagasse was obtained from the Center for Research and Breeding of Sugarcane of the Federal University of Viçosa, Brazil, from which the sugars had already been extracted. In the laboratory this was again washed and dried in an oven at 70°C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) and submitted to alkaline pretreatment prior to being employed in saccharification experiments. Sodium hydroxide 1.5% was used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v); and treatments were performed in an autoclave at 120°C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at -20°C.

Chemical composition of the untreated and alkali-treated sugarcane bagasse samples were determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 (TAPPI, 1999). Extractive-free biomass (0.3 g) was incubated at 30°C with 3 mL of 72% H_2SO_4 for 1 h with occasional mixing. The slurry was then transferred into a penicillin bottle containing 84 mL of deionized water and the flask was sealed with a rubber stopper and aluminum seal. The bottle was placed in an autoclave calibrated at 118 C for 1 h, then the slurry was filtered through a medium coarseness sintered glass filter for gravimetric determination of acid-insoluble lignin. Concentrations of biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate were quantified using high-performance liquid chromatography (HPLC), while acid-soluble lignin was determined by absorption measurements at 205 nm (TAPPI, 1991). The HPLC system Dionex DX-300 (Dionex Co. – Sunnyvale, CA, USA) was equipped with a Carbopac PA1 column and a pulsed amperometric detector with a gold electrode. Prior to injection, samples were filtered through 0.45-mm HV filters and a volume of 20 μL was loaded into the chromatograph system. The column was pre-equilibrated with a NaOH solution, 300 mM, and elution was carried out at a flow rate of 1.0 mL/min at room temperature.

2.6. Enzymatic saccharification and fermentation

Enzymatic hydrolysis was performed in 125 mL Erlenmeyer flasks with 50 mL working volume, subjected to mechanical agitation 150 rpm and an external water bath for maintaining the desired hydrolysis temperature (50°C). Flasks were plugged with rubber stoppers through which a needle was used for sampling at predetermined times. Solids and enzyme loadings in all SHF and HSF assays were 7.5% (w/v) and 10 FPU/g. The pH of the medium was maintained by application of a 1 M sodium acetate buffer (pH 5.0) to result in a final concentration of 50 mM. In the SHF assay tetracycline (40 mg/L) and azide (10 mM) were added to prevent contamination. Yeasts were inoculated to an OD_{600} of 2.0 and flasks were transferred to a rotary shaker at 30°C and 180 rpm where the experiment was conducted. All assays were performed in duplicate.

In the HSF assays, the cultivated yeasts were transferred to the flasks after the predetermined pre-hydrolysis periods. The flasks destined for the HSF analyses were initially loaded with a fermentation medium at the beginning of the saccharification period which included, in g/L, yeast extract, 2.5; peptone, 2.5; NH_4Cl, 2; KH_2PO_4, 1.0;

and $MgSO_4$, 0.3. Nitrogen was bubbled through the medium of all flasks via the sampling needle for five minutes directly after yeast inoculation and the flasks were then transferred to a rotary shaker at 30°C and 180 rpm where the experiment was conducted. All assays were performed in duplicate.

Analysis of hydrolysis efficiency compared the theoretical sugar yield based on full conversion of polysaccharides to monosaccharides with the actual measured monosaccharide concentration. Theoretical monosaccharide concentration is obtained by multiplying the concentration of polysaccharide by 1.11 for six carbon sugars or 1.136 for five carbon sugars.

2.7. Product analysis

Products of the saccharification and HSF assays were analyzed by high performance liquid chromatography (HPLC) with a Shimadzu series 10A chromatograph. The HPLC was equipped with an Aminex HPX-87P column (300 x 7.8 mm) (BioRad, Hercules, CA, USA) and refractive index detector (Shimadzu Corporation, Kyoto, Japan). The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 mL/min and 65°C.

3. Results and Discussion

3.1. Yeast comparison

The yeasts *S. cerevisiae* YRH-400, *K. marxianus* ATCC-8554 and *K. marxianus* UFV-3 were evaluated with respect to their ability to ferment both glucose and xylose. For this purpose, fermentation assays were conducted with pure xylose (40 g/L) or a mixture of glucose and xylose (20 g/L of each) under conditions of hypoxia, at 30°C for 120 hours.

In the fermentation assays comparing the three yeasts, in all cases glucose was rapidly consumed within the first 12 hours (Figure 1-1A, 2A and 3A). After consuming glucose, xylose was partially consumed during a 120 h period (Figure 1 and Table 1). The *S. cerevisiae* YRH-400 strain was by far the efficient for fermentation of both glucose and xylose. When comparing the two strains of *K. marxianus*, the strain ATCC-8554 much more efficiency utilized xylose when glucose/ethanol was present than the

UFV-3 strain, however xylose appeared to be entirely fermented to xylitol (Figure 1 – 2A and 3A). When only xylose was present, strain UFV-3 consumed less xylose but produced nearly the same amount of xylitol as the ATCC-8554 strain (Figure 1 – 2B and 3B). This difference may be due to the higher xylose concentration in the assay, where it has previously been shown that sugar utilization decreases with initial sugar concentration (Kumar, Singh et al. 2009). A study using a pure culture of *Kluyveromyces marxianus* (DSMZ 7239) indicated that for an initial sugar loading of 50 g/L, less than 10% of the sugar was utilized by the yeast, while at a concentration of 200 g/L a total of 90% of sugars were consumed (Ozmihci and Kargi 2007).

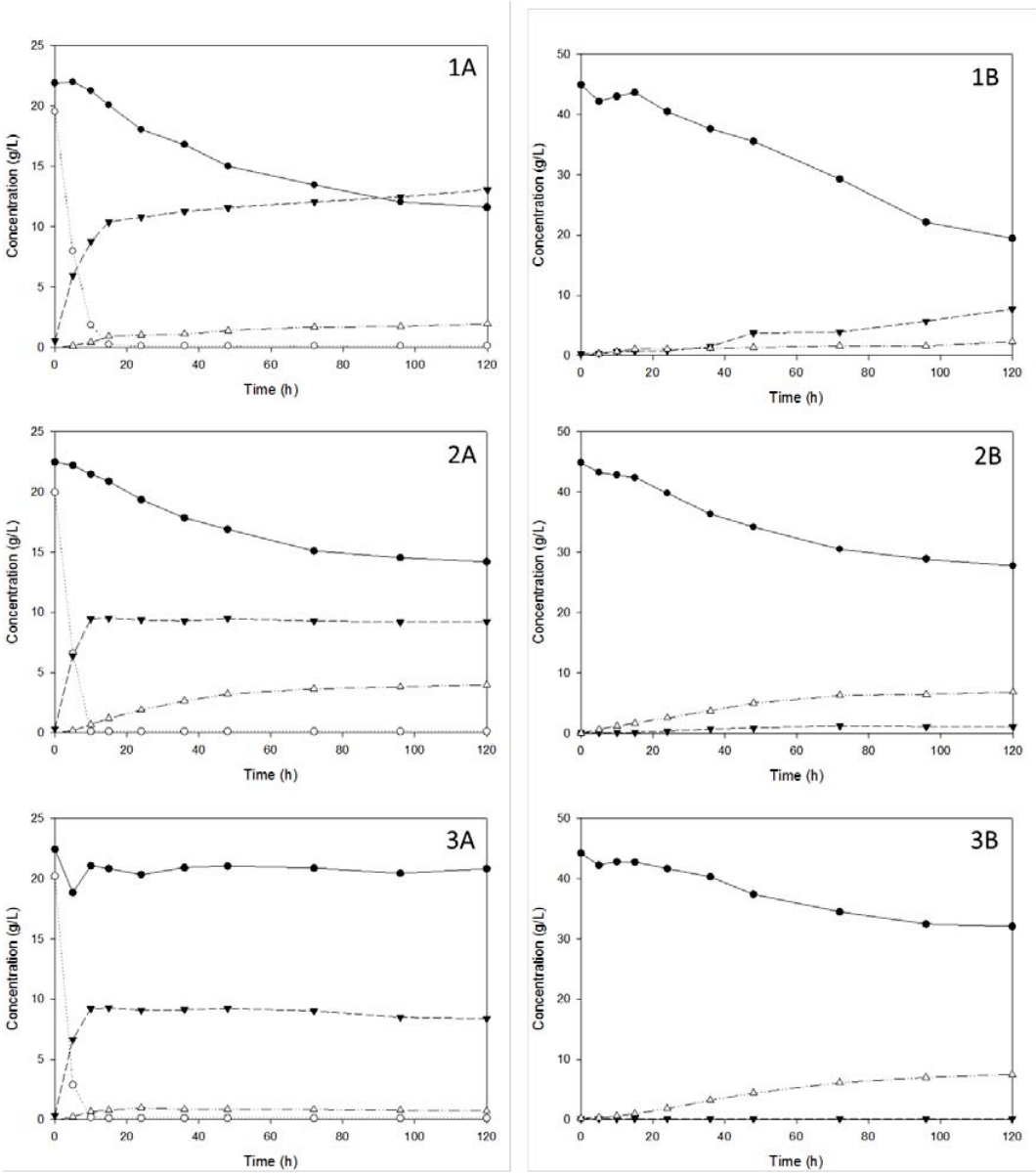

Figure 1. Fermentation of xylose + glucose (A) and xylose alone (B) using the yeasts *S. cerevisiae* YRH-400 (1), *K. marxianus* ATCC-8554 (2) and *K. marxianus* UFV-3 (3). Glucose (open circle), xylose (closed circle), ethanol (closed triangle) and xylitol (open triangle).

In the present study all fermentations were performed at 30°C, however the yeast strain *K. marxianus* was selected due to its tolerance to high temperatures (de Souza, Costa et al. 2012) considering application in HSF. It has been reported that strains of *K. marxianus* are capable of producing ethanol at temperatures up to 45°C, close to the optimal hydrolysis temperature of cellulolytic enzymes, and ethanol

fermentation results have been reported as being the same when performed at 30 or 40°C (Nonklang, Abdel-Banat et al. 2008).

Table 1. Consumed xylose, product concentration and yield from xylose fermentation (40 g/L) using different yeast strains after a 120 hour period.

	Xylose consumed (g/L)	Ethanol produced (g/L)	Xylitol produced (g/L)	Glycerol produced (g/L)	$Y_{ethanol}$ (g/g xylose)	$Y_{xylitol}$ (g/g xylose)	$Y_{glycerol}$ (g/g xylose)
S. cerevisiae YRH-400	25.47	7.70	2.29	0.2	0.30	0.09	0.01
K. marxianus ATCC 8554	17.13	1.09	6.86	0	0.06	0.40	0
K. marxianus UFV-3	12.13	0.13	7.44	0	0.01	0.61	0

The *S. cerevisiae* YRH-400 strain yielded 0.30 g ethanol/g xylose and 0.09 g xylitol/g xylose, being the only yeast to effectively ferment xylose to ethanol. This strain was genetically engineered by chromosomal integration of the *Pichia stipitis* xylose reductase (XYL1), *P. stipitis* xylitol dehydrogenase (XYL2), and *S. cerevisiae* xylulokinase (XKS1) genes for xylose fermentation (Hector, Dien et al. 2011). In the study performed by Hector, Dien et al. (2011) this strain presented greater xylitol yield than ethanol when cultivated under anaerobic conditions (0.24 g ethanol/g xylose and 0.49 g xylitol/g xylose). In the present study with *S. cerevisiae* YRH-400 xylitol yields were significantly lower (0.09 g xylitol/g xylose), however because the main objective was to produce ethanol and remove xylose from the fermentation medium, this was not viewed as a negative point. Varying oxygen concentration in the medium may have triggered increased ethanol yields at the expense of xylitol (Yablochkova, Bolotnikova et al. 2004; Ishtar Snoek and Yde Steensma 2006).

The two *K. marxianus* strains presented minimal ethanol production from xylose (0.06 and 0.01 g ethanol/g xylose for strains ATCC-8554 and UFV-3, respectively) and both consumed significantly less xylose than the modified *S. cerevisiae* strain, however *K. marxianus* ATCC-8554 consumed 30% more xylose than the *K. marxianus* UFV-3 strain for the initial xylose concentration of 40 g/L (17.13 and 12.13 g, respectively). *K. marxianus* UFV-3 more efficiently converted xylose to xylitol, yielding 0.61 g xylitol/g xylose, however the strain *K. marxianus* ATCC-8554 showed more appealing characteristics for application in HSF due to greater consumption of xylose and potential for producing small concentrations of ethanol (Figure 1-2A and 3A). It has previously been reported that variations are encountered among *K. marxianus* strains

with regards to their ethanol fermentation effectiveness, where initial the substrate concentration also plays an important role (Lane and Morrissey 2010).

Table 2. Consumed sugar, product concentration and yield from glucose and xylose co-fermentation (20 g/L glucose and 20 g/L xylose) using different yeast strains after a 120 hour period.

	Glucose consumed (g/L)	Xylose consumed (g/L)	Ethanol produced (g/L)	Xylitol produced (g/L)	Glycerol produced (g/L)	$Y_{ethanol}$ (g/g sugar)	$Y_{xylitol}$ (g/g sugar)	$Y_{glycerol}$ (g/g sugar)
S. cerevisiae YRH-400	20.00	10.29	13.08	1.95	1.8	0.43	0.06	0.06
K. marxianus ATCC 8554	20.00	7.51	9.21	3.98	1.8	0.33	0.14	0.07
K. marxianus UFV-3	20.00	1.61	8.36	0.70	1.6	0.39	0.03	0.07

The strain *S. cerevisiae* YRH-400 again showed to be most efficient for co-fermentation of both glucose and xylose (Table 2). In the co-fermentation study all glucose was rapidly consumed in the first 15 hours (Figure 1-1A). The *S. cerevisiae* strain presented an ethanol yield of 0.43 g per g of total sugar consumed, but when considering the ethanol concentration after only 15 hours of fermentation, when all glucose was consumed along with 1.8 g/L of xylose (10.4 g/L of ethanol), the ethanol yield per sugar consumed was 0.477 g/g, or 93.5% of the theoretical conversion (Figure 1A).

As was observed for *S. cerevisiae* YRH-400, after 15 hours of fermentation the ethanol yields per sugar consumed were 0.45 and 0.44 g/g for *K. marxianus* ATCC-8554 and *K. marxianus* UFV-3, respectively (Figure 1-2A and 3A). After 120 hours the yields decreased significantly because in the case of both yeasts, xylose was not fermented to ethanol. A previous study on these *K. marxianus* strains also reported that they did not ferment xylose to ethanol and reported higher ethanol yields from glucose for *K. marxianus* UFV-3 compared to *K. marxianus* ATCC-8554 (Santos, Bragança et al. 2013). The same authors found ethanol yields from glucose of only 0.25 and 0.13 were encountered for *K. marxianus* UFV-3 and *K. marxianus* ATCC-8554, respectively. In the present study it was also found that both *K. marxianus* strains poorly fermented xylose to ethanol (Table 2), and *K. marxianus* UFV-3 consumed xylose at a significantly slower rate. Moreover, in a study using *K. marxianus* ATCC-8554 for fermentation of inulin, a linear D-fructose polymer with a D-glucose terminal, a

fermentation yield of 0.467 g per g of sugar consumed was obtained (Yuan, Zhao et al. 2008).

3.2. Enzyme saccharification and fermentation

Enzyme saccharification was performed on dilute alkali pretreated biomass using the partially concentrated enzyme extract produced by *C. cubensis*. Alkali pretreated biomass showed a significant reduction in lignin (51% decrease), while preserving the cellulose (glucan) and hemicellulose (xylan + arabinan) fractions (Table 3). This is desirable when considering that the enzyme extract is rich in hemicellulases, especially xylanase.

Table 3. Composition of the raw and pretreated sugarcane bagasse.

Biomass component	Untreated bagasse (%)	Pretreated bagasse (%)
Glucan	52.8	59.2
Xylan	19.1	22.3
Arabinan	1.6	2.1
Lignin	22.1	11.4

The enzyme extract produced by *C. cubensis* showed to be extremely rich in xylanase, 93.43 U/ml. It presented FPase, β-glucosidase and endoglucanase activities of 1.46 FPU/ml, 14.30 U/ml and 12.59 U/ml, respectively. In the initial study on the potential of producing and applying an enzyme extract from *C. cubensis* for degradation of lignocellulosic biomass, the ratios of xylanase, β-glucosidase, and endoglucanase to FPase were even higher, 390.74, 35.74 and 35.02, respectively (Falkoski, Guimaraes et al. 2013) when compared to those obtained in the present study. This may be because a lower moisture content was used for cultivation in an attempt to better induce FPase activity (60% in the present study compared to 70% in the previous study), and may also be result from the concentration process.

