LARISSE APARECIDA RIBAS BATALHA

CARACTERIZAÇÃO E APLICAÇÃO DOS RESÍDUOS DA CANA DE AÇÚCAR VISANDO À PRODUÇÃO DE ETANOL 2G

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Agroquímica, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 13 de junho de 2016.

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SUMÁRIO

RESUMO

BATALHA, Larisse Aparecida Ribas, D.Sc. Universidade Federal de Viçosa, Junho de 2016. **Caracterização e aplicação dos resíduos da cana de açúcar visando à produção de etanol 2G.** Orientador: Jorge Luiz Colodette.

A utilização racional da biomassa tem sido considerada uma prática promissora para a obtenção de produtos de alto valor agregado em uma cadeia de produção sustentável. Neste cenário, o conceito de biorrefinaria surgiu, esse refere à conversão de materiais lignocelulósicos em bioprodutos (celulose, produtos químicos, etc.) e bioenergia (etanol, energia, etc.) com o mínimo de emissões e baixo desperdício. O conceito de biorrefinaria baseia-se em muitos diferentes tipos de biomassa, como resíduos agrícolas e florestais, madeira e gramíneas em geral, etc. Nesse trabalho, nos reportamos uma caracterização da lignina e dos extrativos presentes no bagaço, medula e palha de cana-de-açúcar. Para a caracterização da lignina utilizamos a técnica de pirólise analítica acoplada a cromatografia gasosa e espectrometria de massa (Pi-CG/EM) e para extrativos; a técnica de cromatografia gasosa e espectrometria de massa (CG/EM). Também, foi investigado o uso do bagaço e da palha de cana de açúcar como fonte para produção de etanol de segunda geração, através da realização de processos de pré-tratamento (auto-hidrólise) seguido pela sacarificação. A análise química dos extrativos permitiu a identificação de 45 compostos de *Eucalyptus urograndis*, 47 para palha e 67 para o bagaço. Os principais componentes das matérias-primas estudadas foram os ácidos graxos para *E. urograndis* e carboidratos para palha e o bagaço. Foi possível identificar mais de 40 produtos primários da pirólise para lignina solúvel em ácido e 30 para lignina Klason para os resíduos da cana de açúcar. A pirólise analítica da lignina solúvel em ácido a partir de bagaço de cana (SCB), da palha de cana (SCS), e da medula da cana (SCP) mostrou a prevalência de picos relacionados com produtos derivados de carboidratos. As pirogramas para as amostras de lignina Klason de SCB, SCP e SCS mostraram a prevalência dos picos derivados da lignina. O estudo da produção de etanol de 2G mostrou que um total de 84,4% de açúcar pode ser recuperado a partir do bagaço de cana de açúcar, a 180 ^{0}C durante 20 min com uma dosagem de enzima de 5 FPU/grama de substrato. A análise econômica para o método proposto mostrou que a produção de bioetanol pode ter um retorno financeiro maior do que 12%. Já produção de etanol a partir da autohidrólise da palha da cana parece ser uma abordagem viável financeiramente se um sistema rentável de coleta de palha seja estabelecido. Com esse estudo foi possível verificar que os resíduos da cana de açúcar podem ser fonte promissoras de bioquímicos valiosos os

quais podem ser utilizados na indústria de cosméticos, alimentos ou farmacêutica, como também, na produção do etanol de 2G.

ABSTRACT

BATALHA, Larisse Aparecida Ribas, D.Sc. Universidade Federal de Viçosa, June, 2016. **Characterization and application of sugarcane waste aiming at the production of 2G ethanol.** Adviser: Jorge Luiz Colodette.

In this work, we report the characterization of lignin and extractives present in the bagasse, pith and straw sugarcane. For the characterization of lignin we used the technique of analytical pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC/MS and for extractives to gas chromatography and mass spectrometry (GC/MS). Also, we investigated the use of straw and bagasse sugarcane as a source for production of second generation ethanol, by performing pretreatment (autohydrolysis) processes followed by saccharification. Chemical analysis of extractives allowed the identification of 45 compounds for Eucalyptus urograndis, 47 for straw and 67 for bagasse. The major components from the raw materials studied were fatty acids for E. urograndis and carbohydrates for straw and bagasse. It was possible to identify more 40 primary pyrolysis products for acid-soluble lignin and 30 for Klason lignin for sugarcane residues. Analytical pyrolysis of acid-soluble lignin from sugarcane bagasse (SCB), sugarcane straw (SCS), and sugarcane pitch (SCP), showed prevalence of peaks related to products derived from carbohydrates. The pyrograms for the Klason lignin samples of SCB, SCP and SCS showed the prevalence of lignin derivatives. The study of 2G ethanol production showed a total of 84.4% sugar can be recovered from the sugar cane bagasse, at 180 ^{0}C for 20 min with an enzyme dosage 5 FPU / gram of substrate. The economic analysis for the proposed method showed that the production of bioethanol may have a greater financial return than 12%. Already producing ethanol from sugarcane straw autohydrolysis seems to be a viable approach financially if a profitable system of straw collection is established. With this study it was possible to verify that the wastes sugarcane may be promising feedstocks of valuable biochemical that can be used in cosmetics, foods or pharmaceuticals, as well as the production of the 2G ethanol.

INTRODUÇÃO GERAL

A crescente demanda da sociedade por processos industriais sustentáveis tem motivado a constante busca dos setores acadêmicos e industriais por fontes de energias renováveis, principalmente oriundas de resíduos florestais e agroindustriais. Assim, nos últimos anos, tem havido uma tendência em pesquisas que visem à utilização mais eficiente dos resíduos agroindustriais. Uma fonte significativa desses resíduos pode ser obtida das indústrias sucroenergéticas, por exemplo. A produtividade média da cana no Brasil é de 85 toneladas por hectare. Em média, para cada tonelada de cana processada 140 kg de matéria seca do bagaço e 140 kg de matéria seca da palha são gerados, isto é, 12 toneladas de palha e 12 toneladas de bagaço por hectare (Santos et al., 2012). Em torno de 60% dos resíduos da cana de açúcar, palha e bagaço são constituídos por carboidratos, o restante é principalmente por lignina além de minerais e extrativos (Santos et al, 2012; Batalha et al, 2015). Por serem ricos em polissacarídeos e por esses resíduos estarem presente em grande abundância no Brasil, os mesmos se tornam grandes atrativos para produção de bioetanol.

No que diz respeito ao uso da biomassa para a produção de biocombustíveis, novos avanços científicos tornaram a produção de etanol celulósico uma realidade, mas ainda é preciso um esforço concentrado de pesquisa para tornar a sua conversão ainda mais eficiente. Atualmente, a produção de biocombustíveis provenientes de materiais lignocelulósicos por algumas rotas tecnológicas não se apresenta economicamente viável devido ao alto custo da enzima que é utilizada na hidrólise enzimática. Assim, para tornar biocombustíveis celulósicos mais competitivos, melhoramentos na degradação enzimática da celulose são necessários e essenciais (Lynd et al., 2008; Ertas et al., 2014, Batalha et al, 2015).

Para auxiliar na acessibilidade das enzimas aos carboidratos, uma alternativa seria os estudos que visem à aplicação de estágios de pré-tratamento da biomassa que "afrouxem" sua estrutura uma vez que o mesmo causa a remoção da lignina e/ou hemiceluloses, reduzindo a cristalinidade da celulose, e o aumento da porosidade do material (Sarkar et al., 2012) aumentando a susceptibilidade dos carboidratos as enzimas (Mosier et al., 2005). Um pré-tratamento eficaz deve preservar a utilidade das hemiceluloses e evitar a formação de inibidores (Margéot et al., 2009). Um pré-tratamento é considerado econômico quando utiliza produtos químicos baratos e requer equipamentos e procedimentos simples (Margéot et al., 2009). Em geral, as tecnologias de pré-tratamento pode ser dividido em diferentes categorias: físico (moagem e trituração), físico-químico (pré-tratamento a vapor / autohidrólise,

hidrotérmico, e oxidação), químicos (alcalino, ácido diluído, agentes oxidantes e solventes orgânicos), biológicos, elétrico, ou uma combinação destes (Kumar et al., 2009).

Visando um uso mais eficiente da biomassa lignocelulósica, a aplicação de resíduos agroindustriais em processos de produção de bioetanol bem como na produção de polpa celulósica são interessantes uma vez que se converteriam os mesmos em produtos de maior valor agregado. Neste contexto a compreensão da composição lignocelulósica torna-se um requisito essencial para a obtenção de uma estratégia bem sucedida para utilizações destes resíduos em produtos de maior valor agregado. A lignina é um heteropolímero aromático e, é o segundo biopolímero mais abundante na biomassa após a celulose. Essa tem origem na polimerização oxidativa dos seguintes precursores: álcool coniferílico, álcool sinapílico e, álcool cumárico (Yuan et al., 2011). A eficácia do pré-tratamento da biomassa é altamente dependente tanto do teor quanto da estrutura da lignina e, por conseguinte, um conhecimento da estrutura deste polímero é muito importante. Os extrativos, apesar de se apresentar em menores teores na biomassa, têm grandes efeitos na produção da polpa celulósica, como exemplo causando o *pitch*, e também pode prejudicial às enzimas durante a produção de bioetanol assim sua caracterização é de grande valia.

Assim, esse estudo objetivou identificar e quantificar os extrativos presentes no bagaço e a na palha da cana de açúcar; caracterizar a lignina presente no bagaço, medula e palha de cana de açúcar quanto à sua composição e estrutura, bem como estudar a obtenção de açúcares fermentáveis a partir desses resíduos (bagaço e a palha da cana de açúcar) utilizando os processos de auto-hidrólise e refino como pré-tratamentos. As informações obtidas irão promover o uso dos resíduos da cana de açúcar como matéria-prima para os biocombustíveis e outros produtos de biorrefinaria, tais como alimentos.