Many previously published studies evaluating bioethanol production from sugarcane bagasse have employed purified commercial enzymes which often times do not contain the numerous auxiliary enzymes found in crude extracts produced by fungi (Soares, Travassos et al. 2011; Cao and Aita 2013; Wanderley, Martín et al. 2013). The array of different enzyme activities encountered in crude extracts may also present

synergy effects which further improve biomass hydrolysis. Synergy among cellulase enzymes was indicated by Gottschalk, Oliveira et al. (2010), which exceeded 100% for endoglucanase when blending extracts from *Trichoderma* and *Aspergillus*. The addition of hemicellulase enzymes has also been shown to increase cellulose hydrolysis, even when biomass is previously subjected to pretreatments for hemicellulose removal (eg. dilute acid, steam explosion), where these enzymes appear to increase fiber swelling and fiber porosity (Hu, Arantes et al. 2011; Várnai, Huikko et al. 2011).

Accumulation of glucose and xylose during enzyme hydrolysis is desired since higher sugar concentrations reduce the thermal demand of downstream evaporation processes; however, these monosaccharides also inhibit activity of β-glucosidase and β-xylosidase enzymes. Elevated sugar concentrations also stimulate the Crabtree effect in which yeasts produce ethanol aerobically rather than producing biomass via the tricarboxylic acid cycle. In the present study no accumulation of cellobiose or xylobiose, the substrates of β-glucosidase and β-xylosidase, was observed in either the SHF or HSF assays. This is expected in HSF since the glucose that inhibits β-glucosidases is consumed by the yeast and in SHF assays is likely due to a sufficiently large concentration of β-glucosidase and β-xylosidase enzymes in the extract utilized.

As proposed in numerous other studies, hybrid saccharification and fermentation was proposed as a method to reduce product inhibition of the enzymes, but the same major challenge is faced due to the optimal temperature of yeasts being much lower than that of enzymes (Öhgren, Bura et al. 2007). Application of thermotolerant yeasts is the most cited method for overcoming the difference between optimal saccharification and fermentation temperatures, however rarely do fermentation temperatures exceed 40-42°C (Menon, Prakash et al. 2010; Kwon, Ma et al. 2011).

From the results presented in this study, it was observed that the modified *S. cerevisiae* strain is best suited for fermentation of the glucose/xylose mixture produced by the *C. cubensis* enzyme extract. However a noticeable difference is observed between *S. cerevisiae* and *K. marxianus* in the maximum fermentation temperature, where the former is limited to about 30°C and the latter two may reach 40°C.

Table 5. Conditions of the hydrolysis and HSF experiments, as well as the final concentrations of glucose, xylose and ethanol.

Assay	Pre-hydrolysis time (h)*	Fermentation time (h)	Fermentation organism	Fermentation temperature (°C)	Glucose (g/L)	Xylose (g/L)	Ethanol (g/L)
1	120	-	-	-	22.34	18.5	
2	48	72	*S. cerevisiae* YRH-400	30	2	5.7	9.3
3	12	108	*K. marxianus* ATCC-8554	40	1.81	8.6	8.8
4	12	108	*K. marxianus* UFV-3	40	0.3	7.9	6.0

*Pre-hydrolysis temperature of 50°C
Enzyme loading was identical in all assays (10 FPU/g pretreated biomass)
Solids loading was the same in all assays (7.5% w/v)

Due to this difference in optimal temperatures, different processing techniques were evaluated (Table 5). Because *S. cerevisiae* has a significantly lower fermentation temperature, it was decided to utilize a 48 hour pre-hydrolysis period at 50°C instead of the 12 hour period utilized for the two *K. marxianus* strains. This was performed because at 30°C the enzyme activity is very low and the assay would be limited by the lower enzyme activity. This pre-hydrolysis stage is essential to speed biomass breakdown since the enzyme extract has an optimal temperature of 50°C (Falkoski, Guimaraes et al. 2013), and also to generate an initial sugar load for yeast fermentation. In a study performed on the conversion of spruce to ethanol, a 22 hour pre-hydrolysis period at 48°C prior to HSF at 32°C resulted in a 5-6% increase in conversion efficiency (Hoyer, Galbe et al. 2013). Initial results obtained indicated that when no pre-hydrolysis period is used, HSF with *K. marxianus* ATCC-8554 is more efficient than with *S. cerevisiae* YRH-400 due to the higher operating temperature, despite the fact that *S. cerevisiae* YRH-400 is a more efficient fermenter of xylose to ethanol (data not shown).

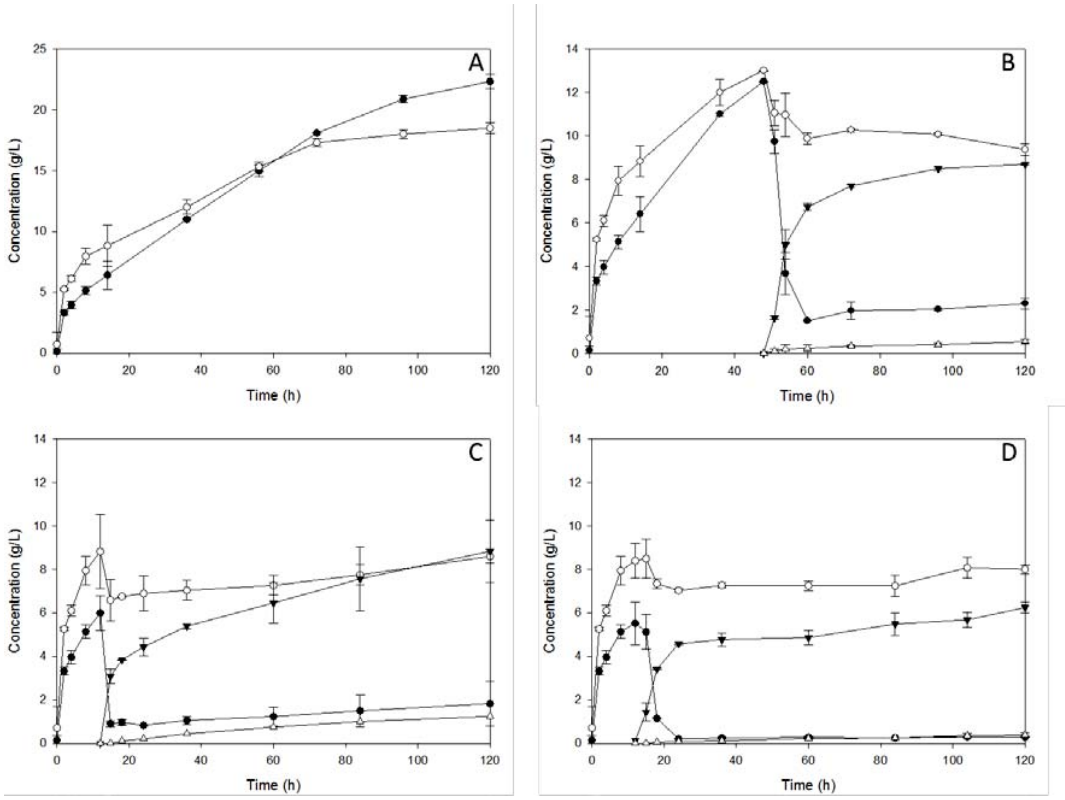

Figure 2. Product generation from enzymatic hydrolysis of sugar cane bagasse (A) and HSF with the yeasts *S. cerevisiae* YRH-400 (B), *K. marxianus* ATCC 8554 (C) and *K. marxianus* UFV-3 (D). Glucose (closed circle), xylose (open circle), ethanol (closed triangle) and xylitol (open triangle).

Based on the biomass composition and theoretical conversion of polysaccharides to monosaccharides, in the saccharification experiment (Assay 1) efficiencies of 45% and 97% were obtained for glucan and xylan hydrolysis, respectively. When considering the ethanol yield of 0.467 g per g of glucose (observed for *S. cerevisiae* YRH-400 in the yeast comparison above), this would yield 10.4 g of ethanol, more than that produced by the HSF assays even when disregarding potential conversion of xylose to ethanol. Based on this information, it appears that enzyme inhibition was not present at the give hydrolysis conditions and SHF would result in the highest ethanol yield.

In all HSF assays (Figure 2-B, C and D), the ethanol concentration gradually increased due to continuous glucose liberation and fermentation to ethanol, maintaining glucose concentrations near to zero. Xylose levels tended to rise in the assays with *K. marxianus* (Figures 2 C and D), indicating that xylose was liberated via enzymatic

hydrolysis faster than it could be consumed by the yeast. Contrarily, xylose concentrations decreased in HSF utilizing *S. cerevisiae* due to the lower temperature, less active enzymes, and greater xylose consumption, yeast with higher xylose affinity. More effective xylose removal from the HSF medium may permit more effective xylan hydrolysis, reducing inhibition of xylose on β-xylosidase enzymes, which in turn accelerates glucan hydrolysis by increasing fiber swelling and porosity (Gao, Uppugundla et al. 2011; Hu, Arantes et al. 2011).

4. Conclusions

Different processing methods for conversion of lignocellulosic biomass to ethanol may provide for improved conversion efficiencies. Although HSF may aid in eliminating inhibition to the enzyme caused by the product generated, in the present study this was not observed. The highest efficiencies were encountered when performing hydrolysis alone, or when using the extended pre-hydrolysis period of 48 hours. This is likely due to the fact that the optimal enzyme activity is found at 50°C and that of fermenting microorganisms rarely exceeds 40°C. Product inhibition may be greater when employing higher solids loading in which product generation is also likely to be greater, in which case HSF or intermittent fermentation steps may be justified.

5. References

Cao, Shuo, and Giovanna M. Aita. 2013. Enzymatic hydrolysis and ethanol yields of combined surfactant and dilute ammonia treated sugarcane bagasse. *Bioresource Technology* 131 (0):357-364.

de Souza, Carlos J. A., Daniela A. Costa, Marina Q. R. B. Rodrigues, Ancély F. dos Santos, Mariana R. Lopes, Aline B. P. Abrantes, Patrícia dos Santos Costa, Wendel Batista Silveira, Flávia M. L. Passos, and Luciano G. Fietto. 2012. The influence of presaccharification, fermentation temperature and yeast strain on ethanol production from sugarcane bagasse. *Bioresource Technology* 109 (0):63-69.

Falkoski, D. L., V. M. Guimaraes, M. N. de Almeida, A. C. Alfenas, J. L. Colodette, and S. T. de Rezende. 2013. *Chrysoporthe cubensis*: A new source of cellulases

and hemicellulases to application in biomass saccharification processes. *Bioresour Technol* 130:296-305.

Gao, Dahai, Nirmal Uppugundla, Shishir Chundawat, Xiurong Yu, Spencer Hermanson, Krishne Gowda, Phillip Brumm, David Mead, Venkatesh Balan, and Bruce Dale. 2011. Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. *Biotechnology for Biofuels* 4 (1):5.

Gnansounou, Edgard, and Arnaud Dauriat. 2010. Techno-economic analysis of lignocellulosic ethanol: A review. *Bioresource Technology* 101 (13):4980-4991.

Gottschalk, Leda Maria Fortes, Raul Alves Oliveira, and Elba Pinto da Silva Bon. 2010. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. *Biochemical Engineering Journal* 51 (1–2):72-78.

Hector, Ronald E, Bruce S Dien, Michael A Cotta, and Nasib Qureshi. 2011. Engineering industrial Saccharomyces cerevisiae strains for xylose fermentation and comparison for switchgrass conversion. *Journal of Industrial Microbiology & Biotechnology* 38 (9):1193-1202.

Hoyer, Kerstin, Mats Galbe, and Guido Zacchi. 2013. The effect of prehydrolysis and improved mixing on high-solids batch simultaneous saccharification and fermentation of spruce to ethanol. *Process Biochemistry* 48 (2):289-293.

Hu, Jinguang, Valdeir Arantes, and Jack Saddler. 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnology for Biofuels* 4 (1):36.

Ishtar Snoek, I. S., and H. Yde Steensma. 2006. Why does *Kluyveromyces lactis* not grow under anaerobic conditions? Comparison of essential anaerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces lactis* genome. *FEMS Yeast Research* 6 (3):393-403.

Kumar, Sachin, Surendra P Singh, Indra M Mishra, and Dilip K Adhikari. 2009. Ethanol and xylitol production from glucose and xylose at high temperature by *Kluyveromyces* sp. IIPE453. *Journal of Industrial Microbiology & Biotechnology* 36 (12):1483-1489.

Kwon, Yong-Jin, An-Zhou Ma, Qian Li, Feng Wang, Guo-Qiang Zhuang, and Chun-Zhao Liu. 2011. Effect of lignocellulosic inhibitory compounds on growth and

ethanol fermentation of newly-isolated thermotolerant *Issatchenkia orientalis*. *Bioresource Technology* 102 (17):8099-8104.

Lane, Melanie M., and John P. Morrissey. 2010. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biology Reviews* 24 (1–2):17-26.

Menon, Vishnu, Gyan Prakash, Asmita Prabhune, and Mala Rao. 2010. Biocatalytic approach for the utilization of hemicellulose for ethanol production from agricultural residue using thermostable xylanase and thermotolerant yeast. *Bioresource Technology* 101 (14):5366-5373.

Nonklang, Sanom, Babiker M. A. Abdel-Banat, Kamonchai Cha-aim, Nareerat Moonjai, Hisashi Hoshida, Savitree Limtong, Mamoru Yamada, and Rinji Akada. 2008. High-Temperature Ethanol Fermentation and Transformation with Linear DNA in the Thermotolerant Yeast *Kluyveromyces marxianus* DMKU3-1042. *Applied and Environmental Microbiology* 74 (24):7514-7521.

Öhgren, Karin, Renata Bura, Gary Lesnicki, Jack Saddler, and Guido 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.

Ozmihci, Serpil, and Fikret Kargi. 2007. Effects of feed sugar concentration on continuous ethanol fermentation of cheese whey powder solution (CWP). *Enzyme and Microbial Technology* 41 (6–7):876-880.

Santos, Valdilene Canazart, Caio Roberto Soares Bragança, Frederico José Vieira Passos, and Flávia Maria Lopes Passos. 2013. Kinetics of growth and ethanol formation from a mix of glucose/xylose substrate by *Kluyveromyces marxianus* UFV-3. *Antonie van Leeuwenhoek* 103 (1):153-161.

Schmitt, Elliott, Renata Bura, Rick Gustafson, Joyce Cooper, and Azra Vajzovic. 2012. Converting lignocellulosic solid waste into ethanol for the State of Washington: An investigation of treatment technologies and environmental impacts. *Bioresource Technology* 104 (0):400-409.

Sills, Deborah L., and James M. Gossett. 2011. Assessment of commercial hemicellulases for saccharification of alkaline pretreated perennial biomass. *Bioresource Technology* 102 (2):1389-1398.

Skoog, Kerstin, and Bärbel Hahn-Hägerdal. 1988. Xylose fermentation. *Enzyme and Microbial Technology* 10 (2):66-80.

Soares, I. B., J. A. Travassos, H. M. Baudel, M. Benachour, and C. A. M. Abreu. 2011. Effects of washing, milling and loading enzymes on the enzymatic hydrolysis of a steam pretreated sugarcane bagasse. *Industrial Crops and Products* 33 (3):670-675.

Suwannarangsee, Surisa, Benjarat Bunterngsook, Jantima Arnthong, Atchara Paemanee, Arinthip Thamchaipenet, Lily Eurwilaichitr, Navadol Laosiripojana, and Verawat Champreda. 2012. Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design. *Bioresource Technology* 119 (0):252-261.

Várnai, Anikó, Laura Huikko, Jaakko Pere, Matti Siika-aho, and Liisa Viikari. 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. *Bioresource Technology* 102 (19):9096-9104.

Wanderley, Maria Carolina de Albuquerque, Carlos Martín, George Jackson de Moraes Rocha, and Ester Ribeiro Gouveia. 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. *Bioresource Technology* 128 (0):448-453.

Watanabe, Itsuki, Naonori Miyata, Akira Ando, Riki Shiroma, Ken Tokuyasu, and Toshihide Nakamura. 2012. Ethanol production by repeated-batch simultaneous saccharification and fermentation (SSF) of alkali-treated rice straw using immobilized *Saccharomyces cerevisiae* cells. *Bioresource Technology* 123 (0):695-698.

Yablochkova, E. N., O. I. Bolotnikova, N. P. Mikhailova, N. N. Nemova, and A. I. Ginak. 2004. The Activity of Key Enzymes in Xylose-Assimilating Yeasts at Different Rates of Oxygen Transfer to the Fermentation Medium. *Microbiology* 73 (2):129-133.

Yuan, Dawei, Kripa Rao, Patricia Relue, and Sasidhar Varanasi. 2011. Fermentation of biomass sugars to ethanol using native industrial yeast strains. *Bioresource Technology* 102 (3):3246-3253.

Yuan, W. J., X. Q. Zhao, X. M. Ge, and F. W. Bai. 2008. Ethanol fermentation with *Kluyveromyces marxianus* from Jerusalem artichoke grown in salina and irrigated with a mixture of seawater and freshwater. *Journal of Applied Microbiology* 105 (6):2076-2083.

Chapter 4

Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse

Accepted for publication in Bioresource Technology

Abstract

Blending of the enzyme extracts produced by different fungi can result in favorable synergetic enhancement of the enzyme blend with regards to the main cellulase activities, as well as the inclusion of accessory enzymes that may not be as abundant in enzyme extracts produced by predominantly cellulase producing fungi. The *C. cubensis*:*P. pinophilum* 50:50 (v/v) blend produced herein presented good synergy, especially for FPase and endoglucanase activities which were 76% and 48% greater than theoretical, respectively. This enzyme blend was applied to sugarcane bagasse previously submitted to a simple alkali pretreatment. Glucan hydrolysis efficiency reached an excess of 60% and xylan conversion exceeded 90%. Increasing the hydrolysis temperature from 45 to 50°C also resulted in a 16-20% increase in conversion of both glucan and xylan fractions. The blended enzyme extract obtained therefore showed great potential for application in the lignocellulose hydrolysis process.

Keywords: synergy; saccharification; sugarcane bagasse; *Chrysoporthe cubensis*; *Penicillium pinophilum*

1. Introduction

Lignocellulosic biomass is the world's most abundant renewable feedstock (Jarvis 2003) and has the potential to be converted to a number of different fuels and chemicals (Jørgensen, Kristensen et al. 2007). Successful biochemical conversion of lignocellulosic biomass requires a combination of efficient and economical pretreatment method, high monosaccharide yields during enzymatic hydrolysis and efficient conversion of these monosaccharides to ethanol or the desired end-product (Jonsson, Alriksson et al. 2013).

For complete cellulose degradation the synergistic action of the four cellulase enzymes is necessary: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.176) and β-glucosidases (EC 3.2.1.21). The hemicellulose fraction hydrolysis requires a more complex group of enzymes, referred to as hemicellulases. Complete enzymatic hydrolysis of xylan, the major polymer founded in hemicelluloses, requires endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and several accessory enzymes including α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-

galactosidase (EC 3.2.1.22), acetylxylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (Van Dyk & Pletschke, 2012; Yeh et al., 2010).

Conversion of cellulose to glucose at high solids concentrations (e.g. >10%) is necessary for the economic viability of commercial processes. Many published studies seek to achieve maximum conversion efficiency, often at the expense of maximizing product yield (Hu, Arantes et al. 2011; Singh, Sharma et al. 2013). Operation at high solids concentration results in decreasing fractional conversion of the feedstock compared to operation at lower concentration, however it also allows for utilization of less water in the process and production of a more concentrated product, ultimately leading to lower downstream processing costs (Hodge, Karim et al. 2008).

Of the complete biochemical conversion process, enzymatic hydrolysis is typically cited as the bottleneck due to the high cost of commercial enzymes and/or low efficiencies. One of various different strategies to decrease the cost of hydrolysis is the application of enzymes produced on-site, which can be used as a crude extract as compared to a purified commercial extract (Falkoski, Guimaraes et al. 2013). Genetic alteration of specific enzyme-producing microorganisms is another strategy commonly applied to maximize enzyme production and subsequent hydrolysis efficiency. An alternative strategy for acquiring a more complete enzyme cocktail includes blending of enzyme extracts with complementing activities so as to obtain a more ample enzyme hydrolysis spectrum for degradation of hemicellulose fractions, which in turn facilitates cellulose hydrolysis (Hu, Arantes et al. 2011).

Matching of enzyme cocktails with the biomass feedstock and pretreatment method is another of the subjects studied in-depth. It appears that the majority of pretreatment methods published in literature are acid-based thermochemical methods, with fewer based on alkali pretreatments (Alvira, Tomás-Pejó et al. 2010). Alkali pretreatment presents the advantage of lignin solubilization and retention of both cellulose and hemicellulose fractions, and when combined with an enzyme cocktail rich in hemicellulase enzymes, presents the potential to hydrolyze nearly all pretreated biomass and also to release hemicellulose-derived pentose sugars in an environment free of strong acids and fermentation inhibitors (furfurals). Many studies also present multiple integrated pretreatment steps, especially steam explosion or acid pretreatments plus a delignification step. These pretreated biomasses may consist of nearly 90%

cellulose and significantly facilitate enzymatic hydrolysis, however processing costs are also significantly increased and may potentially generate more environmentally harmful by-products (de Souza, Costa et al. 2012; Wanderley, Martín et al. 2013).

Numerous studies utilize blends of commercial enzymes, typically blending mixtures of cellulase enzymes (endo and exo-glucanases) with β-glucosidases and additional hemicellulose degrading enzymes (xylanases, arabinases, etc.) (Hu, Arantes et al. 2011); and many others utilize produced enzyme extracts supplemented with the enzyme activities which appear to be in fault (Kovacs, Macrelli et al. 2009). Blending of separate enzyme extracts is an alternative which has received less attention, but shows great potential since no activities are lost in concentration/purification processes. Blending of crude enzyme extracts can result in enzyme synergy, resulting in enzyme activities greater than expected and therefore improved hydrolysis efficiencies (Gottschalk, Oliveira et al. 2010). However enzyme synergy is extremely complex and may be dependent on a number of different factors (Kostylev and Wilson 2011).

Selection of enzyme extracts for blending is typically based on combining those which complement each other, e.g., a cellulase-rich extract with a hemicellulase-rich extract. Strains of *Trichoderma* and *Aspergillus* are the most common cellulase-producing fungi, however *Penicillium pinophilum* has also been studied and its enzyme extract applied for hydrolysis of lignocellulose (Wood, Wilson et al. 1994). Contrarily, little has been published on application of enzymes produced by *Chrysoporthe cubensis* for application in lignocellulose hydrolysis (Falkoski, Guimaraes et al. 2013), but it shows promise, especially in relation to production of accessory enzymes which not only degrade hemicellulose fractions of biomass, but facilitate degradation of cellulose by making it more accessible to cellulase enzymes, as well as increasing synergism among enzymes.

In the present study an enzyme cocktail was produced by blending crude enzyme extracts from the fungi *Chrysoporthe cubensis* and *Penicillium pinophilum*. Enzymes were blended at different concentrations of the two extracts to obtain preparations with different levels cellulase and hemicellulase degrading enzymes, where synergy between the *C. cubensis* and *P. pinophilum* enzyme activities was evaluated. The hydrolytic efficiency of the enzyme preparations was determined by performing saccharification assays and measuring glucose and xylose released from the bagasse.

2. Material and Methods

2.1. Microorganisms and cultivation

2.1.1. *Chrysoporthe cubensis*

The fungus *C. cubensis* LPF-1 used in this study was obtained from the mycological collection of the Forest Pathology Laboratory, Federal University of Viçosa, MG, Brazil (Falkoski, Guimaraes et al. 2013). The fungus was maintained on PDA plates at 28 °C and subcultured periodically. The inoculum was prepared by growing the fungus under submerged fermentation in 250 mL Erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Each flask was inoculated with 10 agar plugs cut out of a 5 day-old colony of *C. cubensis* grown on PDA plates and incubated in a rotary shaker for 5 days, at 150 rpm and 28 °C. The culture obtained was aseptically homogenized with Polytron® and immediately used to inoculate the solid culture media.

For enzyme production via solid state fermentation, 250 mL Erlenmeyer contained 12.5 g of wheat bran and 18.75 mL of the culture media (final moisture of 60%) consisting of, in g/L, NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2. Furthermore, $MnCl_2$ (0.1 mg/L), H_3BO_3 (0.075 mg/L), Na_2MoO_4 (0.02 mg/L), $FeCl_3$ (1.0 mg/L) and $ZnSO_4$ (3.5 mg/L) also were added to the medium as trace elements. The flasks were autoclaved at 120°C for 20 min and then inoculated with 5 mL (containing 1.5 X 10^7 spores/mL) of inoculum obtained as aforementioned. The flasks were maintained at 28 °C in a controlled temperature chamber and the enzymatic extraction was performed after 7 days of fermentation. Enzymes secreted during SSF were extracted with sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate), under agitation of 150 rpm for 60 min at room temperature. Solids were separated by filtration through nylon cloth followed by centrifugation at 15000*g* for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis.

2.1.2. *Penicillium pinophilum*

The fungus *P. pinophilum* was isolated from agroindustrial residues at a cattle production facility in Minas Gerais, Brazil as part of a bioprospecting study and selected due to its elevated cellulase production (data not shown). It was maintained at the mycological collection of the Laboratory of Biochemical Technology at Federal University of Viçosa, MG, Brazil. The fungus was identified by the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as *Penicillium pinophilum*. It was routinely propagated on potato dextrose agar slants at 28 °C for 7 days and maintained at 10 °C for 2-10 days.

P. pinophilum was cultivated under submerged fermentation in 250 mL Erlenmeyer flasks with 125 mL of a culture medium consisting of (in g/L): KH_4PO_4, 1.5; NH_4NO_3, 1.0; $MgSO_4$, 0.5; yeast extract, 1.5; peptone, 0.5;$CaCl_2$, 0.2; $MnCl_2$, 0.03; $FeSO_4$, 0.025; and $ZnSO_4$ 0.04, where knife-milled elephant grass (30 mesh) was used as the carbon source at a concentration of 3% (w/v), previously defined as the carbon source that best induced enzyme production (data not shown). The flasks were autoclaved at 120°C for 20 min. Each flask was inoculated with 9 agar plugs cut out of a 5 day-old colony of *P. pinophilum* grown on PDA plates and incubated in a rotary shaker for 7 days at 150 rpm and 28°C. Solids were separated by filtration through nylon cloth followed by centrifugation at 15000g for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis.

2.2. Protein determination

Protein concentration in the enzymatic extracts was determined by the Coomassie Blue binding method using bovine serum albumin as the standard (Bradford 1976).

2.3. Enzyme assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50°C, and in triplicate so the mean values were calculated and reported. Relative standard deviations of measurements were below 5%. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions (Ghose 1987). The total reducing sugar liberated during the enzymatic assays was quantified by the dinitrosalicylic acid (DNS) method (Miller

1959) using glucose as a standard. Xylanase, mannanase and pectinase activities were determined using xylan from birchwood (1% w/v at final concentration), locust bean gum (0.4% w/v) and poligalacturonic acid (0.2% w/v) as substrates, respectively. The enzymatic reactions were initiated by the addition of 100 µL of the appropriately diluted enzyme solution to 400 µL of the polysaccharide substrate solution prepared in buffer. Reaction mixtures were incubated for 30 min and the total reducing sugar released was determined via the DNS method using xylose, mannose and galacturonic acid as standards. β-Glucosidase, β-xylosidase, β-mannosidase, α-galactosidase α-arabinofuranosidase and cellobiohydrolase activities were measured using ρPNGlc, ρNPXyl, ρNPMan, ρNPGal, ρNPAra and ρNPCel as substrates, respectively. The reaction mixtures contained 100 µL of the appropriately diluted enzyme solution, 125 µL of the synthetic substrate solution (1 mM at final concentration) and 275 µL of buffer. The reaction mixture was incubated for 30 min and stopped by addition of 0.5 mL sodium carbonate solution (0.5 M). Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated by a standard curve. Cellobiase activity was assessed using 100 µL of the appropriately diluted enzyme solution, 125 µL of a cellobiose solution (8 mM at final concentration) and 275 µL of buffer. After 30 min the reaction was stopped by boiling for 5 min and submitted to a glucose-oxidase assay for glucose quantification. For all activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 µmol of the corresponding product (glucose equivalent, xylose, mannose, galacturonic acid and ρ-nitrophenol) per minute, under the assay condition used.

2.4. Biomass pretreatment and composition analysis

Sugarcane bagasse was obtained from the Center for Research and Breeding of Sugarcane of the Federal University of Viçosa, Brazil, from which the sugars had already been extracted. In the laboratory this was again washed and dried in an oven at 70°C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) and submitted to alkaline pretreatment prior to being employed in saccharification experiments. Sodium hydroxide 1.5% was used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v); and treatments were performed in an autoclave at 120°C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid

fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at -20°C.

Chemical composition of the untreated and alkali-treated sugarcane bagasse samples were determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 (TAPPI 1999). Extractive-free biomass (0.3 g) was incubated at 30°C with 3 mL of 72% H_2SO_4 for 1 h with occasional mixing. The slurry was then transferred into a penicillin bottle containing 84 mL of deionized water and the flask was sealed with a rubber stopper and aluminum seal. The bottle was placed in an autoclave calibrated at 118 C for 1 h, then the slurry was filtered through a medium coarseness sintered glass filter for gravimetric determination of acid-insoluble lignin. Concentrations of biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate were quantified using high-performance liquid chromatography (HPLC), while acid-soluble lignin was determined by absorption measurements at 205 nm (TAPPI 1991). The HPLC system Dionex DX-300 (Dionex Co. – Sunnyvale, CA, USA) was equipped with a Carbopac PA1 column and a pulsed amperometric detector with a gold electrode. Prior to injection, samples were filtered through 0.45-μm HV filters and a volume of 20 μL was loaded into the chromatograph system. The column was pre-equilibrated with a NaOH solution, 300 mM, and elution was carried out at a flow rate of 1.0 mL/min at room temperature.

2.5. Preparation of *C. cubensis* and *P. pinophilum* enzyme blends and enzyme activity synergism

Enzyme blends were obtained by mixing the supernatants of the two fungi cultures at the following *C. cubensis*:*P. pinophilum* ratios: 25:75, 50:50 and 75:25. FPase, endoglucanase, β-glucosidase and xylanase activities were measured for the individual *C. cubensis* and *P. pinophilum* extracts as well as in the blends. The blended enzyme extracts were concentrated using a Micron ultrafiltration unit (Millipore Corporation, Bedford, MA) for later application in sugarcane hydrolysis assays.

To investigate the presence of synergy of enzyme activity in the blends, the different theoretical activities were calculated based on the equation: (*C. cubensis* ratio x *C. cubensis* enzyme activity) + (*P. pinophilum* ratio x *P. pinophilum* enzyme activity). This theoretical value was compared the actual measured activity, and synergism was expressed as a percentage of the theoretical activity.

2.6. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 125 mL Erlenmeyer flasks with 50 mL working volume, subjected to mechanical agitation and an external water bath for maintaining the desired hydrolysis temperature. Flasks were loaded with the predetermined amounts of biomass and enzyme, as well as a calculated volume of 1 M sodium acetate buffer (pH 5.0) to result in a final concentration of 50 mM. Tetracycline (40 mg/L) and azide (10 mM) were added to prevent contamination. The flasks were plugged with rubber stoppers through which a needle was used for sampling at predetermined times.

Analysis of hydrolysis efficiency compared the theoretical sugar yield based on full conversion of polysaccharides to monosaccharides with the actual measured monosaccharide concentration. Theoretical monosaccharide concentration is obtained by multiplying the concentration of polysaccharide by 1.11 for six carbon sugars or 1.136 for five carbon sugars.

2.7. Hydrolysis product analysis

Products of the saccharification assays were analyzed by high performance liquid chromatography (HPLC) with a Shimadzu series 10A chromatograph. The HPLC was equipped with an Aminex HPX-87P column (300 x 7.8 mm) and refractive index detectors. The column was eluted with water at a flow rate of 0.6 mL/min and 80°C.

3. Results and discussion

3.1. Protein content and activities of the enzyme cocktail

Initially the protein concentrations and activities of the different enzyme mixtures analyzed for the assessment of synergy were determined and compared (Table 1). As was expected, the crude enzyme extracts from *Chrysoporthe cubensis* and *Penicillium pinophilum* showed substantial differences. Protein concentration of the extract from *C. cubensis* was more than two times that of *P. pinophilum*, and this may be due to the fact that *C. cubensis* was grown in solid state conditions which permitted greater protein concentration. One of the major advantages of solid state fermentation is the acquisition of a concentrated enzymatic product (Hölker, Höfer et al. 2004).

Table 1. Protein concentrations and activities of the crude enzyme extracts and the different blends.

	Total proteins (mg/mL)	Activities (U/ml)			
		FPase	Endoglucanase	β-glucosidase	Xylanase
C. cubensis	0.393	0.179	2.946	5.602	19.765
P. pinophilum	0.178	0.507	5.549	1.666	22.765
25:75 *C. cubensis*:*P. pinophilum*	0.23	0.71	6.25	2.73	22.64
50:50 *C. cubensis*:*P. pinophilum*	0.28	0.61	6.36	3.67	26.45
75:25 *C. cubensis*:*P. pinophilum*	0.34	0.32	4.02	5.87	24.41

C. cubensis produces a crude enzyme extract exceptionally rich in β-glucosidase when cultivated in solid state (roughly three times greater than *P. pinophilum* per total volume), while the extract from *P. pinophilum* produced in submersed culture presents higher activities for FPase (about 2.8 times greater) and endoglucanase (approximately 1.9 times greater).

Synergy is defined as the interactions that occur between two or more hydrolytic components, producing a total effect greater than the sum of the effects of the individual components. Synergy of the two crude enzyme extracts was evaluated by comparing the measured activities of FPase, endoglucanase, β-glucosidase and xylanase with the theoretical activities expected based on simple mixing. Activities of the different blends of *C. cubensis* and *P. pinophilum* indicated synergetic action between the two substrates, especially with respect to FPase and endoglucanase in the 50:50 blend (Figure 1).