Para isso, este trabalho foi dividido em quatro capítulos a saber*: (1) Comparative chemical composition of extractives from eucalyptus versus straw and bagasse sugarcane;(2) Characterization of Klason and acid-soluble lignins from sugarcane residues by pyrolysis-gas chromatography/mass spectrometry (PY - GC/MS); (3) Production of fermentable sugars from sugarcane bagasse by enzymatic hydrolysis after autohydrolysis and mechanical refining e (4) Autohydrolysis pretreatment followed by refining as method to enhance sugar recovery from sugarcane straw for bioethanol production*.

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Capítulo 1: Comparative chemical composition of extractives from eucalyptus versus straw and bagasse sugarcane

ABSTRACT

Eucalypts are one of the most important source of lignocellulosic biomass for paper production, however alternative raw materials has being studied to analyze its potential in the biorefinery processes. In this context, bagasse and straw sugarcane are examples of alternative sources and can be explored because they are sugar industry waste and they are rich in lignocellulosic components. Among their main chemical constituents, the extractives deserve a special attention because they are in part responsible to the pitch origins and the low quality of the final product. This study aimed to quantify and identify the chemical constituents of extractives presents in *Eucalyptus urograndis* and straw and bagasse sugarcane. These raw materials were extracted with a sequence of solvents in order of increasing polarity, ethanol/toluene (1:2 v/v), ethanol and water, were analyzed by FT-IR and gas chromatography coupled to mass spectrometry. The results showed a greater amount of extractive in straw (11.33% w/w) and bagasse (7.91% w/w) samples in relation to *E. urograndis* (2.44% w/w). Chemical analysis of the extractives by infrared and gas chromatography-mass spectrometry enabled the identification of 45 compounds for *E. urograndis*, 47 for straw and 67 for bagasse. The major components for the raw materials studied were fatty acids for *E. urograndis* and carbohydrates for straw and bagasse. These data are important for industries to develop new technologies for the disposal of extractive pitches and improving the pulping processes, as well as contributes for the knowledge of the chemistry of straw and bagasse from sugarcane.

Keywords: Extractives, *Eucalyptus urograndis*, sugarcane bagasse, sugarcane straw, GC-MS, FT-IR.

1. Introduction

Eucalypts are one of the most important source of cellulose for paper production, however alternative raw materials has being studied to analyze its potential in the biorefinery processes (Batalha et al., 2012; Requejo et al., 2012; Gomes et al., 2013; Andrade and Colodette, 2014). In this context, bagasse and straw sugarcane are examples of alternative sources and can be explored since they are sugar-alcohol industry waste and are rich in lignocellulosic components. The sugarcane straw (or trash) is divided in three principal components, that is, fresh leaves, dry leaves, and tops. The sugarcane bagasse is residual fraction from the sugarcane stem milling (Canilha et al., 2012).

In Brazil, the sugarcane bagasse and straw are an abundant source of raw materials, e.g., the average sugar cane productivity in the country is 85 tons per hectare. In average for each ton of sugar cane processed 140 kg dry-matter of bagasse and 140 kg dry-matter of straw are generated, i.e, 12 tons of straw and 12 tons of bagasse per hectare (Santos et al., 2012). Generally, the sugarcane straw is handles three different ways: (1) a small fraction is transported to the mills for heat/power generation, (2) the largest part is left over in the fields where it serves as nutrient source, but causes all sorts of difficulties to the subsequent crop, (3) is simply burnt in the fields to enable manual harvesting in places where mechanical harvesting is not possible (da Silva et al., 2010). Normally, sugarcane bagasse is used as the main source of the energy required in sugar mills and ethanol distilleries and also for generating electricity to be sold to the grids. Nevertheless, a significant portion of the produced bagasse is underexplored in terms of alternative application. It has been reported that upon the technological improvements made to the boilers it is possible to satisfy the energy requirements of the plants with only half of the produced bagasse. Due to the amount of this biomass as an industrial waste, there is great interest in developing methods for the production chemicals from this important raw material in a sustainable way.

Among their main chemical constituents of the lignocellulosic materials, the extractives deserve a special attention as they are primary responsible for the pitch deposits formed in the in the pulp and paper industries (Hillis and Sumimoto, 1989; del Río et al. 2000; Gutiérrez et al. 2001; Freire et al. 2002a; Cruz et al., 2006; Barbosa et al., 2008). These pitch results in reduction of production levels, higher operating costs and an increased incidence of quality defects in pulps and papers (Hillis and Sumimoto, 1989). Also, these can impair the bioethanol production from lignocellulosic material since some extractive components may decrease the enzymatic activity during the enzymatic hydrolysis process (Cherdchim et al., 2012).

Nevertheless in the biorefinery concept, all organic fractions derived from lignocellulosic materials can potentially be used, including the extractives (Prinsen et al., 2012). The extractives may lead to the generation of valuable products since these are raw materials for a substantial chemicals industry including printing inks, flavours and fragrances (Hergert et al., 2012; Raimo et al., 2015). Tall oil and turpentine, extractives recovered as byproducts of the kraft pulp industry, can be utilized primarily in the manufacture of specialty chemicals and a wide range of industrial products, including solvents, adhesives, polymers, emulsifiers, coatings, and paper sizing. For example, polyphenolic compounds valued for their high

antioxidizing efficiency, have been examined and different processes for isolation from wood have been suggested (Hergert et al., 2012; Raimo et al., 2015). Exploitation of extractive-rich knotwood (if separated from wood prior to pulping) has been suggested as a pathway for recovering lignans from softwoods and flavonoids from hardwoods (Amidon et al., 2011). Also, production of triterpenic acids from eucalypts, valued for their biological effects and pharmacological activities (Domingues et al., 2010, 2011).

Lipophilic extractives are difficult to remove in the pulp washing line and they often form sticky deposits on process equipment, e.g. screens and wires, and may give rise to spots in paper. Many extractives are surface-active compounds and in paper they affect the surface properties, such as the binding between fibers, the water adsorption and friction. Further, the smell of the paper, especially important in food contact applications, is affected by the extractives. Foaming is also another problem in process liquors that is often connected with extractives. In the kraft process the main part of the wood extractives are dissolved in the black liquor and either burnt to give energy or separated, and used as a source for production of speciality chemicals (Jansson and Nilvebrant, 2009).

The eucalypts lipophilic extractives fraction has been the subject of a significant number of investigations (Gutiérrez and del Río, 2001; Freire et al. 2002ab, 2004, 2005, 2006; Manji et al., 2005; Silvestre et al., 2005; Silvério et al., 2007). However, detailed chemical characterization study has not been published about straw and bagasse sugarcane extractives. So, this study aimed to quantify and identify the chemical constituents of the extractives from straw and bagasse sugarcane, using a *Eucalyptus urograndis* sample as reference, as well as to report the difference in extractives composition for the studied species.

2. **Material and methods**

2.1 Samples

Eucalyptus urograndis (Eucalyptus grandis x Eucalyptus urophylla) hybrids samples were obtained from an industrial plantation. Straw and bagasse sugarcane was obtained from UFV. The samples were chopped into small pieces, and air-dried at ambient temperature for three days. It was then ground to pass a 1 mm sieve in a Wiley mill, screened in a vibratory sieving apparatus, and the 40-60 mesh fractions were used for chemical analysis.

2.2 Extraction

The milled samples were extracted successively with ethanol/toluene (1:2 v/v), ethanol and finally water according following the Tappi method T264 cm-97. The organic solvents were removed under reduced pressure in a rotary evaporator and the water was lyophilized, and each extract was weighed. All extractions were carried out in triplicate, and the extraction yields were expressed in percentage in relation to the wood's dry weight.

Ethanol/toluene, ethanol and water extracts were analyzed by infrared spectroscopic. The spectra were recorded on a Varian 660-IR instrument, equipped with GladiATR scanning from 4000 to 500 cm^{-1}.

To isolate the lipophilic fraction, the extracts were dissolved in dichloromethane (3 x 2 mL) and filtered off, as described by del Río et al. (1998). The dichloromethane lipophilic residues were derivatized and analyzed by GC-MS, before and after hydrolysis as described below.

2.3 Alkaline hydrolysis

10 mg of the dichloromethane extract were added to a two-neck round-bottomed flask, followed by 1.8 mL aqueous solution of KOH (3 mol L^{-1}) and 0.2 mL of methanol. The mixture was refluxed under nitrogen atmosphere for 1h. It was then cooled down to room temperature, acidified with aqueous HCl (3 mol L^{-1}) to pH~2 and extracted with dichloromethane (3 x 2 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered off, and the solvent was completely removed under reduced pressure in a rotary evaporator.

2.3 Derivatization

Aliquots of hydrolyzed and non-hydrolyzed dichloromethane extracts (2.0 mg) were dissolved in pyridine (60 μL) in capped vials followed by the addition of 100 μL bis(trimethylsilyl)trifluoroacetamide containing 1% chlorotrimethylsilane. The reaction mixture was heated at 70 ºC for 30 min. It was then cooled down to room temperature before GC-MS analysis (Cruz et al., 2006).