The 50:50 blend presented the highest levels of synergism for FPase, endoglucanase and xylanase with synergy values of 76%, 50% and 24%, respectively, but nearly no synergism for β-glucosidase enzymes (Figure 1). It is known that β-glucosidase plays an important role in filter paper activity, but because β-glucosidase activity was relatively high in the enzyme blend (5.8 times FPase activity), it did not limit filter paper hydrolysis. The 25:75 blend also presented high synergy for FPase (67%). Contrarily, the 75:25 cocktail presented a β-glucosidase synergy value of 27%, the highest among the different cocktails, but the lowest values of both FPase and endoglucanase. Due to the high synergy of FPase in the 50:50 cocktail, this was selected as the most promising for utilization in biomass hydrolysis. Despite the poor synergy among β-glucosidase enzymes, posterior analyses of the hydrolysis potential of the 50:50 blend showed that it was efficient.

Many studies on the synergistic effects of enzymes in the lignocellulose hydrolysis process are based on the hydrolysis process itself, typically supplementing a specific enzyme or enzyme cocktail with another individual enzyme, which may also be considered as addition effects (Hu, Arantes et al. 2011; Zhang, Pakarinen et al. 2013). Studies that directly assess enzyme activities when blending different enzyme extracts are less common in literature. A study performed by Gottschalk, Oliveira et al. (2010) presented activities of the same four enzymes as in the present study when blending enzyme extracts from *Trichoderma reesei* and *Aspergillus awamori* at different proportions. These authors also found significant enzyme synergy, especially with relation to filter paper and carboxymethyl cellulase activities which for some blends exceeded 100%. However, in the same study a trend was observed in enzyme synergy, where FPase and carboxymethyl cellulase activity decreased linearly when decreasing the concentration of the *Trichoderma reesei* extract. In the present study there were no linear trends, but significant synergetic effects were observed. Synergy of FPase activity may be partially due to the fact that the *C. cubensis* extract showed significantly higher cellobiohydrolase activity than that of *P. pinophilum*, where upon blending, an optimal mixture of cellulase enzymes was obtained. The specific reasons for these synergetic effects are unknown, and filter paper activity is considered even more complex since a number of different enzymes may affect FPase activity.

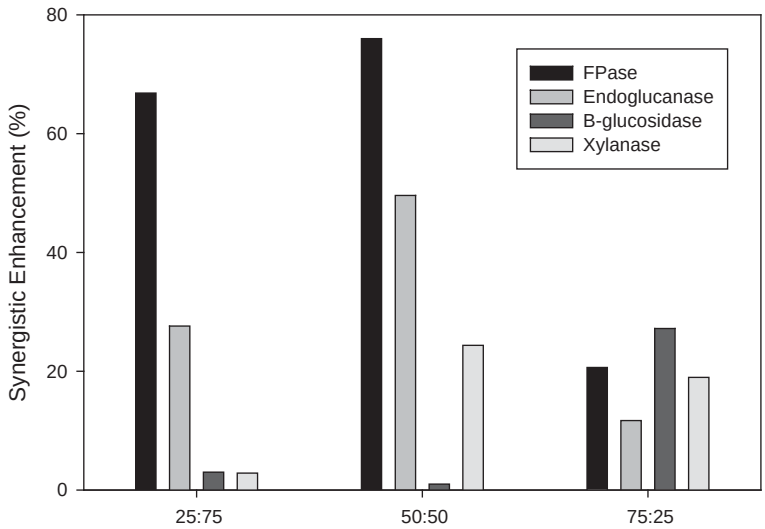

Figure 1. Synergistic enhancement of the FPase, CMCase, β-glucosidase and xylanase activities using mixtures of the *C. cubensis* and *P. pinophilum* preparations at ratios of 25:75, 50:50 and 75:25.

Upon determining that the most attractive enzyme cocktail for lignocellulosic biomass degradation was that composed of 50:50 *C. cubensis*:*P. pinophilum*, this blend was produced in larger quantity and partially concentrated (≈ 10 times). At this point the enzyme activities were again assessed, including a broader spectrum of activities (Table 2). The total protein concentration was 0.89 mg/mL. Few studies present such an extensive characterization of an enzyme extract, however a prior study of the fungus *Chrysoporthe cubensis* indicated that when comparing enzyme activities on a 1:1 FPU basis, the *C. cubensis* extract was more efficient for sugarcane bagasse hydrolysis than a commercial cellulase product (Falkoski, Guimaraes et al. 2013).

Comparing the activities of cellulases and accessory enzymes from the *P. pinophilum* and *C. cubensis* extracts indicated that *C. cubensis* was a greater producer of cellobiohydrolase, cellobiase, α-arabinofuranosidase, β-mannosidase, mannanase and pectinase, presenting activities that were 9.4, 2.2, 1.9, 15.6, 1.4 and 1.2 times greater than those of the *P. pinophilum* extract. *P. pinophilum* presented greater activities of β-xylosidase and α-galactosidase that were 1.7 and 4.6 times greater than those of *C. cubensis*, respectively. This confirms the fact that *C. cubensis* plays a fundamental role in providing enzymes to the blend presented in Table 2 that assist in breaking down hemicellulose and providing greater access to cellulose, especially with respect to cellobiohydrolase activity which plays an important role in the glucose liberation process. The enzyme activities presented in Table 2 presents a more extensive indication of enzyme synergy, as well as the effects of ultrafiltration for concentration of the enzyme blend.

Table 2. Enzymatic activities of the 50:50 enzyme blend.

	C. cubensis	*P. pinophilum*	Unconcentrated 50:50 blend	Concentrated 50:50 blend
Protein concentration (mg/ml)				
Total proteins	0.39	0.18	0.28	2.89
Enzyme activities (U/ml)				
Fpase	0.18	0.51	0.61	4.45
Endoglucanase	2.95	5.55	6.36	41.67
Cellobiohydrolase	1.09	0.12	0.20	1.79
β-glucosidase	5.60	1.67	3.67	21.15
Cellobiase	1.70	0.78	1.67	16.50
Xylanase	19.76	22.76	26.45	282.88
β-xylosidase	0.01	0.01	0.07	0.23
α-galactosidase	0.14	0.63	0.23	1.98
α-arabinofuranosidase	0.52	0.27	0.25	2.14
β-mannosidase	0.02	0.00	0.02	0.19
Mannanase	1.06	0.76	1.42	13.93
Pectinase	1.58	1.25	1.79	17.54

The good synergy results obtained in the present study may also be partially attributed to the different cultivation techniques of the two fungi (submersed and solid state), since these different methods may induce the production of enzymes with different properties (Singhania, Sukumaran et al. 2010). Solid state fermentation (SSF) for production of enzymes with potential to degrade lignocellulose presents multiple advantages compared to submersed fermentation (SmF), highlighting lower water demand, high concentration of the product (Hölker, Höfer et al. 2004) and reduced costs of up to 10 fold (Tengerdy 1996). Based on the data obtained in the present study, where one enzyme extract is obtained via SmF and the other by SSF, enzymes produced via SSF may be directly solubilized in the SmF extract, thus minimizing downstream processing costs for enzyme concentration.

3.2. Application of the Enzyme Cocktail

3.2.1. Biomass pretreatment

Sugarcane bagasse was utilized as a model substrate for saccharification experiments due to its availability as a potential lignocellulosic feedstock in Brazil. Prior to enzymatic hydrolysis, it was submitted to thermochemical pretreatment with 1.5% NaOH at 120°C in which approximately 50% of lignin was removed, thus facilitating enzyme attack of the cellulose and hemicellulose fractions (Table 3).

Table 3. Composition of the raw and pretreated sugarcane bagasse.

Biomass component	Untreated bagasse	Pretreated bagasse
Glucan	52.8	59.2
Xylan	19.1	22.3
Arabinan	1.6	2.1
Lignin	22.1	11.4

3.2.2. Saccharification

Previous studies have indicated the importance of hemicellulases, especially xylanases, in enzyme cocktails to improve hydrolysis of both cellulose and hemicellulose fractions to their respective monosaccharides (Hu, Arantes et al. 2011). Therefore, saccharification assays were performed using the 50:50 enzyme blend previously defined in section 2.1. The initial enzyme hydrolysis tests compared different enzyme loading and solid loading levels on the rate of sugar release (g/g/h) and the total yield defined as sugar (glucose or xylose) produced per unit of feedstock (g/g).

The major beneficial effect of supplementing hemicellulase enzymes to cellulase enzyme mixtures has frequently been suggested to increasing cellulose accessibility to the cellulase enzymes due to the removal of hemicellulase fibers on the outer surface of the pretreated pulp fibers (Várnai, Huikko et al. 2011). Cellulase + xylanase enzyme blends are not as efficient as the blend acquired in the present study (Hu, Arantes et al. 2011; Sills and Gossett 2011); and this is probably due to the great extent of accessory enzymes provided by the *C. cubensis* enzyme extract. These additional enzymes assist in hydrolysis of hemicellulose ramifications (Rakotoarivonina, Hermant et al. 2012). *C. cubensis* is a producer of large quantities of hemicellulase and other accessory enzymes, as proven when comparing the two enzyme extracts and in the study performed by Falkoski et al. (2013) when comparing enzyme activities as a function of FPase activity. Furthermore, the fact that *C. cubensis* was a major producer of cellobiohydrolase activity when compared with *P. pinophilum* resulted in synergy of FPase activity due to the importance of this enzyme for glucose liberation. Additionally, when combined with an alkali pretreatment this supplementation permits for liberation of additional monosaccharide sugars from hydrolysis of the preserved hemicellulase fraction, and due to the minimal solid lignin concentration nearly complete breakdown of the pretreated biomass is achieved (Alvira, Tomás-Pejó et al. 2010).

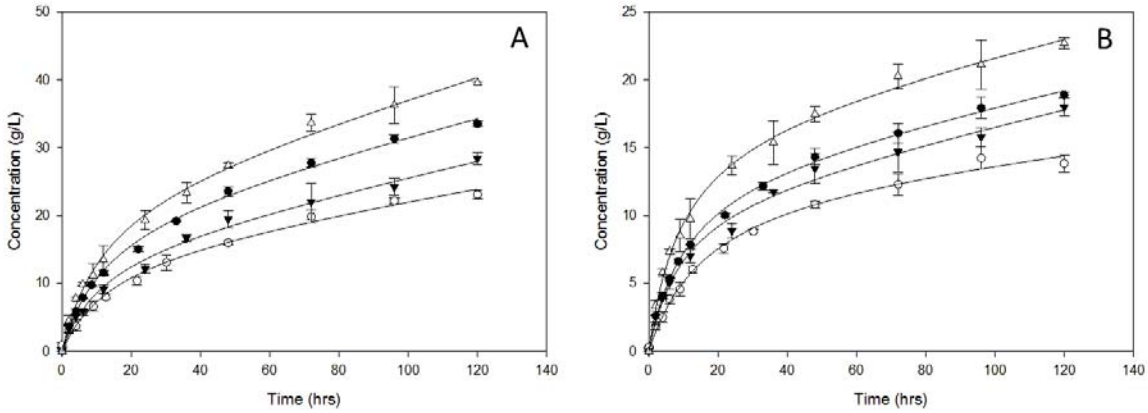

Figure 2. Glucose (A) and xylose (B) liberation curves from hydrolysis performed at 45°C and 200 rpm magnetic agitation with the following enzyme and solid loadings: 10 FPU/g and 8% biomass (open circle), 10 FPU/g and 12% biomass (filled triangle), 20 FPU/g and 8% biomass (filled circle), and 20 FPU/g and 12% biomass (open triangle).

Enzyme and solids loading had significant effects on both hydrolysis efficiency and yield of product sugars (glucose and xylose). Based on the pretreated biomass composition, theoretical conversion of glucan would yield 47.4 and 71.1 g/L of glucose for the solid loadings of 8 and 12%, respectively, while theoretical yields of xylose from xylan are 17.8 and 26.8 g/L for the same solids loadings. Small concentrations of cellobiose were observed in the initial hours of hydrolysis, but after 24 hours no accumulation was observed. Figure 2 indicates the importance of these variables by comparing the glucose and xylose production curves over the hydrolysis period of 120 hours.

Robustness of the enzyme cocktail produced in the present study can also be verified when comparing hydrolysis efficiency at similar enzyme and solid loadings. In a study utilizing a commercial cellulase extract supplemented with β-glucosidase, it was necessary to utilize a two-step pretreatment stage (steam explosion + delignification via NaOH) to acquire significant hydrolysis efficiencies in the range of 70% (Table 4). Without the delignification step conversion efficiency was limited to 40%, obtained after about 40 hours with minimal increase from 40-120 hours (Wanderley, Martín et al. 2013). In the cited study the glucan percentage in biomass submitted to both pretreatment steps was 87%, which significantly improves hydrolysis efficiency because enzymes have greater access to cellulose and lignin does not build up in fed-batch type processing. In another study utilizing a Formaline pretreatment process,

pretreated biomass consisted of 86% glucan, where even for enzyme and solid loadings of 10 FPU/g and 20%, respectively, in a fed batch process, glucan conversion efficiencies exceeding 70% were obtained (glucose concentrations exceeding 120 g/L) (Zhao, Dong et al.). However, this process requires using formic acid concentrations in the range of 70-90 wt.%, followed by deformylation with a base (Zhao and Liu 2012), again adding significant costs to the overall biomass to liquids process.

As expected, both glucose and xylose liberations were higher when increasing the quantity of the enzyme blend added to the biomass slurry. Maximum glucose and xylose conversions of 63.8% and 93.3%, respectively, were therefore obtained for the conditions of 8% solids loading and 20 FPU/g enzyme loading. The lowest conversions were obtained for the solids loading of 12% and enzyme loading of 10 FPU/g (36.0% and 59.2%). In all cases the final percent yield of xylose was 20-30% greater than that of glucose. This is one of the advantages of combining a delignification pretreatment (alkali) with utilization of a hemicellulase rich enzyme cocktail, where it is possible to obtain a much larger soluble monosaccharide concentration when compared to acid pretreatment (hemicellulose removal) due to the presence of xylan and absence of lignin.

Table 4. Pretreated sugarcane bagasse hydrolysis treatments and efficiencies.

	Treatments				
Enzyme loading (FPU/g)	20	20	10	10	10
Solids loading (%)	8%	12%	8%	12%	12%
Temperature (°C)	45	45	45	45	50
Glucan conversion (%)	63.8%	50.1%	43.9%	36.0%	41.9%
Xylan conversion (%)	93.3%	74.6%	68.3%	59.2%	71.6%

The acquisition of elevated hydrolysis efficiencies is the objective of numerous studies published in literature, but often this is at the expense of increased solids loading which ultimately results in lower monosaccharide concentrations (Kristensen, Felby et al. 2009). Gottschalk, Oliveira et al. (2010) presented glucan and xylan hydrolysis efficiencies of 80% and 90%, respectively, when using an enzyme cocktail from fungi extracts, however a solids loading of only 2% was used. The same was also observed in the study performed by Hu, Arantes et al. (2011) who showed elevated hydrolysis efficiencies when supplementing a cellulase cocktail with xylanase enzymes at a solids loading of only 2%. These authors used a total protein loading of 35 mg/g cellulose, further indicating the great potential of the cocktail produced in the present study since

protein loadings were significantly lower. High sugar concentrations obtained via hydrolysis decrease the energy demands required to further concentrate these sugars prior to fermentation. In the case of ethanol production, development of pentose fermenting microorganisms would permit significant increases in ethanol yields since a mixture of pentose and hexose sugars is generated. However, there are additional bioprocessing routes that can be utilized for pentose sugar processing (Dumon, Song et al. 2012; Bondesson, Galbe et al. 2013).

3.2.3. Assessment of Temperature

The saccharification temperature of 45°C was selected as a function of thermostability of the *P. pinophilum* enzyme extract, since at 50°C the FPase activity decreased to half that at 45°C in less than 50 minutes (data not shown). However, to determine if the same behavior would be observed for the *C. cubensis*:*P.pinophilum* 50:50 blend a saccharification assay was performed at 50 °C, using the same conditions that lead to the poorest hydrolysis yields in the prior experiment performed at 45 °C.