2.4 GC-MS Analysis

GC-MS analyses were performed on a Shimadzu PQ5050A GC-MS equipped with an AOC-5000 autoinjector and a DB-1 J&W capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness), using helium as carrier gas (35 cm/s). The chromatographic conditions were as follows: injector temperature 290 ºC; oven initial temperature 80 ºC held for 5 min; temperature rate 4 ºC/min; final temperature 285 ºC held for 40 min. The transfer-line temperature was 290 ºC, and a split ratio of 1:10 was used. The mass detector was operated at electron impact mode (70 eV) with a scan range of 30 to 600 a.m.u. For semi-quantitative analysis, the GC-MS equipment was calibrated with pure reference compounds, representative of the major extractives components, namely, hexadecanoic acid, hexadecan-1-ol, 16-hydroxyhexadecanoic acid, 2-hydroxyoctanoic acid, tetracosane, β-sitosterol and *trans*-ferulic acid) (Silvério et al., 2007; Maltha et al., 2011). For this quantification hexanedioic acid and tetracosane were used as internal standards, as described by Freire et al. (2002a). The corresponding response factors needed to obtain correct quantifications were calculated as an average of sixteen GC-MS runs.

Compounds were identified as TMS derivatives by comparing their mass spectra with the GC-MS spectral in the library Willey 333.000 and NIST libraries, by mass fragmentography.

3. Results and discussion

The extractives comprise a large class of chemical compounds which can be removed using organic solvents or water (Gutiérrez et al., 2001). Fatty acids, resin acids, waxes, fatty alcohols, steryl esters, sterols, carbohydrates, tannins are some example of extractives. Due to heterogeneity and inherent complexity of the extractives composition is difficult to use only one solvent to completely remove all of the polar and nonpolar extractive components (Burkhardt et al., 2013).

Nowadays, the determination of the total extractives contents is commonly carried out by extraction with organic solvents using a Soxhlet apparatus in accordance with Tappi method T264 cm-97. The Tappi method consists of successive extraction with ethanol/toluene or benzene (1:2 v/v), ethanol and hot water. Due to the toxicity of benzene, it was replaced by toluene. Ethanol-toluene is used to extract waxes, fats, some resins, and possibly some portions of wood gums. Ethanol is efficient in extracting resin acids, fatty acids, steroids, terpenes, resin oxidation products and degradation products of cellulose and lignin. Hot water

can extract tannins, carbohydrates, starches, and coloring matter (Tappi 2000; Sarto and Sansigolo, 2010).

Table 1 shows the extractive contents obtained after each extraction step. The percentage of total extractives found in *E. urograndis* was similar with results found by others authors that reported values in the range of 2% to 6% (Gominho et al., 2001 and Silvério et al., 2007). The amounts of extractives for the straw and bagasse were close to those obtained by Pitarelo (2007) who found values of 11.5% and 6.8%, respectively, and by Carvalho et al. (2013) that got in their studies 12.2% for sugarcane straw. Such as other chemicals components of lignocellulosic materials, the large variation in the extractives content may be observed for straw, 5.3–11.5% and for bagasse, 4.6–9.1% (Canilha et al., 2012). This depend on many factors including plant genetics, growth environmental and processing conditions as well as methods used for the compositional analysis (Amores et al., 2013).

Considering the percentage of extractives obtained in ethanol/toluene extraction step, the crushed cane sugar has a greater potential for the formation of pitch in case of its use in a pulp and paper industry. This is expected since it has a higher content of extractives compared with the other materials investigated.

Table 1 - Values in percentage of extractives removed at each step and total extractives.

Samples	**Ethanol/toluene extrac. (%)**	**Ethanol extrac.(%)**	**Water extrac.(%)**	**Total extrac.(%)**
E. urograndis	0.81	0.11	1.52	2.44
Sugarcane Straw	2.15	0.42	8.76	11.33
Sugarcane Bagasse	2.67	0.38	4.86	7.91

3.1 Analysis by infrared spectroscopy

The extracts obtained were initially analyzed by infrared spectroscopy in order to get some preliminary information about the chemical composition of their many constituents. Figure 1 shows the spectra for the samples obtained in ethanol/toluene, ethanol and water.

The FT-IR spectra of ethanol/toluene extracts showed the presence of -OH stretching, typical of alcohols and phenols which can be evidenced by absorption bands between 3400-3200 cm^{-1}. There were also narrow bands around 2916 cm^{-1} and 2848 cm^{-1}, characteristic of the CH aliphatic hydrocarbons stretching. Absorption bands between 1711 cm^{-1} and 1725 cm^{-1}, characteristic of C=O stretching of carboxylic acids and esters. The signals between 1605

cm^{-1} and 1511 cm^{-1} typical for C=C stretching of alkenes and aromatics. Ether compounds were evidenced by bands between 1221 cm^{-1} and 1024 cm^{-1} due to stretching C-O. It was also observed bands between 926 cm^{-1} and 830 cm^{-1} characteristics of aromatic angular stretching (-CH) (Barbosa, 2007).

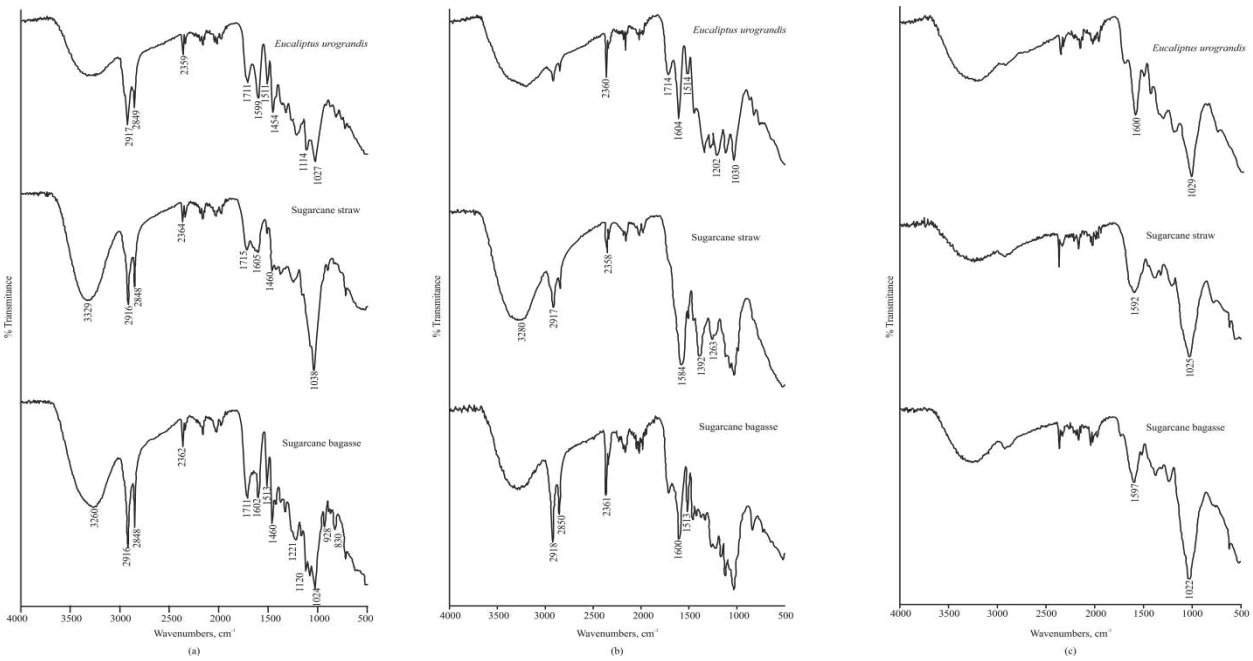

Figure 1. FT-IR spectra of the extractives of *E.urograndis*, straw and bagasse of sugarcane obtained (a) ethanol/toluene extraction, (b) ethanol extraction, and (c) water extraction.

From the analyses of FT-IR spectra of ethanol extracts were observed the presence of alcohols, phenols and aromatic compounds, alkenes, carboxylic acid, ether and ester. The main difference among the samples was in the ethanol extract of *E. urograndis* that showed small bands between 2916 cm $^{-1}$ and 2848 cm $^{-1}$, corresponding to -CH stretching of aliphatic compounds. Regarding the water extracts, three major bands were observed. The signal typical alcohols (-OH) stretching, the bands between 1605 cm^{-1} and 1511 cm^{-1} typical for C=C stretching of alkenes and aromatics; and the peak around 1020 cm $^{-1}$ corresponding to C-O stretching.

The infrared spectroscopy technique is very effective in identifying the functional groups, but does not allow the identification of specific organic compounds present in the extracts of very complexity as those obtained in the present work. Despite this limitation the information obtained from the IR analyses was important since the results clearly indicated

that the samples had to be derivatized prior to the gas chromatography-mass spectrometer analyses.

3.2 Qualitative analysis of the extract constituents by gas chromatography and mass spectrometry

The GC-MS chromatograms for some samples are depicted in Figure 2. All peaks identified were subjected to a semi-quantitative analysis as described in the experimental and the results are presented in Table 2.

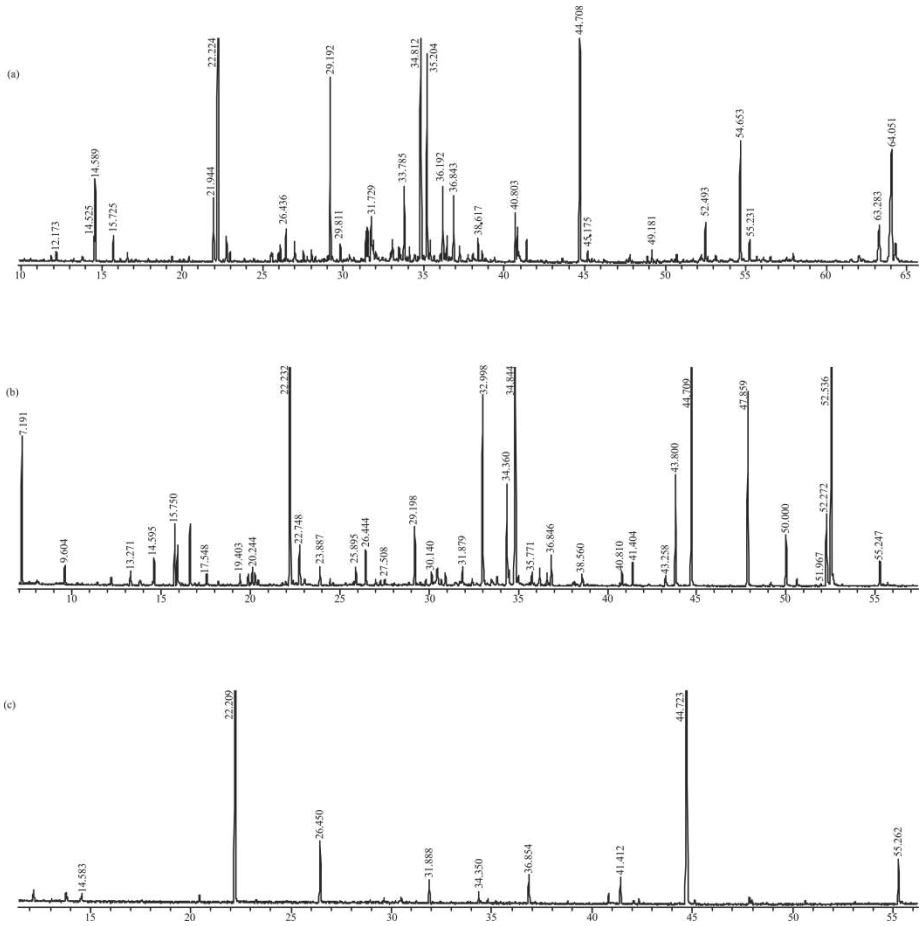

Figure 2. CG-MS chromatograms of (a) ethanol/toluene extract of *E. urograndis* (b) ethanol extract of straw, and (c) water extract of bagasse.

3.2.1 Ethanol/toluene

For the ethanol/toluene extraction the major chemical compounds extracted were carbohydrates. Since the fragmentation patterns of several carbohydrates in the mass spectra are similar, it was not possible to fully identify each one of the. This identification would require the injection of various standard carbohydrates, and we found that this would not be worth doing considering the porpoise of this work. The fatty acids were also extracted in large

quantities, mainly hexadecanoic acid (palmitic acid) from bagasse sugarcane (340.86 mg/Kg). Among the aromatic compounds, *p*-hydroxycinnamic acid (*p*-coumaric acid) was the major one (132.22 mg/kg).

The chemical compounds classes extracted by ethanol/toluene had molecular structures consisting of long carbon chains, responsible for the low polarity of this fraction. As expected, toluene extracted predominantly low polar compounds. This justifies the removal of alcohols, fatty acids and aromatic compounds. Another factor that contributed to the extraction of these compounds at this stage is the presence of ethanol, which gives the mixture a polar character. Thus, hydroxylated compounds were also removed through the intermolecular interactions with ethanol. This occurred with acids, aromatic compounds, and especially with the carbohydrates present mostly in samples of straw and bagasse of sugarcane.

Significant amount of steroid was extracted from *E. urograndis*, in agreement with previous reports by Charlet et al. (1997), Freire et al. (2002a) and Silvério et al., (2007) that found β-sitosterol as the main component of *E. urograndis* extractives. Carbohydrates represented 32.79% of *E. urograndis* extracts in ethanol/toluene, 15.05% of fatty acids, 3.15% of long chain aliphatic alcohols, 18.39% of aromatics, 27.33% of steroids and 3.3% of unidentified constituents. The percentage of fatty acids (15.05%) and aromatics (18.39%) found for the *E. urograndis* extract were in the range of values previously reported by Silvestre et al. (2005) and Gutiérrez et al. (1998) for *E. globulus*. The straw extract constituted of 43.8% of carbohydrates, 22.72% by of long chain aliphatic alcohols alcohols, 16.28% of fatty acids, 6.77% of aromatic compounds and 6.4% for steroids. Bagasse extract was constituted by 74.72% of carbohydrates, 6.83% of acids, 15.41% by alcohols and 3.04% of aromatics.

3.2.2 Ethanol

The compounds obtained from the ethanol extraction showed molecular structures constituted of shorter carbon chains than those extracted by ethanol/toluene, thus the polar character of the compounds removed at this stage was more pronounced.

It was noted that fatty acid compounds are removed in larger quantity, and (*Z*)-prop-1-ene-1,2,3-tricarboxylic acid (aconitic acid) derived from sugarcane bagasse was most significant representative (110.56 mg/Kg). The carbohydrates were extracted in large quantities, mainly for straw and bagasse biomass. Among the identified compounds, fatty

acids were removed in the largest amount in straw and bagasse, however for the *E. urograndis* were extracted mainly aromatics. In addition, the polar characteristics of ethanol also contributed for removal of hydroxyl and carbonyl compounds.

Table 2 - Major components (expressed in mg/kg of dry biomass) of the extracts of *Eucalyptus urograndis*, straw and bagasse sugarcane in ethanol/toluene, ethanol and water.

Rent. Time (min)[1]	**Identification**	**Concentration (mg/Kg)**								
		Ethanol/Toluene Extract			**Ethanol Extract**			**Water Extract**		
		Euc	**Straw**	**Bag**	**Euc**	**Straw**	**Bag**	**Euc**	**Straw**	**Bag**
7.173	Hydroxyacetic acid (glycolic acid)	5.37	-	180.34	0.50	36.14	21.64	-	-	-
7.547	2-oxy-3-hydroxypropanoic acid	-	-	-	-	-	6.28	-	-	-
9.217	Ethanedioic acid (oxalic acid)	-	-	-	0.93	-	-	-	-	-
9.592	3-hydroxypropanoic acid	-	-	108.51	-	-	-	-	-	-
10.378	2-hydroxy-3-methoxybutanoic acid	-	-	34.77	-	5.02	-	-	-	-
11.824	Propanedioic acid	-	-	-	-	-	7.19	-	-	-
12.173	NI	2.34	-	-	0.40	2.56	8.29	7.48	-	-
13.237	Diethyleneglycol				1.03	4.43	24.03	-	-	-
14.525	Phosphoric acid	11.09	-	-	2.87	-	-	-	-	-
14.589	Glycerol	37.95	70.24	2513.56	1.74	34.83	17.29	79.23	-	110.60
14.742	hept-2-enoic acid				-	-	8.90	-	-	-
15.725	Butanedioic acid (Succinic)	9.67	-	270.72	-	20.01	18.06	-	-	-
16.589	2,3-dihydroxypropanoic acid	-	-	34.72	-	20.40	20.51	-	-	-
17.075	2-methylenebutanedioic acid				-	-	15.68	-	-	-
17.541	4-hydroxybenzaldehyde	-	-	48.28	-	3.89	10.98	-	-	-
19.383	2,4-dihydroxybutanoic acid	-	-	-	-	3.75	-	-	-	-
19.817	Maleicdimethyl ester	-	-	-	-	2.97	-	-	-	-
20.075	3,4-dihydroxybutanoic acid	-	-	-	-	4.44	-	-	-	-
21.267	3-hydroxy-3-methoxipentanedioic acid	-	-	-	-	-	11.16	-	-	-
21.944	Hydroxybutanedioic acid (malic acid)	32.91	-	-	-	-	19.36	-	-	-
22.714	1-hydroxy-5-oxo-L-proline	-	-	-	-	16.80	14.46	-	-	-
22.748	Alditol[2]	11.11	-	150.55	-	-	-	-	-	-
22.878	L-aspartic acid	-	-	-	-	-	12.62	-	-	-
22.969	4-hydroxy-3-methoxybenzaldehyde (vanillin)	3.80	-	-	-	-	-	-	-	-
23.861	2,3,4-trihydroxybutyric acid	-	-	-	-	6.22	15.74	-	-	-
24.435	2,3,4-trihydroxybutyric acid isomer	-	-	-	-	2.37	17.99	-	-	-
24.565	2-hydroxypentanedioic acid	-	-	-	-	-	13.35	-	-	-
25.017	L-Asparagine	-	-	-	-	-	13.08	-	-	-
25.517	2-hydroxybenzoic acid	3.95	-	-	-	-	-	-	-	-