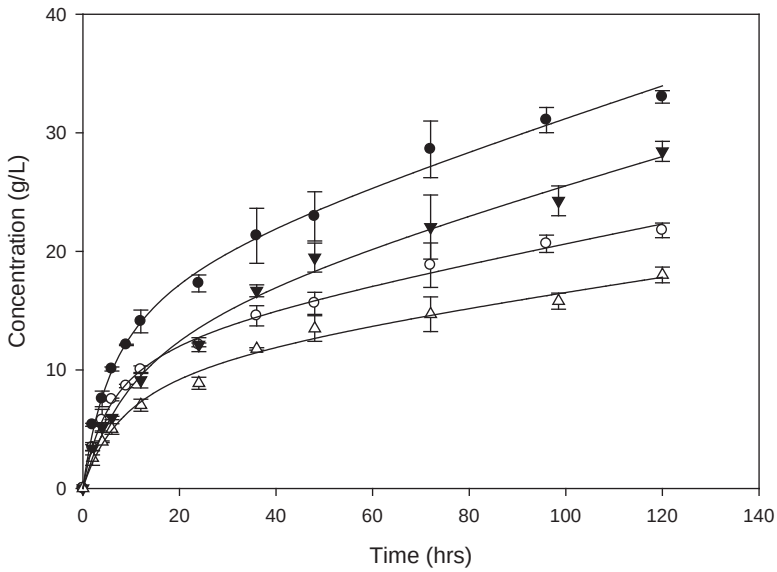

Figure 3. Comparison of the enzymatic hydrolysis profile for glucose (solid shapes) at xylose (hollow shapes) at the temperatures of 45°C (triangles) and 50°C (circles) with enzyme loading of 10 FPU/g biomass and solids loading of 12%.

The increase in temperature appeared to show no change in enzyme activity loss in comparison with that performed at 45°C, since the slope of the line for the assay performed at 50°C is slightly more accentuated for the period from 40 to 120 hours

(Figure 3). As expected, over the course of time the treatments with increased enzyme loadings showed higher reaction velocities (Figure 4). However, it is interesting to note the effect of temperature in this case. The initial hydrolysis velocity of the saccharification assay at 50°C utilizing the lower enzyme loading (10 FPU/g) and higher biomass concentration was the greatest (2.3 g/L/h), however between 10 and 30 hours the curves for both assays with 10 FPU/g enzyme loading and 12% biomass performed at 45°C and 50°C were nearly identical. Even more striking is the fact that while all treatments conducted at 45°C tended to show slight declines in reaction velocity during the final hours of hydrolysis (from 80 to 120 hours), the reaction conducted at 50°C maintained a steady reaction velocity (Insert of Figure 4).

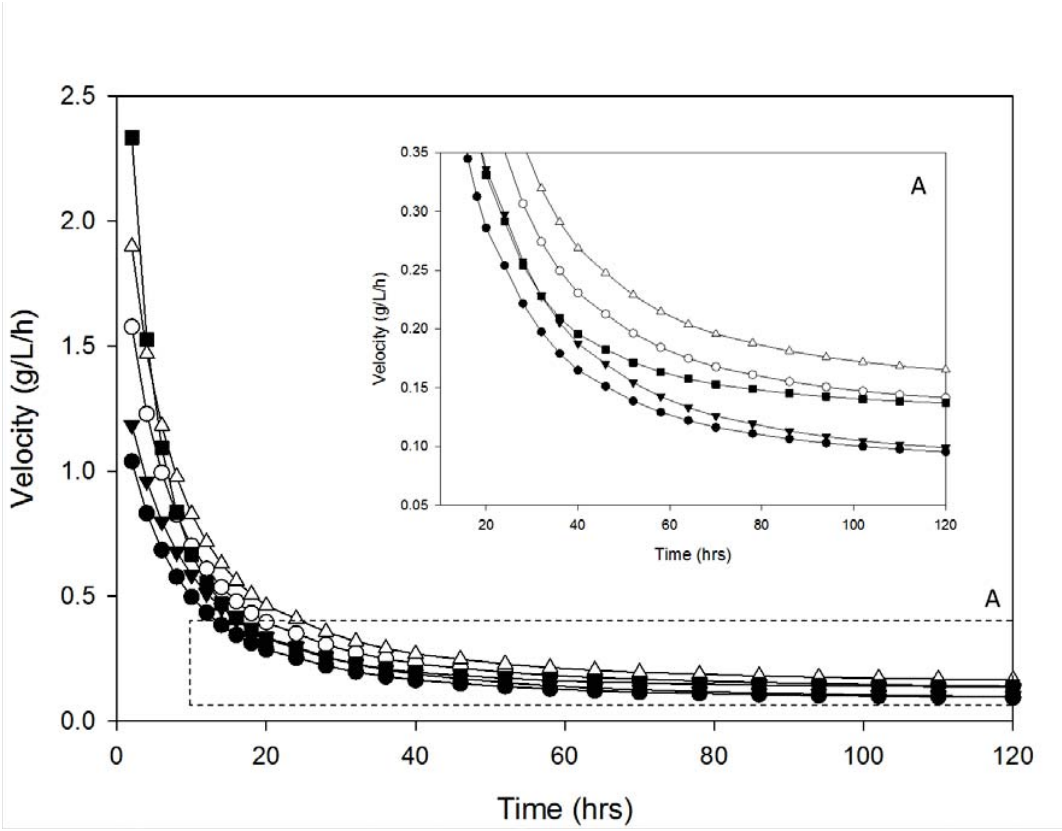

Figure 4. Reaction velocity of glucose liberation over the 120 hour hydrolysis period. Insert A corresponds to the dashed region to provide greater detail: 20 FPU/g, 12% biomass and 45 °C (Δ); 20 FPU/g, 8% biomass and 45°C (○); 10 FPU/g, 12% biomass and 50°C (■); 10 FPU/g, 12% biomass and 45°C (▼); and 10 FPU/g, 8% biomass and 45°C (●).

4. Conclusion

The synergetic action of the two enzyme extracts from *Chrysoporthe cubensis* and *Penicillium pinophilum* was confirmed to be beneficial with respect to enzyme

activities, especially FPase and endoglucanase. Hydrolysis yields obtained with the *P. pinophilum:C. cubensis* 50:50 blend were considered good based on utilization of a weak alkali pretreatment, low enzyme loading and high solids loading. Increasing temperature from 45°C to 50°C resulted in significantly higher hydrolysis yields, indicating potential for even higher monosaccharide yields. Future studies may seek to evaluate higher enzyme loadings since the hydrolysis curves showed continued enzyme activity even after 120 hours.

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6. References

1. Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.* 101, 4851-4861.
2. Bondesson, P.-M., Galbe, M., Zacchi, G. 2013. Ethanol and biogas production after steam pretreatment of corn stover with or without the addition of sulphuric acid. *Biotechnol. Biofuels*. 6, 11.
3. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
4. de Souza, C.J.A., Costa, D.A., Rodrigues, M.Q.R.B., dos Santos, A.F., Lopes, M.R., Abrantes, A.B.P., dos Santos Costa, P., Silveira, W.B., Passos, F.M.L., Fietto, L.G. 2012. The influence of presaccharification, fermentation

temperature and yeast strain on ethanol production from sugarcane bagasse. *Bioresour. Technol.* 109, 63-69.

5. Dumon, C., Song, L., Bozonnet, S., Fauré, R., O'Donohue, M.J. 2012. Progress and future prospects for pentose-specific biocatalysts in biorefining. *Process Biochem.* 47, 346-357.
6. Falkoski, D.L., Guimaraes, V.M., de Almeida, M.N., Alfenas, A.C., Colodette, J.L., de Rezende, S.T. 2013. Chrysoporthe cubensis: A new source of cellulases and hemicellulases to application in biomass saccharification processes. *Bioresour. Technol*. 130, 296-305.
7. Ghose, T.K. 1987. Measurement of Cellulase Activities. *Pure Appl. Chem.* 59, 257-268.
8. Gottschalk, L.M.F., Oliveira, R.A., Bon, E.P.d.S. 2010. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by Trichoderma and Aspergillus act synergistically in the hydrolysis of sugarcane bagasse. *Biochem. Eng. J.* 51, 72-78.
9. Hodge, D.B., Karim, M.N., Schell, D.J., McMillan, J.D. 2008. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.* 99, 8940-8948.
10. Hölker, U., Höfer, M., Lenz, J. 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Appl. Microbiol. Biotechnol.* 64, 175-186.
11. Hu, J., Arantes, V., Saddler, J. 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnol. Biofuels*. 4, 36.
12. Jarvis, M. 2003. Chemistry: Cellulose stacks up. *Nature*. 426, 611-612.

13. Jonsson, L., Alriksson, B., Nilvebrant, N.-O. 2013. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels*. 6, 16.

14. Jørgensen, H., Kristensen, J.B., Felby, C. 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioprod. Biorefin.* 1, 119-134.

15. Kostylev, M., Wilson, D. 2011. Synergistic interactions in cellulose hydrolysis. *Biofuels*. 3, 61-70.

16. Kovacs, K., Macrelli, S., Szakacs, G., Zacchi, G. 2009. Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with Trichoderma atroviride enzymes produced in-house. *Biotechnol. Biofuels*. 2, 14.

17. Kristensen, J., Felby, C., Jorgensen, H. 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnol. Biofuels*. 2, 11.

18. Miller, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* 31, 426-428.

19. Rakotoarivonina, H., Hermant, B., Monthe, N., Remond, C. 2012. The hemicellulolytic enzyme arsenal of Thermobacillus xylanilyticus depends on the composition of biomass used for growth. *Microb. Cell Fact.* 11, 159.

20. Sills, D.L., Gossett, J.M. 2011. Assessment of commercial hemicellulases for saccharification of alkaline pretreated perennial biomass. *Bioresour. Technol.* 102, 1389-1398.

21. Singh, A., Sharma, P., Saran, A.K., Singh, N., Bishnoi, N.R. 2013. Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. *Renew. Energ.* 50, 488-493.

22. Singhania, R.R., Sukumaran, R.K., Patel, A.K., Larroche, C., Pandey, A. 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme Microb. Tech.* 46, 541-549.

23. TAPPI. 1991. Technical Association of the Pulp and Paper Industry Tappi Useful Methods (UM-250), Norcross.

24. TAPPI. 1999. Technical Association of the Pulp and Paper Industry. Tappi Standard Methods (T-222 om-98), Atlanta.

25. Tengerdy, R.P. 1996. Cellulase production by solid substrate fermentation. *J. Sci. Ind. Res.* 55, 313-316.

26. Van Dyk, J.S., Pletschke, B.I. 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnol. Adv.* 30, 1458-1480.

27. Várnai, A., Huikko, L., Pere, J., Siika-aho, M., Viikari, L. 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. *Bioresour. Technol.* 102, 9096-9104.

28. Wanderley, M.C.d.A., Martín, C., Rocha, G.J.d.M., Gouveia, E.R. 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. *Bioresour. Technol.* 128, 448-453.

29. Wood, T.M., Wilson, C.A., McCrae, S.I. 1994. Synergism between components of the cellulase system of the anaerobic rumen fungus Neocallimastix frontalis and those of the aerobic fungi Penicillium pinophilum and Trichoderma koningii in degrading crystalline cellulose. *Appl. Microbiol. Biotechnol.* 41, 257-261.

30. Yeh, A.-I., Huang, Y.-C., Chen, S.H. 2010. Effect of particle size on the rate of enzymatic hydrolysis of cellulose. *Carbohydr. Polym.* 79, 192-199.
31. Zhang, J., Pakarinen, A., Viikari, L. 2013. Synergy between cellulases and pectinases in the hydrolysis of hemp. *Bioresour. Technol.* 129, 302-307.
32. Zhao, X., Dong, L., Chen, L., Liu, D. Batch and multi-step fed-batch enzymatic saccharification of Formiline-pretreated sugarcane bagasse at high solid loadings for high sugar and ethanol titers. *Bioresour. Technol.* 135, 350-356.
33. Zhao, X., Liu, D. 2012. Fractionating pretreatment of sugarcane bagasse by aqueous formic acid with direct recycle of spent liquor to increase cellulose digestibility–the Formiline process. *Bioresour. Technol.* 117, 25-32.

Chapter 5

Increased enzymatic hydrolysis of biomass from enzyme recycling

Abstract

Development of efficient methods for production of renewable fuels from lignocellulosic biomass is necessary to maximize yields and reduce operating costs. One of the main challenges to industrial application of the lignocellulosic conversion process is the high costs of cellulolytic enzymes. Recycling of enzymes may present a potential solution to alleviate this problem. In the present study enzymes associated with the insoluble fraction were recycled after enzymatic hydrolysis of pretreated sugarcane bagasse, utilizing different processing conditions, enzyme loadings and solid loadings. It was found that the enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* was efficient for enzymatic hydrolysis and that a significant portion of enzyme activity could be recovered upon recycling of the insoluble fraction. Enzyme productivity values (g glucose/mg enzyme protein) over all recycle periods were 2.4 and 3.7 for application of 15 and 30 FPU/g glucan, representing an increase in excess of 10 times that obtained in a batch process with the same enzyme blend and even greater increase compared to commercial cellulase enzymes. Increasing lignin concentrations throughout the recycle period did not negatively influence hydrolysis efficiency, but instead conversion efficiencies continuously improved. Recycling the entire insoluble solids fraction composed of enzymes and biomass therefore showed to be an effective method to increase enzyme productivity.

1. Introduction

Production of bioethanol from lignocellulosic biomass is a subject of great interest because cellulosic ethanol presents the potential to substitute gasoline, promote rural development and reduce greenhouse gases, while utilizing material not fit for human consumption (Dwivedi, Alavalapati, and Lal 2009). The hydrolysis process for conversion of cellulose to glucose can be either acid or enzyme catalyzed. Acid-catalyzed hydrolysis yields sugars from highly complex biomass, but requires either high temperature or high acid concentrations which can often make the process economically infeasible (El-Zawawy et al. 2011). However, the major bottleneck of enzyme-catalyzed hydrolysis is the high cost of enzymes and relatively low yields.

To overcome low hydrolysis efficiencies, multiple studies have assessed various pretreatment techniques, however often at the expense of higher energy costs and

concentrated chemicals which complicate the process and may make it uneconomical (Zhao and Liu 2012; Wanderley et al. 2013). These pretreatment methods generally seek to remove lignin and hemicellulose from the biomass, leaving cellulose which is more readily hydrolyzed when free of the hemicellulose and lignin fractions. Utilization of hemicellulase-rich enzyme extracts may be a potential solution to the use of pretreatments seeking to hydrolyze the hemicellulose fraction via thermo-chemical methods, where enzymatic hydrolysis would result in an inhibitor-free blend of pentose and hexose sugars.

Not only is the enzymatic hydrolysis step of the lignocellulosic biomass to ethanol conversion process economically considered the overall bottleneck, technically it is also the most complex. Low sugar concentrations resulting from enzyme hydrolysis are associated with subsequent lower ethanol concentrations obtained via fermentation, which increases energy consumption in ethanol distillation processes. It has previously been shown that a 3.7% (v/v) ethanol solution requires 2.4 times more steam than a 12% solution for separation to obtain anhydrous ethanol (Patzek 2007). To overcome this problem and obtain a higher soluble sugar concentration and consequently higher ethanol concentration, high solid loadings must be employed. However, additional challenges are encountered when utilizing high solids loading related to the insolubility and heterogeneous characteristics of the substrate, resulting in slurries that are increasingly dense and difficult to process (Kristensen, Felby, and Jorgensen 2009). It has also been observed that cellulose hydrolysis is dramatically reduced at high solids loadings, where this is often referred to as the main bottleneck affecting the total ethanol yield (Lau and Dale 2009).

Fed-batch processes have been utilized in an attempt to improve product yields which may suffer from high solids loadings, in which the solid substrate is continuously or intermittently fed with the solid substrate. Strategies for using this process are typically categorized into three main groups: (i) enzyme recycling, (ii) fed-batch SSF processes for mitigation of the inhibitory effect caused by hydrolysis products, and (iii) increase the cumulative substrate in a hydrolysis reactor (Hodge et al. 2009). These fed-batch have been applied for enzyme saccharification and sometimes fermentation of various different biomasses to increase final concentrations of sugars or ethanol (Chang et al. 2012; Zhao et al. 2013; Gupta et al. 2012).

The low cellulose content of pretreated biomass also contributes to complicate the acquisition of high product concentrations. Lignin is typically viewed as one of the major inhibitors to enzymatic hydrolysis, and also accumulates in fed-batch processes since it is not broken down by cellulase enzymes and therefore remains in an insoluble form (Zhao et al. 2013). For this reason more intense pretreatment methods are utilized, however in these cases additional factors must be considered, including the additional energy and material costs, as well as environmental issues related to the use of concentrated chemicals.

Blending of enzyme extracts is a strategy used to improve enzyme hydrolysis. Numerous studies have used blends of commercial enzymes and others have blended crude fungi-produced enzyme extracts with specific commercial enzymes to make up low activities of a specific enzyme (Hu, Arantes, and Saddler 2011; Kovacs et al. 2009). Blending of crude enzyme extracts from different fungi has received less attention, but shows great potential since no activities are lost in concentration/purification processes, maintaining a wide spectrum of enzyme activities. Synergy among enzymes from individual enzyme extracts is also another advantage to utilization of these enzyme blends (Gottschalk, Oliveira, and Bon 2010; Visser et al. 2013).

The objective of the present study was to determine if recycling of the insoluble solids fraction a significant portion of enzyme activity could be reutilized, thus resulting in an increased overall yield or decrease in the quantity of enzyme required for conversion. Two experiments were therefore performed to evaluate recycling of the solids fraction, in which one assessed successive additions of a predefined biomass quantity, and in the other biomass was added in order to maintain a determined solids loading. An enzyme blend consisting of extracts from the fungi *Chrysoporthe cubensis* and *Penicillium pinophilum* was used which previously showed excellent lignocellulose hydrolysis potential and synergetic action. Different enzyme loadings were assessed to evaluate the effect of the recycled enzymes adhered to the solids fraction. The influence of the recycled lignin-rich residue was also assessed with respect to its effect on enzyme hydrolysis.