25.879	Glutamic acid	-	-	144.46	-	7.61	30.15	-	-	-
25.972	Carbohydrate[2]	2.48	-	53.80	-	-	-	-	-	-
26.085	Carbohydrate[2]	5.36	-	95.97	-	-	-	-	-	-
26.309	Ácid	-	-	-	-	-	18.50	-	-	-
26.436	Dodecanoic acid (lauric acid)	17.66	44.54	76.40	2.22	11.54	10.20	55.65	111.70	47.52
26.804	16-hydroxyhexadecanoic acid	-	58.98	21.45	-	-	-	-	161.51	-
26.985	Carbohydrate[2]	6.96	-	113.14	-	-	-	-	-	-
27.058	Carbohydrate[2]	-	-	12.66	0.34	2.13	3.78	-	-	-
28.033	4-hydroxy-3,5-dimethoxybenzaldehyde	5.35	-	-	-	-	-	-	-	-
28.269	n-hydroxyundecanoic acid	-	-	-	-	-	44.58	-	-	-
28.638	n-hydroxyundecanoic acid isomer	-	-	-	-	-	36.81	-	-	-
28.714	Carbohydrate[2]	-	-	314.70	-	-	-	-	-	-
28.845	Alditol[2]	-	-	43.88	-	-	-	-	-	-
29.108	Tricarbalic acid	-	-	-	-	-	16.06	-	-	-
29.192	Alditol[2]	63.67	123.31	244.26	-	6.74	-	-	-	-
29.308	Carbohydrate[2]	-	-	32.53	-	-	-	-	-	-
29.626	*p*-hydroxycinnamic acid	-	-	132.22	-	-	-	-	-	-
29.641	*(Z)-prop-1-ene-1,2,3-tricarboxylic acid*	-	-	-	-	-	51.31	-	-	-
29.811	4-hydroxy-3-methoxybenzoic acid	8.72	-	-	-	-	-	-	-	-
29.821	(*E*)-prop-1-ene-1,2,3-tricarboxylic acid	-	-	-	-	-	15.76	-	-	-
30.255	Carbohydrate[2]	-	-	365.70	-	1.59	-	-	-	-
30.258	Carbohydrate[2]	-	-	-	-	-	18.41	-	-	-
30.667	2,3,4-trihydroxybutyric acid	-	-	-	-	2.21	-	-	-	-
30.671	Carbohydrate[2]	-	-	-	-	-	16.78	-	-	-
30.891	Carbohydrate[2]	-	-	-	-	2.69	22.04	-	-	-
31.233	2-(3,4-dihydroxyphenyl)ethanol	-	-	-	-	-	-	-	74.23	-
31.404	3,4-dihydroxybenzoic acid	16.69	-	-	-	-	-	-	-	-
31.505	Carbohydrate[2]	14.04	-	367.72	-	-	-	-	-	-
31.729	Carbohydrate[2]	27.47	-	444.03	-	-	-	-	-	-
31.741	2-hydroxy-1,2,3-propanetricarboxylic acid	-	-	-	1.26	-	16.88	-	-	-
31.818	Carbohydrate[2]	-	-	271.84	-	-	-	-	-	-
31.875	Tetradecanoic acid	12.74	-	-	1.14	5.03	18.66	-	85.49	-
32.974	Carbohydrate[2]	-	-	120.41	-	-	-	-	-	-
32.985	2-hydroxydodecanoic acid	-	-	-	-	60.89	22.04	-	-	-
33.270	Carbohydrate[2]	-	-	-	-	-	18.21	-	-	-
33.473	4-hydroxy-3,5-dimethoxybenzoic acid	13.61	-	-	-	-	18.26	-	-	-
33.785	Carbohydrate[2]	35.72	-	822.95	0.55	-	28.87	-	-	-
34.102	Carbohydrate[2]	7.85	-	49.27	-	-	56.63	-	-	-
34.343	*p*-hydroxycinnamic acid (coumaric	-	131.80	436.78	-	10.70	18.04	-	-	33.52

	acid)									
34.384	Carbohydrate[2]	-	-	-	-	44.98	21.00	-	-	-
34.658	Ethyl 3,4,5-trihydroxybenzoic ester	-	-	-	0.61	72.98	19.86	-	-	-
34.812	Alditol[2]	133.6	930.38	10745.5	-	-	23.50	-	-	-
35.167	1,2-benzenedicarboxylic acid	-	-	-	-	-	-	-	56.21	-
35.204	3,4,5-trihydroxybenzoic acid	151.0	39.58	98.77	19.78	1.70	40.78	-	-	-
35.402	Carbohydrate[2]	8.59	-	-	-	-	22.61	-	-	-
35.753	Carbohydrate[2]				-	6.57	-	-	-	-
36.192	Carbohydrate[2]	27.04	-	783.68	0.63	5.61	12.65	-	-	-
36.314	Palmitoleic acid				-	-	18.16	-	-	-
36.434	Carbohydrate[2]	9.92	-	-	-	-	18.23	-	-	-
36.600	Carbohydrate[2]	-	-	-	0.24	2.88	18.30	-	-	-
36.843	Hexadecanoic acid (palmitic acid)	31.66	139.77	340.86	1.85	13.20	18.42	33.38	59.40	62.86
36.967	Carbohydrate[2]	-	-	-	-	-	18.48	-	-	-
37.117	Carbohydrate[2]	-	-	-	-	-	18.56	-	-	-
37.232	Carbohydrate[2]	8.62	-	-	-	-	18.64	-	-	-
38.050	Carbohydrate[2]	4.67	-	-	-	-	-	-	-	-
38.617	Alditol[2]	4.46	-	32.45	-	2.04	19.31	-	-	-
39.029	Alditol[2]	-	-	-	-	-	19.51	-	-	-
40.692	*cis*-octadecen-9,12-dienoic acid (linoleic acid)	19.57	-	31.30	-	5.47	20.34	-	-	-
40.803	*cis*-octadec-9-enoic acid (oleic acid)	14.93	-	52.98	-	-	20.40	8.80	-	-
41.386	Octadecanoic acid (stearic acid)	20.96	66.75	59.06	1.41	11.10	20.69	19.98	51.42	49.25
42.633	2-hydroxydodecanoic acid	-	-	52.83	-	-	-	-	-	-
43.239	Carbohydrate[2]	-	-	-	-	3.05	-	-	-	-
43.787	Carbohydrate[2]	-	-	-	-	23.96		-	-	-
45.175	Carbohydrate[2]	3.33	52.84	49.92	-	-	22.59	-	-	-
47.800	1,2-benzenedicarboxylic acid	-	-	-	3.31	79.16	23.90	-	-	-
48.446	Carbohydrate[2]	-	-	-	-	-	24.22	-	-	-
49.181	4-hydroxy-3-methyl-phenylethyleneglycol	4.55	-	-	-	-	-	-	-	-
50.579	Carbohydrate[2]	-	-	-	-	7.61	25.29	-	-	-
51.938	*trans*-2-hydroxyoctadec-9-enoic acid	-	-	-	-	4.92	-	-	-	-
52.478	Carbohydrate[2]	-	-	-	-	46.41	26.24	-	-	-
52.493	Carbohydrate[2]	17.40	123.48	49.04	0.62	12.09	26.10	-	-	-
52.800	Carbohydrate[2]				-	-	26.40	-	-	-
54.653	Catechin	89.97	-	-	-	-	-	-	-	-
54.825	Sugar	-	-	-	1.51	-	27.41	-	-	-
54.972	Hexacosanol	-	-	29.72	-	-	-	-	-	-
55.231	*p,p*-dioctyldiphenylamine	17.24	58.74	-	55.24	9.64	27.61	-	133.51	88.81
55.575	Carbohydrate[2]	-	-	-	-	-	27.79	-	-	-
55.683	24-hydroxytetracosanoic acid	-	35.12	-	-	-	-	-	-	-
56.646	Tridecanal	-	-	100.59	-	-	-	-	-	-

58.521	Octacosanol	-	238.12	212.11	0.61	-	29.24	-	-	-
60.574	Octacosanoic acid	-	-	-	-	-	-	-	-	-
64.051	β–sitosterol	213.64	211.34	-	-	-	-	-	-	-
64.303	24-Ethylcoprostanol	23.42	180.98	-	-	-	-	-	-	-
70.142	Dotriacontanol	-	171.57	-	-	-	-	-	-	-
	Total of identified compounds	**766.45**	**1447.53**	**4835.98**	**94.50**	**452.39**	**736.31**	**197.04**	**739.47**	**392.55**
	Total of partially identified compounds	**392.29**	**1230.01**	**15188.1**	**3.29**	**168.35**	**555.87**	**0.00**	**0.00**	**0.00**
	Total of non identified compounds	**39.37**	**112.30**	**0.00**	**1.59**	**11.84**	**67.50**	**7.48**	**0.00**	**0.00**
[1]Retention time for TMS derivatives compounds										
[2] Carbohydrates and aditols or other isomers										

Ethanol extraction also removed significant amounts of alcohols and carbohydrates in the sugarcane waste. However for *E. urograndis* a small amount of steroids was extracted, probably traces that were not removed in the previous step in the sequential extraction.

The percentage of each chemical class for each raw material was calculated and 5.09% of the *E. urograndis* extract removed at this stage consisted of carbohydrates, 5.92% of long chain aliphatic alcohols, 26.05% of fatty acids, 55.11% of aromatics, 3.23% of steroid and 4.60% of unidentified compounds. Straw ethanol extract contained 31.57% of carbohydrates, 6.20% of alcohols, 38.17% of fatty acids, 22.4% of aromatics, and 1.65% of unidentified compounds. The chemical composition of bagasse ethanol extract was 33.85% of carbohydrates, 2.88% of alcohols compounds, 49.9% of fatty acids, 12.64% of aromatic and 0.63% of unidentified compounds.

3.2.3 Water

Water extraction removed few compounds, including mainly acids, aromatic and carbohydrates. The fatty acids were the most removed compounds; some examples are dodecanoic acid (lauric acid), hexadecanoic (palmitic acid) and octadecanoic (stearic acid), mainly from straw. The aromatic compounds were removed as the 3,4-dihidroxybenzeneacetic acid and *p*-dioctyldiphenylamine, both also derived from straw.

It was found that the *E. urograndis* extract contained 30.50% of alcohols, 54.62% of fatty acids, 11.99% of aromatics and 2.88% of unidentified compounds. It was observed that 40.95% of the straw extract composition was fatty acids and 59.05% was related as aromatic compounds. The chemical constitution of bagasse was divided in 24.60% of alcohol, 48.19% of fatty acids and 27.21% of aromatic compounds.

3.3 Alkaline hydrolysis

The present study has considered the analysis of the ethanol/toluene extracts before and after alkaline hydrolysis to verify the amount of lipophilic compounds present in the samples in free and esterified forms, as has been reported by others (Swan and Akerblom, 1967; Santos et al. 1997; Gutiérrez et al. 1999; Freire et al. 2002a, 2004; Silvestre et al. 2005; Silvério et al. 2007).