2. Material and Methods

2.1. Biomass pretreatment and composition analysis

Sugarcane bagasse was obtained from the Center for Research and Breeding of Sugarcane of the Federal University of Viçosa, Brazil, from which the sugars had already been extracted. In the laboratory this was again washed and dried in an oven at 70°C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) in a knife mill (Marconi, Piracicaba, SP, Brazil) and submitted to alkaline pretreatment prior to being employed in saccharification experiments. Sodium hydroxide 1.5% (w/v) was used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v); and treatments were performed in an autoclave at 120°C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at -20°C.

Chemical composition of the untreated and alkali-treated sugarcane bagasse samples were determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 (TAPPI 1999) (Table 1). Extractive-free biomass (0.3 g) was incubated at 30°C with 3 mL of 72% H_2SO_4 for 1 h with occasional mixing. The slurry was then transferred into a penicillin bottle containing 84 mL of deionized water and the flask was sealed with a rubber stopper and aluminum seal. The bottle was placed in an autoclave calibrated at 118 C for 1 h, then the slurry was filtered through a medium coarseness sintered glass filter for gravimetric determination of acid-insoluble lignin. Concentrations of biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate were quantified using high-performance liquid chromatography (HPLC), while acid-soluble lignin was determined by absorption measurements at 205 nm (TAPPI 1991). The HPLC system Dionex DX-300 (Dionex Co. – Sunnyvale, CA, USA) was equipped with a Carbopac PA1 column and a pulsed amperometric detector with a gold electrode. Prior to injection, samples were filtered through 0.45-μm HV filters and a volume of 20 μL was loaded into the chromatograph system. The column was pre-equilibrated with a NaOH solution, 300 mM, and elution was carried out at a flow rate of 1.0 mL/min at room temperature.

2.2.Production of the enzyme blend

The enzyme extract utilized in the hydrolysis experiments was a 50:50 (v:v) blend of enzyme extracts from the filamentous fungi *Chrysoporthe cubensis* and *Penicillium pinophilum*. These fungi were cultivated according to the conditions stated in a previous study reporting synergy of this enzyme blend and its potential for application in sugarcane bagasse hydrolysis (Visser et al. 2013). The obtained enzyme blend was concentrated using a Micron ultrafiltration unit (Millipore Corporation, Bedford, MA) for later application in sugarcane hydrolysis assays. FPase, endoglucanase, β-glucosidase and xylanase activities were measured according to previously published methods (Falkoski et al. 2013), and are presented in results section.

Chrysoporthe cubensis and *Penicillium pinophilum* were cultivated and enzymes were recovered according to the methods presented by Visser et al. (2013). Enzyme blends were obtained by mixing the supernatants of the two fungi cultures at a ratio of 1:1. The blended enzyme extracts were concentrated using a Micron ultrafiltration unit (Millipore Corporation, Bedford, MA) for later application in sugarcane hydrolysis assays. FPase, endoglucanase, β-glucosidase and xylanase activities were measured for the enzyme blend along with enzyme protein content.

2.3. Enzyme assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50°C, and in triplicate so the mean values were calculated and reported. Relative standard deviations of measurements were below 5%. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions (Ghose 1987). The total reducing sugar liberated during the enzymatic assays was quantified using the dinitrosalicylic acid (DNS) reagent (Miller 1959) with glucose as a standard. Xylanase activity was determined using xylan from birchwood (1% w/v at final concentration). The enzymatic reactions were initiated by the addition of 100 μL of the appropriately diluted enzyme solution to 400 μL of the polysaccharide substrate solution prepared in buffer. Reaction mixtures were incubated for 30 min and the total reducing sugar released was determined with the DNS reagent using xylose. β-Glucosidase activity was measured using ρPNGlc as substrate. The reaction mixtures contained 100 μL of the appropriately diluted enzyme solution, 125

μL of the synthetic substrate solution (1 mM at final concentration) and 275 μL of buffer. The reaction mixture was incubated for 30 min and stopped by addition of 0.5 mL sodium carbonate solution (0.5 M). Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated by a standard curve. One enzyme unit (U) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute

2.4.Protein determination

Protein concentration in the enzymatic extracts was determined by the Coomassie Blue binding method using bovine serum albumin as the standard (Bradford 1976).

2.5.Enzyme hydrolysis experiments

Two sets of enzymatic hydrolysis experiments were performed where the first sought to assess the viability of recycling insoluble solids in sequential hydrolysis assays and the objective of the second was to simulate a potential scenario for industrial application. These two experiments were referred to as those of "Increasing biomass concentration" and "Consistent biomass concentration".

2.5.1. Increasing biomass concentrations

Enzymatic hydrolysis was performed in 2 mL micro-tubes with initial working volume of 1 mL. The hydrolysis medium consisted of alkali-pretreated biomass at concentration of 8% or 12% (w/v), distilled water, sodium acetate buffer pH 5 (final concentration of 50 mM), enzyme blend (20 FPU/g biomass), tetracycline (40 mg/L) and sodium azide (10 mM). The prepared micro-tubes were sealed and mounted horizontally in an orbital shaker at 200 rpm and 50°C.

At 96 hour intervals the tubes were temporarily removed from the shaker and submitted to centrifugation at 8,000 *g* for 5 minutes (Eppendorf Centrifuge 5424). After this period the supernatant was transferred to a separate tube for sugar analysis and the tube containing the residual biomass was reloaded with the predetermined biomass and enzyme loadings so that hydrolysis may be repeated. In the second hydrolysis the hydrolysis conditions were modified to evaluate the addition of the same enzyme loading, addition of half the original enzyme loading, no additional enzyme loading and

addition of the original enzyme loading but with no additional biomass. The complete set of reaction conditions for the sequential hydrolysis assays is shown in Table 1.

Table 1. Experimental assays for analyzing the effect of insoluble solids recycling

	Treatment															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Enzyme loading 1 (FPU/g)	20	20	10	10	20	20	10	10	20	20	10	10	20	20	10	10
Solids loading 1 (% w/v)	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%
Enzyme loading 2 (FPU/g)	20	20	10	10	10	10	5	5	0	0	0	0	20	20	10	10
Solids loading 2 (% w/v)	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	0%	0%	0%	0%
Enzyme loading 3 (FPU/g)	20	20	10	10	10	10	5	5	0	0	0	0	20	20	10	10
Solids loading 3 (% w/v)	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	0%	0%	0%	0%
Enzyme loading 4 (FPU/g)	20	20	10	10	10	10	5	5	0	0	0	0	20	20	10	10
Solids loading 4 (% w/v)	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	12%	8%	0%	0%	0%	0%

2.5.2. Consistent biomass concentrations

Enzymatic hydrolysis was performed in 50 mL Erlenmeyer flasks with working volume of 12 mL and submitted to mechanical agitation. The hydrolysis medium consisted of alkali-pretreated biomass at concentration of 12% (w/v), distilled water, sodium acetate buffer pH 5 (final concentration of 50 mM), enzyme blend (15 or 30 FPU/g glucan), tetracycline (40 mg/L) and sodium azide (10 mM). The prepared flasks were placed in a water bath at 50°C set upon magnetic agitators at 200 rpm.

Sugar content of the hydrolysis medium was monitored at 12 hour intervals by acquiring samples of the liquid-solid mixture. The percentage of biomass converted to a soluble form was estimated based on the quantity of sugar liberated. At 48 hour intervals additional biomass was added to the hydrolysis medium to maintain a solids loading of 10%. At 96 hour intervals the hydrolysis medium was centrifuged at 8,000 x *g* (Beckman J2-MI), the supernatant removed for analysis and the solids fraction reloaded into the Erlenmeyer flasks along with the calculated fresh biomass, water,

enzyme blend (15 or 30 FPU/g glucan), buffer, tetracycline and sodium azide. This procedure was repeated 4 times over a period of 480 hours.

2.6.Hydrolysis product analysis

Products of the saccharification assays were analyzed by high performance liquid chromatography (HPLC) with a Shimadzu series 10A chromatograph. The HPLC was equipped with an Aminex HPX-87P column (300 x 7.8 mm) (BioRad, Hercules, CA, USA) and refractive index detector (Shimadzu Corporation, Kyoto, Japan). The column was eluted with water at a flow rate of 0.6 mL/min and 80°C.

3. Results and Discussion

3.1.Characterization of the biomass and enzyme blend

Sugarcane bagasse was utilized as a model substrate for the saccharification experiments due to its availability as a potential lignocellulosic feedstock in Brazil. Prior to enzymatic hydrolysis, the bagasse was submitted to thermochemical pretreatment with 1.5% NaOH at 120°C for one hour after which approximately 50% of lignin was removed (Table 2), thus facilitating enzyme attack of the cellulose and hemicellulose fractions.

Table 2. Composition of the raw and pretreated sugarcane bagasse

Biomass component	Untreated bagasse (%)	Pretreated bagasse (%)
Glucan	52.8	59.2
Xylan	19.1	22.3
Arabinan	1.6	2.1
Lignin	22.1	11.4

*Values are the average of three repetitions and standard deviations did not exceed < 10% of the mean.

The enzyme cocktail utilized in the saccharification assays with enzyme recycling was the same as that developed in a previous study evaluating synergism between enzyme extracts produced by the fungi *Chrysoporthe cubensis* and *Penicillium pinophilum* (Visser et al. 2013). In the cited study it was found that synergy of the FPase and endoglucanase activities among the two extracts was 76% and 48% greater than theoretical, respectively. The concentrated enzyme blend utilized in the present study presented activities of 5.7 U/mL, 32.3 U/mL, 23.21 U/mL and 176.95 U/mL for

FPase, endoglucanase, xylanase and β-glucosidase activities, respectively. Total enzyme protein determined according to the Coomassie Blue binding method was 2.2 mg/mL. From previously publication on this same enzyme blend as well as enzyme production from *Chrysoporthe cubensis*, it is clear that an array of enzyme activities are present in the blend utilized (Falkoski et al. 2013; Visser et al. 2013).

3.2. Ability of recycled cellulase and xylanase enzymes to hydrolyze freshly added biomass

Based on the amount of glucose and xylose produced during the course of the saccharification reactions, it could be observed that significant quantities of these sugars were produced from the fresh and remaining substrate when insoluble solids were recycled. Figure 1 indicates the although the fresh glucan conversion decreased overall as a function of increasing solids loading, it was observed that in some cases the conversion efficiency increased in the second hydrolysis period resulting from recycled enzyme activity with the solids fraction. This increase in conversion efficiency is best observed in the treatments receiving additional enzymes at the concentration of 1X and for the lower solids loading (8%) (Figure 1-B). The treatments in which no fresh biomass was added are not included in Figure 1 since there was no biomass accumulation. Glucan and xylan conversion efficiencies maintained a linear relationship, as can be seen in Figure 2.

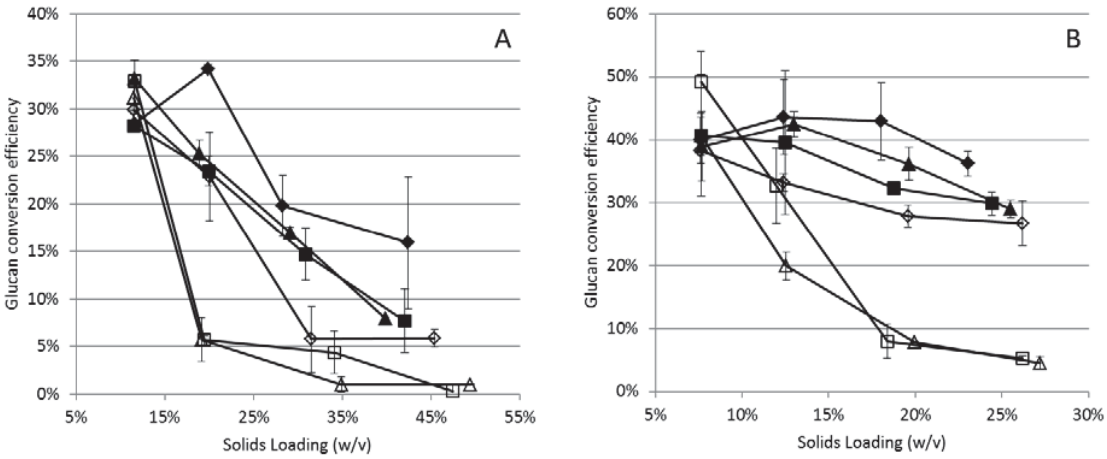

Figure 1. Conversion efficiencies of fresh glucan as a function of biomass loading for treatments receiving an additional 12% (A) and 8% (B) solids loading at each hydrolysis cycle. In figure A the treatments are represented as: 1 (solid diamond), 3 (solid square), 5 (solid triangle), 7 (open diamond), 9 (open square) and 11 (open triangle). In figure B the treatments are represented as: 2 (solid diamond), 4 (solid square), 6 (solid triangle), 8 (open diamond), 10 (open square) and 12 (open triangle).

As has been observed in diverse other studies, solids loading negatively affected enzyme hydrolysis efficiency (Kristensen, Felby, and Jorgensen 2009). Treatments receiving additional biomass at the concentration of 12 % (Figure 1-A) showed a much more rapid decline in glucose efficiency than the treatments receiving 8% biomass (Figure 1-B). It was also visually verified that when exceeding 25% solids, the slurry was paste-like which limits enzyme mobility (Jørgensen et al. 2007).

Xylan conversion efficiency was always greater than that of glucan, as observed in Figure 2. This is surely due to the high xylanase activity of the enzyme blend. The enzyme extract produced by *Chrysoporthe cubensis* has previously been shown to be an excellent source of xylanase enzymes, as well as additional accessory enzymes that aid in the biomass hydrolysis process (Falkoski et al. 2013). Synergy has also been confirmed when blending the *C. cubensis* extract with that of *P. pinophilum*, where *P. pinophilum* produces greater quantities of cellulase enzymes (Visser et al. 2013). This is one of the advantages of blending crude enzyme extracts, since no accessory enzymes are lost in purification steps which may have synergetic effects (Van Dyk and Pletschke 2012).

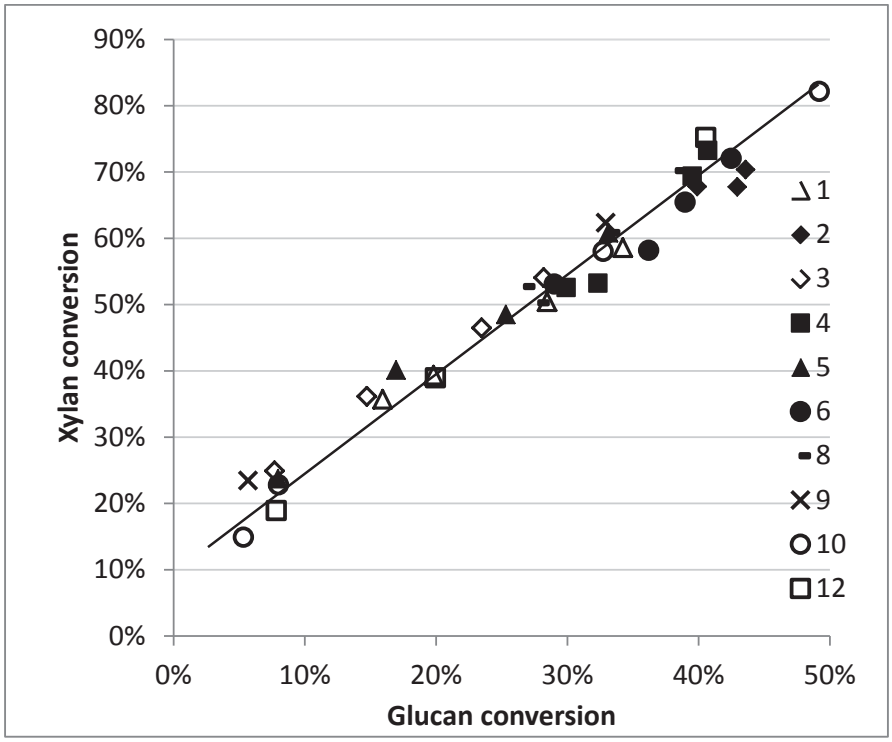

Figure 2. Relationship between glucan and xylan conversion to their respective monosaccharides. Points reflect the diverse recycle numbers for the different treatments.

Xylose liberation was directly correlated with that of glucose, indicated by a linear trend between glucan and xylan conversion efficiencies (Figure 2). This same effect has been reported in previous studies evaluating the effect of supplementing hemicellulases for saccharification of lignocellulosic biomass (Hu, Arantes, and Saddler 2011). Therefore, efficiency of xylan hydrolysis directly affects glucan hydrolysis efficiency, indicating the importance of removing xylan from the biomass structure for maximizing glucose yields. The more rapid liberation of xylose than glucose may be due to the fact that the enzyme blend utilized was rich in xylanase and other hemicellulase enzymes, and also because xylose has a much lower degree of polymerization when compared to cellulose which makes it more accessible to enzymes (Xiao, Sun, and Sun 2001).

To best observe the effects of recycled enzyme activity, the four different enzyme loading treatments were compared for the same biomass loading, where the highest initial enzyme loading (20 FPU/g) and lowest biomass loading (8%) best compared the results (Figure 3 showing treatments 2, 6, 10 and 14 according to Table 1).

In the second and third round of hydrolysis both glucose and xylose were produced in all treatments receiving additional biomass, independent of the additional enzyme loading (1X, 1/2X or 0). The fact that glucose and xylose were continuously liberated even when no fresh enzyme extract was added indicated recycling of enzyme activity; and even more remarkable was the fact that sugar liberation was nearly identical in the treatment receiving only half of the original enzyme loading upon recycling compared with that receiving the full enzyme loading. In the treatment receiving the fresh enzyme extract with no additional biomass (Figure 3-D), the reduced liberation of hydrolysis products is more due to substrate availability than lack of the enzyme catalyst (Gupta and Lee 2009).

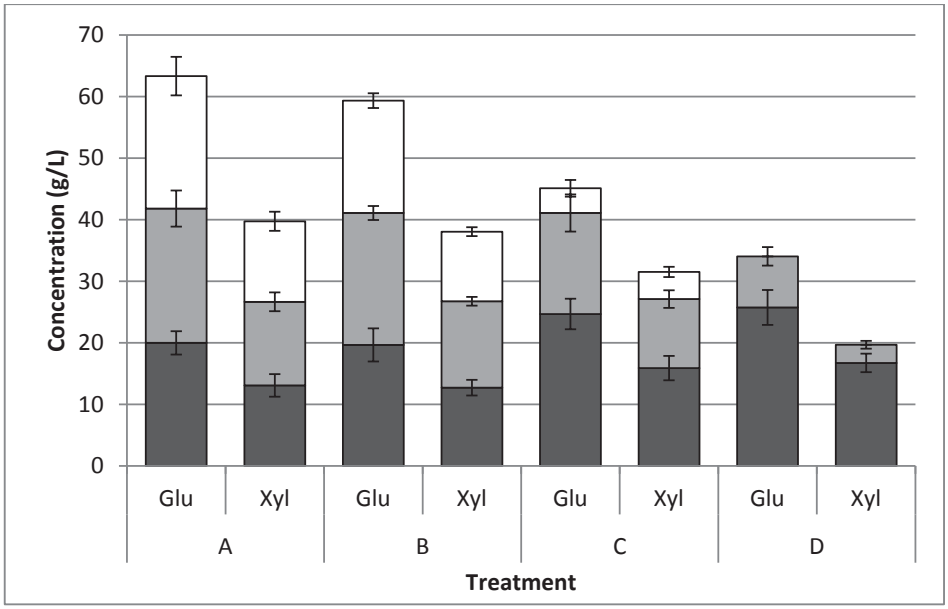

Figure 3. Glucose and xylose produced in treatments 2, 6, 10 and 14, according to Table 1, represented by the letters A, B, C and D. Bottom bars (black) represent the glucose and xylose produced in the first hydrolysis period, the middle bars (gray) are the concentrations of sugars produced in the second hydrolysis period and the top bars (white) represent the sugar masses produced in the third hydrolysis period. Error bars represent ± one standard deviation.

Recycling of the lignocellulolytic enzymes occurs because the enzymes readily adsorb to the solid substrates. Exceptions are the enzymes which have soluble substrates, such as β-glucosidase and β-xylosidase (Weiss et al. 2013; Várnai et al. 2011). In the experimental no accumulation of cellobiose was observed, even in the assays receiving no fresh enzymes upon recycle, indicating that β-glucosidase may have been recycle by other forms. Cellulase adsorption by lignin has also been reported when assessing

enzymatic hydrolysis, further preventing that enzymes are removed with the liquid fraction (Berlin et al. 2006; Nakagame et al. 2011). Further information on the effects of celluase adsorption by lignin are presented in the results section 3.5.

3.3.Fixed solids concentration

Having observed the potential for enzyme recycling together with insoluble solids, it was opted to perform a hydrolysis test with constant product monitoring which permitted for calculating the percentage of biomass hydrolyzed and therefore the biomass concentration. In this experiment it was desired to maintain a solids loading of 12% (w/v), where at 48 hour intervals biomass was added to the hydrolysis medium to maintain this concentration. At 96 hour intervals the enzyme cocktail was added as well, at concentrations of 15 or 30 FPU per gram of glucan (7 or 14 mg enzyme protein per gram of glucan). Enzyme loading was defined as a function of glucan mass instead of total biomass due to the ever changing composition of the solid fraction. By maintaining a constant solids loading proper mechanical agitation could be maintained, not inhibiting enzyme activity; however as in any recycle loop there would be potential for buildup of contaminants, in this case lignin. This method is also more industrially attractive since it allows for greater process control, as well as arrangement of multiple reactors to minimize capital and operational costs while maximizing yields.

Results of recycling the entire insoluble solids fraction permitted for increasing sugar concentrations (Figure 4). Large decreases in sugar concentration were observed at 96 hour intervals when the hydrolysis mixture was centrifuged and the sugar containing supernatant removed, followed by addition of the fresh enzyme blend, buffer and biomass which diluted the residual sugar content. The small drops in sugar concentration at 48, 144, 240, 336 and 432 hours were resultant of biomass addition, where the biomass contained 60% water and thus resulted in a small dilution of the hydrolysis medium.

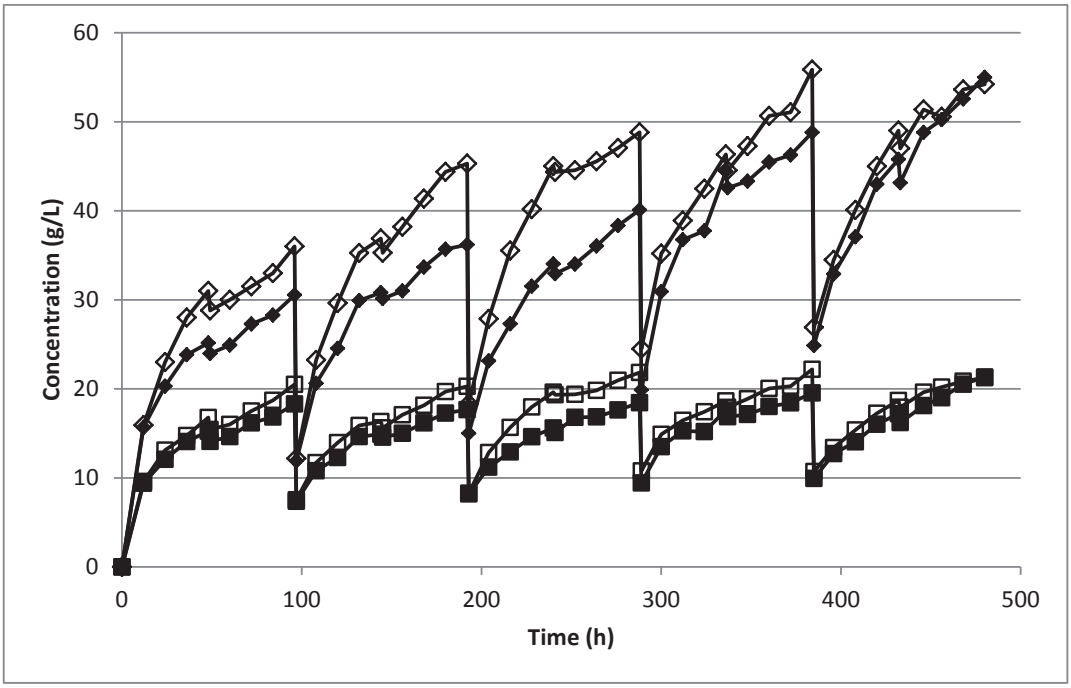

Figure 4. Production profile of glucose and xylose for the two enzyme loadings. The 30 FPU/g treatment is represented by open shapes and the 15 FPU/g treatment is represented by filled shapes (diamonds indicate glucose and squares indicate xylose).

Cellobiose did not accumulate in either of the experiments. Because the substrate for the enzyme β-glucosidase is soluble (cellobiose), it would be expected that this enzyme is not linked to the solid fraction when recycling, but instead lost with the liquid fraction. It is possible that β-glucosidase activity was encountered in multienzyme complexes which have been observed for different fungi (de Almeida et al. In Press; da Silva et al. 2012). However, β-glucosidase has also been found to strongly bind to lignin-rich residues (Zhang et al. 2013). Cellulase binding to lignin has commonly been thought to be one of the major forms of enzyme inhibition in the process of converting lignocellulose to soluble sugars (Rahikainen et al. 2011). In a study evaluating cellulase adsorption by lignin, cellulase adsorption was maximized in the first hour when glucan hydrolysis rates were maximized, followed by decreasing cellulase adsorption (Zheng et al. 2013).

A small decrease in glucan conversion efficiency was observed in the last recycle period for the treatment receiving 30 FPU/g glucan, however the conversion of fresh glucan added was still approximately 90% (Figure 5). This slight decrease in

conversion efficiency may be related to excessive buildup of contaminants from the multiple recycles. In the last recycle period the quantities of glucose and xylose liberated were nearly identical. The reduced glucose liberation in the treatment receiving the higher enzyme loading may be due to the greater glucan concentration in the biomass submitted to the 15 FPU treatment (52%) compared with the 30 FPU treatment (48%).

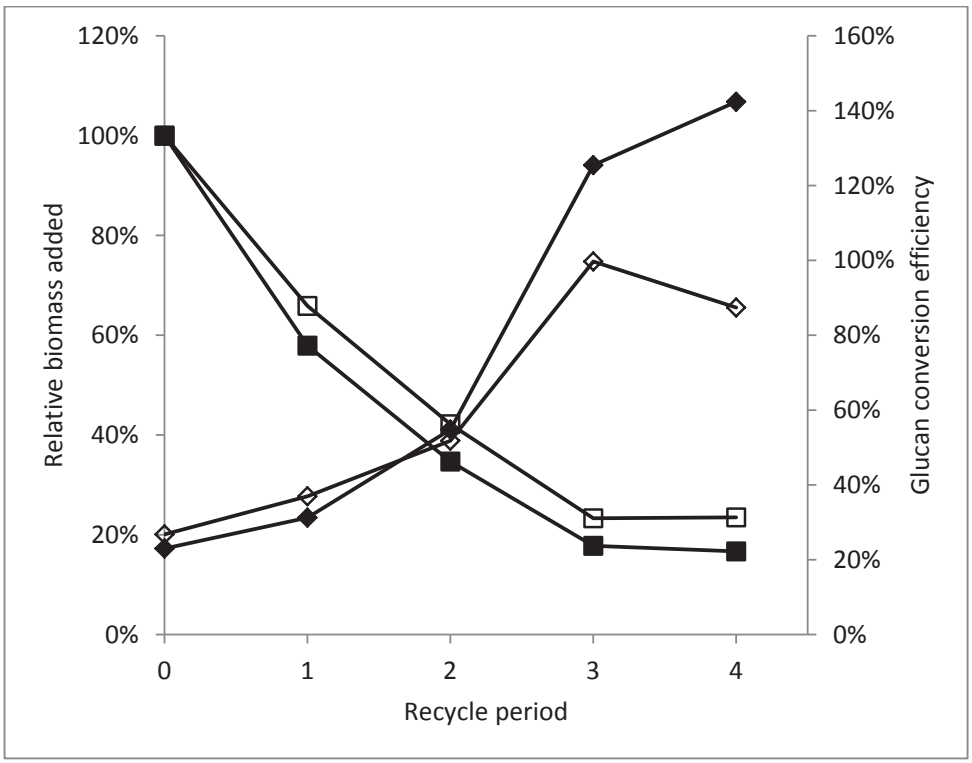

Figure 5. Glucan conversion efficiencies (diamonds) and relative biomass added per recycle period (squares). Filled shapes denote the enzyme loading of 15 FPU/g glucan and open shapes denote the enzyme loading of 30 FPU/g glucan.

3.4.Comparison of batch and fed batch hydrolysis

A previous study was performed utilizing the same enzyme blend on alkali pretreated sugarcane bagasse in a 120 hour batch hydrolysis (Visser et al. 2013). In that study it was observed that the hydrolysis rate decreased to less than 0.2 g/L/h after 120 hours for all processing methods evaluated. In the present fed-batch study the hydrolysis rate was maintained higher, as observed in Figure 6, which shows the glucose and xylose liberation rates over 48 hour intervals. Over the full 488 hour test period, the average glucose liberation rates were 0.31 and 0.33 g/L/h for the treatments with 15

FPU/g glucan and 30 FPU/g glucan, respectively, and xylose was liberated at rates of 0.15 and 0.16 g/L/h. The proximity of these values indicated that extending the hydrolysis period would be more effective than increasing the enzyme loading, since when utilizing only half the enzyme loading the glucose liberation rate was only 6% lower.

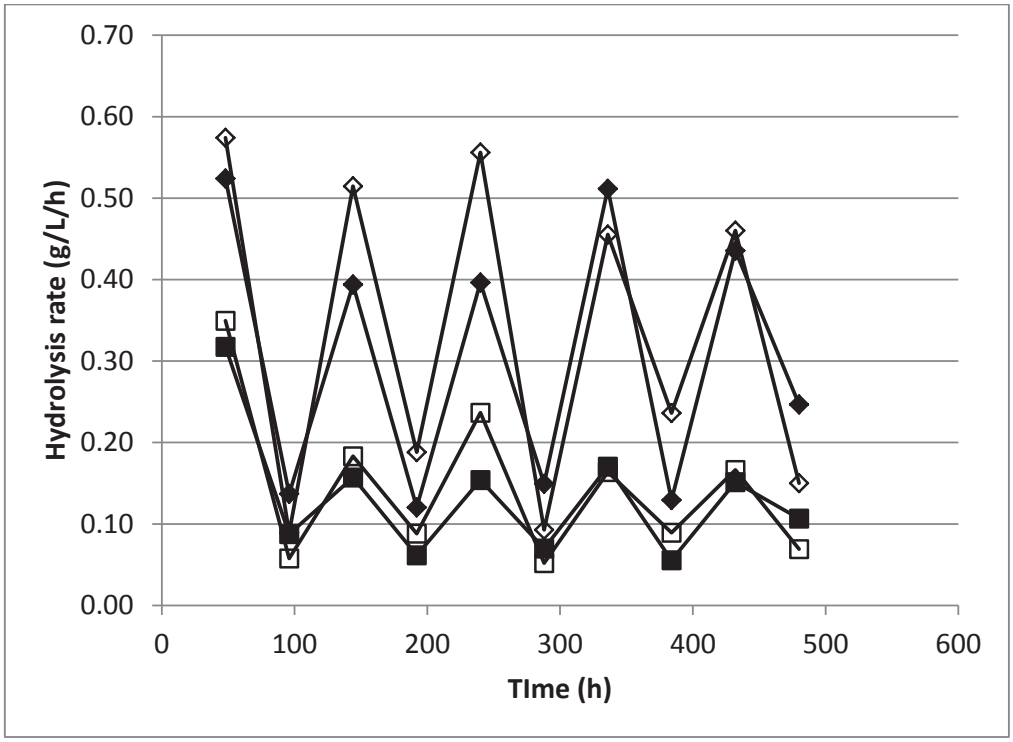

Figure 6. Rates of glucose and xylose liberation for the two enzyme loadings. Glucose liberation rates are represented by diamonds and xylose liberation rates are represented by squares (filled shapes denote the enzyme loading of 15 FPU/g glucan and open shapes denote the enzyme loading of 30 FPU/g glucan).

Enzyme productivity (quantity of sugar produced per quantity of enzyme applied) is one of the best methods for comparison of enzyme efficiency. This parameter was utilized in another enzyme recycling study when applying a commercial enzyme extract, with significantly higher enzyme loadings (Weiss et al. 2013). The study performed by these authors presented a maximum enzyme productivity in the range of 0.3 g glucose/mg enzyme protein. In the present study this number was drastically higher, where the greatest productivity was obtained when applying 15 FPU/g of glucan (3.74 g glucose/mg enzyme protein). When using this same enzyme cocktail in a batch experiment, a maximum productivity of 0.37 g glucose/mg enzyme

protein was obtained, indicating the efficiency of the fed-batch process to significantly increase productivity (Visser et al. 2013). The high productivity values obtained are likely due to the complex nature of the enzyme extract utilized. Inclusion of hemicellulase enzymes in the extract has shown to greatly improve enzymatic hydrolysis of both xylose and also cellulose (Zhang, Tang, and Viikari 2012; Hu, Arantes, and Saddler 2011). Although not reported, it is likely that the enzyme blend contains some lignin degrading enzymes (laccases, lignin peroxidase, manganese peroxidase, etc.) that may have also facilitated breakdown of the lignocellulosic structure.