3.3.1 Analysis by infrared spectroscopy of hydrolyzed samples

The ethanol/toluene extracts obtained from *E. urograndis*, straw and bagasse from sugarcane were submitted to alkaline hydrolysis and the products analyzed by infrared spectroscopy (Figure 3).

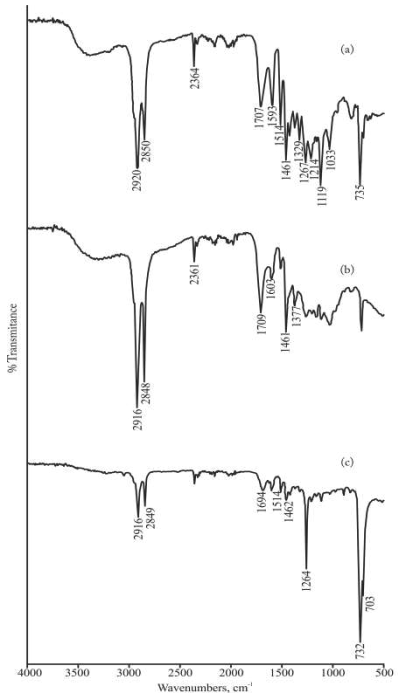

Figure 3. FT-IR spectra of hydrolysed ethanol/toluene extracts of *E. urograndis* (a), straw (b) and bagasse (c) of sugarcane.

Concerning the FT-IR spectra data obtained from the hydrolyzed ethanol/toluene extracts it was observed the presence of -OH stretching, which is typical of alcohols and phenols. The presence of -OH stretching is evidenced by absorption bands between 3400-3200 cm^{-1} for both *E. urograndis* and bagasse samples evaluated. It was also observed a narrow band between 2920-2848 cm^{-1}, assigned to the -CH aliphatic hydrocarbons stretching. Additionally, the absorption bands between 1711 cm^{-1} and 1715 cm^{-1} indicates the presence of C=O stretching of carboxylic acid compounds and ester. The observed signals between 1603

cm^{-1} and 1514 cm^{-1} are typical for C=C stretching of alkenes and aromatics groups. Ether compounds were evidenced by bands between 1267 cm^{-1} and 1033 cm^{-1} due to stretching C-O. From these data the presence of aliphatic compounds is evident, but the identification of some polar groups like OH shows that a derivatization is required for the GC-MS analyses.

3.3.2 Qualitative and semi-quantitative analysis by GC-MS of the constituents from extracts of E. urograndis, straw and bagasse after alkaline hydrolysis

The major classes of identified compounds and their abundances, before and after hydrolysis, are shown in Fig. 4.

After the alkaline hydrolysis, fatty acids were the mainly identified compounds, with its main representatives being hexadecanoic, linoleic, octadecanoic and octadecenoic acids. The aromatic compounds extracted were identified *p*-hydroxycinnamic acid, dioctyl phthalate, ferulic acid, 3-methoxy-4-hydroxybenzoic, all derived from crushed cane sugar.

The *E. urograndis* extract after hydrolysis constituted by 0.79% alcohol, 23.53% of fatty acids, 16.88% of aromatics, 55.04% of steroid, 0.96% of hydrocarbons and 2.79% of unidentified compounds. The extract of straw was constituted by 16.61% of alcohol, 41.68% of fatty acids, 11.76% of aromatics, 22.38% of steroids, 5% of oil and 2.5% of unidentified compounds. For bagasse, after hydrolysis, the extract was composed of 22.56% of alcohol, 38.08% of fatty acids, 31.04% of aromatics, 6.5% of steroid and 1.8% of unidentified compounds.

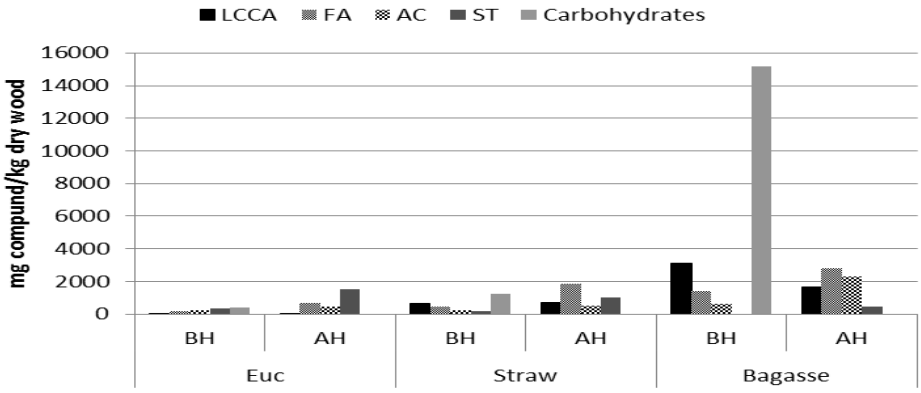

Figure 4. Major classes of compounds identified in the lipophilic extracts, before (BH) and after hydrolysis (AH), of *E. urograndis*, sugarcane straw and bagasse. LCAA: long chain aliphatic alcohols, FA: fatty acids, ST: sterols, AC: aromatic compounds.

Acids and especially the steroid were the major compounds removed, indicating that they were esterified, except for the bagasse. A justification for the presence of fatty acids and

alcohols is the fact that they are the products of the hydrolysis reaction, thus indicating the presence of ester compounds in the samples (Cruz et al.,2004).

A comparison of non-hydrolyzed and hydrolyzed extracts revealed that some common components appeared in different proportions. There was an increase in the quantification of the chemical components after hydrolysis for all classes except carbohydrates, thus revealing the presence of lipophilic compounds esterified. Steroids increased significantly, mainly in samples of *E. urograndis* and straw sugarcane as well as acids derived from the reactions of alkaline hydrolysis. Carbohydrates did not follow this trend because the carbohydrates are polar compounds and thus were poorly soluble in solvent extractor used after hydrolysis (dichloromethane). The increase observed for aromatic compounds may be due to hydroxycinnamic acid might appear esterified with lignin and carbohydrates, fatty alcohols and hydroxyl fatty acids (Freire et al., 2002a). Regarding the total amount of extractives, it was observed that the straw and bagasse residues showed higher amounts compared to *E. urograndis*. However, this does not diminish the potential of this biomass for the production of pulp and paper, because most of the extractives are removed during the pulping stage.

This study can contribute to the research and development of industrial methodologies that can optimize the removal of extractives, thus minimizing the consequences of the presence of these compounds in the pulp and the formation of pitch.

Besides this study also contributed with the biorefinery field, since that were identified many compounds in the extractives fraction which may generate biochemical of high added value, for example: (1) glycolic acids (hidroxy acids), which have been used in clinical for decades in pharmaceutical and cosmetic to treat a variety of skin conditions (Hornhauser et al. 2010); (2) Glycerol or also known as glycerine or propane-1,2,3-triol is a chemical which has a multitude of uses in pharmaceutical, cosmetic, and food industries (Tan et al, 2013); (3) Alditol, have widespread commercial applications, often as a result of its hygroscopic properties (Brimacombe and Webber, 1972).

4. Conclusion

The present work constitutes, to our knowledge, one of the first complete studies of the total extractives composition of sugarcane straw and bagasse, and its comparison with the extractives from *E. urograndis*. Chemical analysis of the extracts by infrared and gas chromatography-mass spectrometry enabled the identification total of 45 compounds for *E. urograndis*, 47 to 67 for straw and bagasse. The major components for the raw materials

studied were fatty acids and carbohydrates for *E. urograndis* and straw and bagasse, respectively. The major chemical classes extracted by ethanol/toluene were carbohydrates for all raw materials. For sugarcane straw and *E. urograndis* were also removed fatty acids and aromatic compounds, beyond these for *E. urograndis* it can be highlighted steroids, mainly β-sistosterol. The ethanol solvent provided the higher extraction of aromatic compounds for *E. urograndis* sample, and acids substances for sugarcane straw and bagasse. It was also found removal of significant amounts of carbohydrate compounds in the waste sugarcane. The classes more removed in extraction step with water were acids and alcohol for *E. urograndis* sample and fatty acids and aromatic compounds for waste sugarcane. The data obtained in this study are important for industries to develop new technologies for the disposal of extractive pitches and improving the pulping processes, as well as contributes to the knowledge of the chemistry of *E. urograndis*, sugarcane straw and bagasse.

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Capítulo 2: Characterization of Klason and acid-soluble lignins from sugarcane residues by pyrolysis-gas chromatography/mass spectrometry (PY - GC/MS)

Abstract

Currently, there has been an increasing research interest in the lignocellulosic materials recovered from residual agricultural and agro-industrial activities such as sugarcane bagasse and straw. These materials are being considered as feedstocks to the biorefineries platforms which aim to convert the biomass into value added products, e.g., biofuels, biochemical, bioproducts, etc. Thereby, the understanding of lignocellulosic composition is a requirement for obtaining a successful strategy of use. Cellulose, lignin, and hemicelluloses are the most abundant components in these materials, respectively. Among these components, the scientific community believes that the lignin is still an undervalued and underdeveloped feedstock mainly due to its complex nature. Therefore, studies for obtaining more information on the lignin structural composition are desirable. In this way, this study aimed to investigate the lignin structures formed in the acid soluble and Klason lignins of sugarcane residues (bagasse, picth, and straw) by pyrolysis-gas chromatography/mass spectrometry. It was possible to identify 40 primary pyrolysis products for acid-soluble lignin and 30 for Klason lignin for sugarcane residues. Analytical pyrolysis of acid-soluble lignin from SCB, SCS and SCP showed prevalence of peaks related to products derived from carbohydrates. The pyrograms for the Klason lignin samples of SCB, SCP and SCS showed the prevalence of lignin derivatives. Also, the Py-GC/MS technique allowed to identify the presence of derivatives lignin in Klason and acid-soluble lignins from sugarcane residues.

Keywords: acid soluble lignin, Klason lignin, sugarcane residues, Py-GC/MS

1. INTRODUCTION

The wood, mainly of the Pinus and Eucalyptus genus, are the most important feedstocks for pulp and paper production worldwide. These are also the main raw materials for the biorefineries processes. However, the cost of wood has grown largely in the last decades mainly due to the increase in labor cost and land price. Thus, as a consequence of it, alternative raw materials have being studied to analyze their potential in the biorefinery processes (Batalha et al., 2012; Requejo et al., 2012; Gomes et al., 2013; Andrade and Colodette, 2014). In this context, sugarcane bagasse and straw are examples of lignocellulosic alternative sources, since they are available in huge volume of these materials which are

sugar-alcohol industry waste and are rich in lignocellulosic components. The sugarcane straw (or trash) is divided in three principal components: fresh leaves, dry leaves, and tops. The sugarcane bagasse is a residual fraction from the sugarcane stem milling (Canilha et al., 2012). These residues materials, coming from agricultural biomass, constitute a potential source for the production of biochemicals, such as ethanol, sugars and furfural, using enzymes or by acid-catalyzed hydrolysis, for example.

Currently, sugarcane bagasse is burned for obtaining energy in the sugar mills and ethanol distilleries, and also for generating electricity to be sold to the grids. Nevertheless, a significant portion of the produced bagasse is underexplored in terms of alternative application. It has been reported the possibility of satisfying the mills energy demands using approximately 50% of the produced bagasse (Rabelo et al., 2011), which can be achieved after technological improvements in the boilers. Due to the amount of this biomass as an industrial waste, there is a great interest in developing methods for producing chemicals from this important raw material in a sustainable way.

Agricultural and agro-industrial residues are mainly constituted by three structural components (lignin, cellulose and hemicellulose) and each compound has specific properties destined for different uses for chemicals production. Lignins are complex aromatic heteropolymers produced by the oxidative combinatorial coupling of mainly three p-hydroxycinnamyl alcohol monomers differing in their degree of methoxylation, the p-coumaryl, coniferyl, and sinapyl alcohols (Boerjan et al., 2003) and the method used to determine the total lignin is the method Klason.

The Klason method is the most typical lignin determination procedure. The procedure separates lignin as an insoluble material by depolymerization of cellulose and hemicellulose in 72% sulfuric acid (SA) followed by hydrolysis of the dissolved polysaccharides in boiling at 3% SA solution. However, part of the lignin is dissolved in the filtrate as the so-called acid-soluble lignin (ASL). For quantification of ASL, it is necessary to use UV technique (Goldschimid, 1971). So, for quantitative determination of lignin content, the Klason method is considered good (Beramendi-Orosco et al., 2004), but it does not apply for the study of structural composition of lignin.

An alternative analytical tool that aims the structural characterization of lignin is the pyrolysis technique coupled to the gas chromatography/mass spectrometry (Py-GC-MS) (Silvério et al., 2008). This technique is based on thermal degradation of lignin, producing components that can be analyzed by gas chromatography/mass spectrometry. This analysis

requires very small samples, without any preparation, over short periods of time (Ishida et al., 2007). Therefore, the purpose of this study was to characterize the structures formed in the acid soluble and Klason lignins of sugarcane (bagasse, picth, and straw) by pyrolysis-gas chromatography/mass spectrometry.

2. MATERIAL AND METHOD

2.1 Raw material

Sugarcane bagasse (SCB) and sugarcane straw (SCS) were obtained from Universidade Federal de Viçosa experimental plantation. Sugarcane pitch (SCP) was acquired from an industrial plantation. The raw materials were dried at room temperature and grinded in a laboratory mill, then sieved and classified according to TAPPI T257 cm-12 standard procedure. All samples were extracted with acetone and subjected to moisture determination in accordance with TAPPI T204 cm-07.

2.2 Quantitative Chemistry Characterization

The lignin S/G ratio was obtained by nitrobenzene oxidation according to Lin and Dence (1992). Extractives in acetone were analyzed according to TAPPI T204 cm-07.

The Klason method of raw materials was determined after pre-treatment (30 °C, 1 h) of the materials with aqueous 72% H_2SO_4 followed by hydrolysis with 3% H_2SO_4 in an autoclave (100 °C, 3 h). The solid residue after hydrolysis was considered as Klason lignin according to TAPPI T222 cm-11 standard procedure. The filtrate part was considered as acid-soluble lignin (ASL) and analyzed by UV technique according to Goldschimid (1971) and was determined according to Equation 1, where 215 nm is the absorbance values of the lignin filtrate and the 280 nm is used as correction for the potential interference from furfural and hydroxymethylfurfural formation from carbohydrates during the acid hydrolysis.

$$\text{ASL\%} = \frac{4.53(A215 - A280)}{300} \; x \; 100 \quad (1)$$

2.3 Preparation of lignins for Pyrolysis

The Klason lignin and ASL were prepared according to Goldschimid (1971), as shown in Figure 1 for further analysis of pyrolysis.

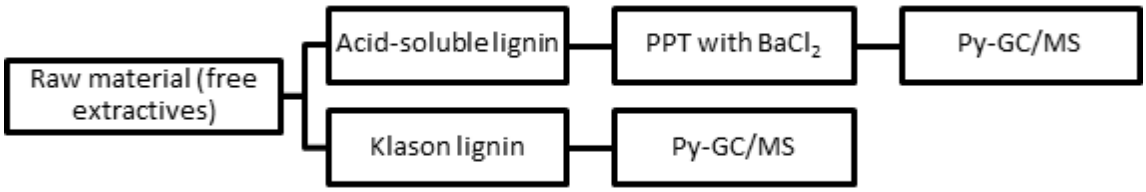

Figure 1. The lignin preparation scheme for pyrolysis

2.4 Pyrolysis-gas chromatography/mass spectrometry (PY - GC/MS)

The pyrolysis of the samples were performed in duplicate with a micro furnace pyrolyser Pyr A-4 model (Shimadzu) coupled to a GC–MS apparatus (Shimadzu, model PQ5050A), using a fused silica capillary column (DB-5, 30 m x 0.25 mm ID, 0.25 μm film thickness). A finely divided sawdust sample (100 μg) was deposited in a small platinum cup that was then inserted into a quartz tube (2 x 40 mm) placed in the pyrolysis chamber. The pyrolysis was carried out at 550 °C for 10s, as previously described (Del Río et al., 2005; Barbosa et al., 2008). The pyrolysis chamber was kept at 250°C and purged with helium to transfer the pyrolysis products as quickly as possible to the GC column. The chromatograph oven was ramped from 40 °C (4 min) to 300°C at a rate of 4 °C min^{-1}. The final temperature was kept for 40 min. The injector and GC–MS interface were maintained at 200°C. Mass spectrometer was operated by the electron impact ionization mode at 70 eV and mass scan range was 40–600 Da. The temperatures of the detector and the GC–MS interface were 250 and 290 °C, respectively. Compounds were identified by comparing their mass spectra with the GC–MS spectral library (Willey 333,000), with data from the literature (Ralph and Hatfield, 1991; Del Río et al., 2005; Barbosa et al.,2008; Oudia et al., 2007) and when necessary by mass fragmentography.

3. RESULTS AND DISCUSSION

3.1 Chemistry composition of raw materials

The chemistry compositions of SCB, SCP, and SCS (percentage on oven-dry weight basis) are shown in Table 1, as well as the standard deviation of data based on two replicate determinations. Because of the current importance of lignin as a raw material for the production of bioproducts and biofuels (Buranov, 2008), samples were also analyzed for their Klason lignin, acid-soluble lignin and lignin S/G ratio.

Table 1. Chemistry content of raw materials

Raw Material	Klason lignina, %	Acid-soluble lignina, %	Total lignina, %	S/G ratio	Acetone extractives, %
SCB	18.9	2.5	21.4	1.0	2.9
SCP	27.0	2.4	29.4	0.9	3.1
SCS	23.1	2.9	26.0	0.5	4.4

The total lignin content of SCB was 21.4%. This value was lower than the ones observed which have been previously reported in the literature (Batalha et al., 2015). For SCP and SCS, the total lignin content was 29.4% and 26.0%, respectively. The lignin S/G ratio for bagasse and straw were similar to the results previously reported by Carvalho et al. (2013). The straw (SCS) showed the higher extractive content (4.4%).

3.2 Identification of fragments of acid-soluble (ASL) lignin pyrolysis

The tree samples from sugarcane (bagasse, pitch, and straw) were pyrolized and the pyrolysis products were separated and characterized by GC-MS. A typical pyrogram of parts of sugarcane is shown in Figure 2.

It was possible to identify a total of 52, 57 and 49 compounds for SCB, SCP and SCS, respectively (Table 2). These compounds were classified broadly as carbohydrates derivatives products, lignin derivatives and others (extractives and proteins derived).

Around 40% of the total compounds identified for the SCB, SCP, and SCP are constituted by products derived from carbohydrate pyrolysis. Acetic acid, 2-methylfuran, furfural, furan-2(3H)-one, 3-furaldehyde, cyclopentanone, 5-methylfuran, 5-hydroxymethylfurfural, levoglucosan were some of the carbohydrates derivatives found. The main products derived from lignin are phenol, 2-cresol, 4-cresol, guaiacol, 2,6-dimethyl phenol, catechol, syringol, syringaldehyde. These substances were identified in previous works involving lignin degradation by analytical pyrolysis (Del Río et al., 2007).