Table 3. Results of fed-batch processing when maintaining a constant solids loading (12% w/v) and varying the enzyme loading

	15 FPU/g cellulose		30 FPU/ g cellulose	
	Glucose	Xylose	Glucose	Xylose
Average rate (g/L/h)	0.31	0.15	0.35	0.16
Overall conversion efficiency	51%	64%	55%	68%
Enzyme productivity (g glucose/mg enzyme protein)	3.78	1.79	2.56	1.19

3.5.Effect of increased lignin concentrations on enzymatic hydrolysis

It was found that in the treatments with enzyme loadings of both 15 and 30 FPU/g, hydrolysis efficiency increased with increasing lignin concentrations. Because the entire soluble solids fraction was reutilized in all recycle periods, it was expected that lignin would build up in the hydrolysis medium since it is not efficiently broken down and solubilized as occurs with the cellulose and hemicellulose fractions. This can be observed when comparing the lignin percentage to the efficiency for converting fresh glucan added to the reaction medium (Figure 7). This differs significantly from what was expected, because lignin is known to nonspecifically bind to cellulase enzymes and therefore inhibit cellulose hydrolysis (Várnai et al. 2011).

Adsorption of enzymes to the substrate during hydrolysis is desired because productive binding of proteins on the substrate surface has been shown to improve enzyme-substrate interactions. However non-productive binding of enzymes on lignocellulosic substrates also occurs, where the enzymes may remain blocked in the dead ends of the substrate of non-productively bind to either cellulose or lignin (Jalak and Väljamäe 2010). Besides non-productive binding, lignin may cause inaccessibility of cellulase enzymes to its substrate. Pretreatment methods also have different effects

on lignin properties. Lignin in eucalyptus wood chips subjected to steam-explosion pretreatment showed greater capacity to adsorb cellulase enzymes than non-pretreated lignin (Nonaka, Kobayashi, and Funaoka In Press). Since it was shown that cellulase adsorption decreased over a 72 hour hydrolysis period (Zheng et al. 2013), since the total hydrolysis period was significantly longer (total of 488 hours), this effect may have been extrapolated.

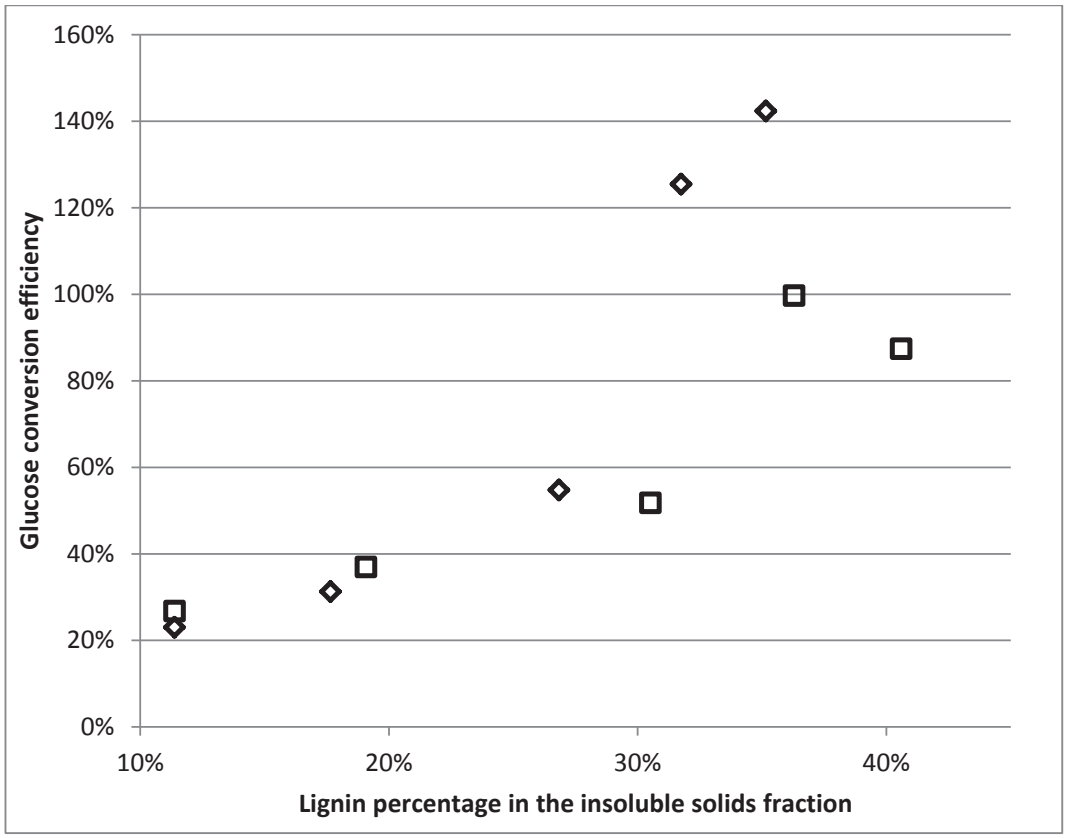

Figure 7. Theoretical glucose yields for the 96 hour hydrolysis reactions on the pretreated sugarcane bagasse as a function of lignin buildup. Diamonds represent the enzyme loading of 15 FPU/g glucan and squares denote the enzyme loading of 30 FPU/g glucan.

This same behavior of lignin was observed in another study evaluating the recycling of insoluble solids (Weiss et al. 2013). These authors suggested that because the lignin residue was previously exposed to the enzyme blend, the binding sites on lignin may have already been occupied by enzymes from the prior hydrolysis. It has already been shown that enzymes remain active associated with the insoluble fraction,

adhered to the cellulose and hemicellulose fractions as well as lignin, and it may also be possible that inactivated enzymes remain adhered to the solids and occupy lignin-protein binding locations. Because glucose concentrations continued to increase (Figure 3), the higher glucose yields cannot be attributed to lower enzyme inhibition. From visual assessment of the solid fraction after the multiple recycle periods it appeared that the biomass which initially showed to be fibrous presented a very fine texture, where this homogeneity and reduce size of solids may have contributed to facilitate enzymatic hydrolysis although the solids concentration was maintained. The results shown in the present study therefore indicate that lignin in the insoluble fraction has no negative effect on glucose and xylose yields.

4. Conclusion

It was possible to efficiency recycle a significant portion of enzymes from blend consisting of enzyme extracts from *Chrysoporthe cubensis* and *Penicillium pinophilum* by recycling the solid fraction after enzymatic hydrolysis of pretreated sugarcane bagasse. Glucose yields remained the same after two hydrolysis periods when biomass was constantly added at the same rate when comparing the treatments receiving the same initial loading, half the initial loading and no additional enzyme. Although efficiencies did decrease when using this method, it was clearly shown that the enzymes could be efficiently recycled.

When basing process control on maintaining a set solids loading, it was found that greater soluble sugar yields can be obtained even when applying fewer enzymes. This represents a significant improvement to the lignocellulosic biomass process as well as reduction in required enzyme loading for hydrolysis. However, when recycling the solid fraction the continued build-up of lignin affects processing parameters. In the present study lignin concentrations exceeded 40%, but apparently had no negative effect on enzymatic hydrolysis since conversion efficiencies continued to increase throughout the recycling period. Enzyme recycling therefore shows potential to reduce enzyme requirements and operating costs for production of bioethanol.

5. Acknowledgements

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6. References

Berlin, Alex, Mikhail Balakshin, Neil Gilkes, John Kadla, Vera Maximenko, Satoshi Kubo, and Jack Saddler. 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. *Journal of Biotechnology* 125 (2):198-209.

Bradford, MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248-254.

Chang, Yi-Huang, Ku-Shang Chang, Cheng-Wei Huang, Chuan-Liang Hsu, and Hung-Der Jang. 2012. Comparison of batch and fed-batch fermentations using corncob hydrolysate for bioethanol production. *Fuel* 97 (0):166-173.

da Silva, A. J., D. P. Gomez-Mendoza, M. Junqueira, G. B. Domont, E. Ximenes Ferreira Filho, M. V. de Sousa, and C. A. Ricart. 2012. Blue native-PAGE analysis of Trichoderma harzianum secretome reveals cellulases and hemicellulases working as multienzymatic complexes. *Proteomics* 12 (17):2729-38.

de Almeida, Maíra N., Daniel L. Falkoski, Valéria M. Guimarães, Humberto Josué de O. Ramos, Evan M. Visser, Gabriela P. Maitan-Alfenas, and Sebastião T. de Rezende. In Press. Characteristics of free endoglucanase and glycosidases multienzyme complex from Fusarium verticillioides. *Bioresource Technology* (0).

Dwivedi, Puneet, Janaki R. R. Alavalapati, and Pankaj Lal. 2009. Cellulosic ethanol production in the United States: Conversion technologies, current production status, economics, and emerging developments. *Energy for Sustainable Development* 13 (3):174-182.

El-Zawawy, Waleed K., Maha M. Ibrahim, Yasser R. Abdel-Fattah, Nadia A. Soliman, and Morsi M. Mahmoud. 2011. Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production. *Carbohydrate Polymers* 84 (3):865-871.

Falkoski, D. L., V. M. Guimaraes, M. N. de Almeida, A. C. Alfenas, J. L. Colodette, and S. T. de Rezende. 2013. Chrysoporthe cubensis: A new source of cellulases and hemicellulases to application in biomass saccharification processes. *Bioresour Technol* 130:296-305.

Ghose, T.K. 1987. Measurement of Cellulase Activities. *Pure and Applied Chemistry* 59 (2):257-268.

Gottschalk, Leda Maria Fortes, Raul Alves Oliveira, and Elba Pinto da Silva Bon. 2010. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by Trichoderma and Aspergillus act synergistically in the hydrolysis of sugarcane bagasse. *Biochemical Engineering Journal* 51 (1–2):72-78.

Gupta, Rajesh, and Y. Y. Lee. 2009. Mechanism of cellulase reaction on pure cellulosic substrates. *Biotechnology and Bioengineering* 102 (6):1570-1581.

Gupta, Rishi, Sanjay Kumar, James Gomes, and Ramesh Kuhad. 2012. Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol. *Biotechnology for Biofuels* 5 (1):16.

Hodge, DavidB, M. Nazmul Karim, DanielJ Schell, and JamesD McMillan. 2009. Model-Based Fed-Batch for High-Solids Enzymatic Cellulose Hydrolysis. *Applied Biochemistry and Biotechnology* 152 (1):88-107.

Hu, Jinguang, Valdeir Arantes, and Jack Saddler. 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnology for Biofuels* 4 (1):36.

Jalak, Jürgen, and Priit Väljamäe. 2010. Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis. *Biotechnology and Bioengineering* 106 (6):871-883.

Jørgensen, Henning, Jakob Vibe-Pedersen, Jan Larsen, and Claus Felby. 2007. Liquefaction of lignocellulose at high-solids concentrations. *Biotechnology and Bioengineering* 96 (5):862-870.

Kovacs, Krisztina, Stefano Macrelli, George Szakacs, and Guido Zacchi. 2009. Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with Trichoderma atroviride enzymes produced in-house. *Biotechnology for Biofuels* 2 (1):14.

Kristensen, Jan, Claus Felby, and Henning Jorgensen. 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels* 2 (1):11.

Lau, Ming W., and Bruce E. Dale. 2009. Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST). *Proceedings of the National Academy of Sciences* 106 (5):1368-1373.

Miller, G. L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* 31 (3):426-428.

Nakagame, Seiji, Richard P. Chandra, John F. Kadla, and Jack N. Saddler. 2011. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. *Bioresource Technology* 102 (6):4507-4517.

Nonaka, Hiroshi, Ai Kobayashi, and Masamitsu Funaoka. In Press. Lignin isolated from steam-exploded eucalyptus wood chips by phase separation and its affinity to Trichoderma reesei cellulase. *Bioresource Technology* (0).

Patzek, T.W. 2007. How can we outlive our way of life? Paper read at 20th Round table on sustainable development of biofuels: is the cure worse than the disease?, at OECD, Paris.

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood—study of enzyme binding and inactivation on lignin-rich surface. *Biotechnology and Bioengineering* 108 (12):2823-2834.

TAPPI. 1991. Technical Association of the Pulp and Paper Industry Tappi Useful Methods (UM-250), Norcross.

———. 1999. Technical Association of the Pulp and Paper Industry. Tappi Standard Methods (T-222 om-98), Atlanta.

Van Dyk, J. S., and B. I. Pletschke. 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnology Advances* 30 (6):1458-1480.

Várnai, Anikó, Liisa Viikari, Kaisa Marjamaa, and Matti Siika-aho. 2011. Adsorption of monocomponent enzymes in enzyme mixture analyzed quantitatively during hydrolysis of lignocellulose substrates. *Bioresource Technology* 102 (2):1220-1227.

Visser, E. M., D. L. Falkoski, M. N. de Almeida, G. P. Maitan-Alfenas, and V. M. Guimarães. 2013. Production and application of an enzyme blend from Chrysoporthe cubensis and Penicillium pinophilum with potential for hydrolysis of sugarcane bagasse. *Bioresour. Technol.*

Wanderley, Maria Carolina de Albuquerque, Carlos Martín, George Jackson de Moraes Rocha, and Ester Ribeiro Gouveia. 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. *Bioresource Technology* 128 (0):448-453.

Weiss, Noah, Johan Borjesson, Lars Saaby Pedersen, and Anne Meyer. 2013. Enzymatic lignocellulose hydrolysis: Improved cellulase productivity by insoluble solids recycling. *Biotechnology for Biofuels* 6 (1):5.

Xiao, B., X. F. Sun, and RunCang Sun. 2001. Chemical, structural, and thermal characterizations of alkali-soluble lignins and hemicelluloses, and cellulose from maize stems, rye straw, and rice straw. *Polymer Degradation and Stability* 74 (2):307-319.

Zhang, Junhua, Ulla Moilanen, Ming Tang, and Liisa Viikari. 2013. The carbohydrate-binding module of xylanase from Nonomuraea flexuosa decreases its non-productive adsorption on lignin. *Biotechnology for Biofuels* 6 (1):18.

Zhang, Junhua, Ming Tang, and Liisa Viikari. 2012. Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. *Bioresource Technology* 121 (0):8-12.

Zhao, Xuebing, Lei Dong, Liang Chen, and Dehua Liu. 2013. Batch and multi-step fed-batch enzymatic saccharification of Formiline-pretreated sugarcane bagasse at high solid loadings for high sugar and ethanol titers. *Bioresource Technology* 135 (0):350-356.

Zhao, Xuebing, and Dehua Liu. 2012. Fractionating pretreatment of sugarcane bagasse by aqueous formic acid with direct recycle of spent liquor to increase cellulose digestibility–the Formiline process. *Bioresource Technology* 117 (0):25-32.

Zheng, Yingfu, Songping Zhang, Shida Miao, Zhiguo Su, and Ping Wang. 2013. Temperature sensitivity of cellulase adsorption on lignin and its impact on enzymatic hydrolysis of lignocellulosic biomass. *Journal of Biotechnology* 166 (3):135-143.

GENERAL CONCLUSION

6.1. General Conclusions

- Based on certain operating conditions, roughly 55% of sugarcane bagasse may be submitted to the second generation ethanol production process while continuing to meet the energy demands of the existing ethanol plant and the increased energy demands for processing/distillation of second generation ethanol
- The genetically modified yeast strain *S. cerevisiae* YRH400 showed to be more efficient for fermenting both glucose and xylose than *K. marxianus* ATCC-8554 and *K. marxianus* UFV-3.
- The reduced temperature of fermentation significantly limited enzymatic hydrolysis of the enzyme extract from *Chrysoporthe cubensis*, even when utilizing the thermotolerant yeast *K. marxianus*, making separate hydrolysis and fermentation more attractive.
- The enzyme blend consisting of the extracts from *Chrysoporthe cubensis* and *Penicillium pinophilum* presented high levels of synergy, represented by values of 76%, 50% and 24% for FPase, endoglucanase and xylanase activities, respectively.
- When utilizing the enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum*, enzymatic hydrolysis performed at 50°C resulted in glucose and xylose yields 16% and 20% greater than the same hydrolysis at 45°C. After the 120 hour hydrolysis period the glucose liberation rate continued to be significantly higher for the higher temperature and also appeared to be constant, while that at 45°C was declining at 120 hours.
- Recycling of cellulases and hemicellulases showed to be feasible, where in certain cases glucose liberation was equal for the cases of adding 1x (the same amount), 1/2x or no additional enzyme in the second hydrolysis period. However when adding the same amount of biomass (8 or 12%) during each recycle period the solids concentration increased and hydrolysis efficiency decreased significantly.
- Maintaining a constant solids loading (12%) allowed for efficient hydrolysis and maximizing of enzyme productivity. Application of enzyme loadings of 15 and 30 FPU/g glucan resulted in glucose hydrolysis efficiencies of 51% and 55%, and enzyme productivity values of 3.8 and 2.6 g glucose per mg enzyme productivity, respectively.