Other compounds also detected ethylene glycol, vinylbenzene, indanone, lauric acid, 2-(methylthio)benzothiazole, ethyl tetradecanoate, hexadecane, isopropyl myristate, phthalic acid, nonadecane, palmitic acid, hexadecane and 2-methylnonadecane. These may be generated by degradation of extractives and proteins.

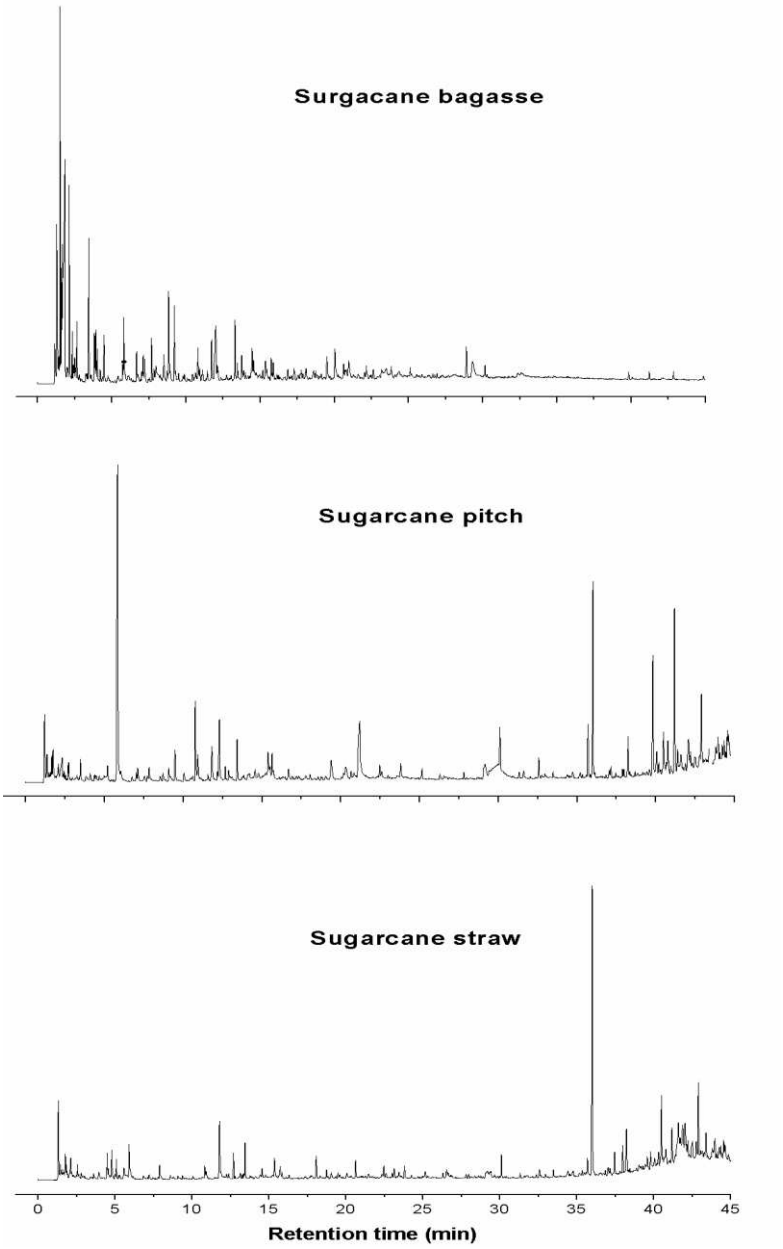

Figure 2. Pyrograms of acid-soluble lignin of Sugarcane bagasse, Sugarcane pitch and Sugarcane straw. Peak identities for compounds are shown in Table 2.

The percentage relative area for ASL at carbohydrate, lignin and others components from SCB, SCP and SCS are showed in Figure 3. It was observed that the content of lignin derivatives in bagasse is greater than in the other raw materials. Although not commonly investigated, the amount of acid soluble lignin seems to be related to the lignin S/G ratio. Amounts of syringyl monomers will condense less during the strong acid hydrolysis treatment, since the C5 position in the aromatic ring is blocked in the syringyl units. These blocked C5 positions prevent C5 condensation. Therefore, lignin containing high S/G ratio,

that is, high number of syringyl units, will condense less during the acid hydrolysis procedure and produce more soluble lignin in the filtrate (Gomes et al., 2015). However, when it was observed the soluble lignin content (Table 1), the bagasse does not have present the highest value compared to other materials. This result demonstrates that the quantification of acid soluble lignin by UV method (Goldmisth) seems to be not consistent. Despite the existing correction for compounds derived from carbohydrates by Goldmisth method, it is evident that there are others components that affect the result of the acid soluble lignin content.

Table 2. Main compounds identified by Py-GC-MS in acid-soluble lignin of parts Sugarcane (SCB, SCP and SCS)

Retention time (min)	Compound	% Relative area			Origin
		SCB	SCP	SCS	
1.187	Chloracetic acid	1.12	2.52	4.91	C, L
1.340	NI	3.87	0.73	0.69	-
1.643	Ethylene glycol	12.07	0.44	1.13	O
1.725	2-Methylfuran	0.42	0.53	0.32	C
2.062	Propylene aldehyde	4.75	0.17	-	-
2.298	Acetic acid	21.41	1.2	1.4	C, L
2.424	3,4-Dihydropyran	1.36	0.16	-	C
2.516	Pentane-2,3-dione	1.47	0.11	-	-
2.691	2,5-Dimethylfuran	0.24	0.33	0.41	C
2.737	Dimethylnitrosamine	-	0.1	0.2	O
3.458	5-Methylfuran	4.86	0.75	0.3	C
4.083	Cyclopentanone	1.99	0.15	-	C
5.078	(2*E*)-3-Methyl-2-heptene	-	-	1.31	C
5.182	3-Furaldehyde	0.46	0.36	0.71	C
5.818	Furfural	2.34	16.39	2.49	C
6.740	Furfuryl alcohol	1.02	0.14	0.11	C
7.009	2-Propylfuran	0.39	0.26	-	C
7.097	Furan-2(3H)-one	0.77	0.35	0.19	C
7.701	1,3-Cyclopentenedione	1.6	0.14	-	C
7.821	Vinylbenzene	0.51	0.39	0.74	O
8.507	2-Methyl-2-cyclopentenone	0.95	0.18	0.21	C
8.702	2-Acetylfuran	0.3	0.25	0.08	C
9.080	2(5H)-Furanone	3.28	0.35	0.18	C
9.469	1,2-Cyclopentanedione	0.32	1.34	0.18	C
10.745	5-Methylfurfural	1.32	2.95	0.86	C
11.805	Phenol	1.82	2.03	4.88	L
12.281	NI	4.21	2.44	0.14	-
12.863	3-Hydroxy-1-methylcyclopenten-3-one	2.87	0.34	-	C
13.417	2-Hydroxy-1-methylcyclopenten-3-one	0.4	1.91	2.7	C
14.554	2-Cresol	0.42	0.25	0.51	L
15.369	4-Cresol	1.1	0.89	1.77	L
15.628	Guaiacol	0.83	0.78	0.4	L
16.669	2-Methyl-3-hydroxypyrone	0.4	0.33	-	C
17.171	3-methyl-2,4(3H,5H)-Furandione	0.88	0.14	-	C
18.051	2,6-Dimethyl phenol	0.68	0.19	1.76	L
18.549	Succinyl anhydride	-	0.12	-	C
18.746	3-Ethylphenol	-	0.07	0.54	L
19.383	NI	1.31	1.17	0.5	-
20.163	Catechol	1.62	0.17	0.34	L
20.824	2,3-Anhydro-d-mannosan	0.48	0.23	1.33	C
21.204	5-Hydrxoymethylfurfural	1.23	5.45	0.08	C
22.590	Indanone	0.61	0.21	0.85	O
23.122	2-Methyl-5-isopropylphenol	0.41	-	0.75	L
23.800	4-Vinylguaiacol	0.36	0.52	0.93	*
25.155	Syringol	0.32	0.33	0.2	L
26.521	Vanillin	0.19	-	0.54	L

26.626	2-Methoxy-1,4-benzenediol	-	-	0.13	L
27.807	2,6-Di-tert-butylphenol	-	0.22	0.23	L
29.152	NI	1.35	1.84	-	-
29.424	Levoglucosan	2.06	0.11	0.2	C
30.098	NI	0.49	1.67	1.88	-
31.600	Lauric acid	0.2	0.28	0.39	O
32.571	2-(Methylthio)benzothiazole	0.57	0.69	0.73	O
33.482	1,3,5-Tri-s-butylbenzene	-	-	0.58	O
34.416	Syringaldehyde	0.12	0.2	0.31	L
35.689	2-ethylhexyl benzoate	-	2.14	1.37	O
36.003	2-(1-Phenylethyl)phenol	-	8.78	26.66	O
37.878	Ethyl tetradecanoate	0.12	0.29	-	O
37.971	Hexadecane	0.25	0.23	2.49	O
38.234	5,5-Dibutylnonane	-	1.86	4.93	O
38.683	Isopropyl myristate	0.15	0.18	0.29	O
39.805	Phytalic acid	0.31	5.76	1.38	O
40.489	Nonadecane	0.59	1.62	5.83	O
40.767	NI	0.5	1.24	-	-
41.181	Palmitic acid	0.33	7.2	3.28	O
42.062	NI	-	2.18	-	-
42.890	Hexadecane	0.29	2.79	6.07	O
44.539	2-Methylnonadecane	0.22	0.78	-	O

C: carbohydrates; L: lignin; O: others; **p*-Hydroxycinnamates derivatives

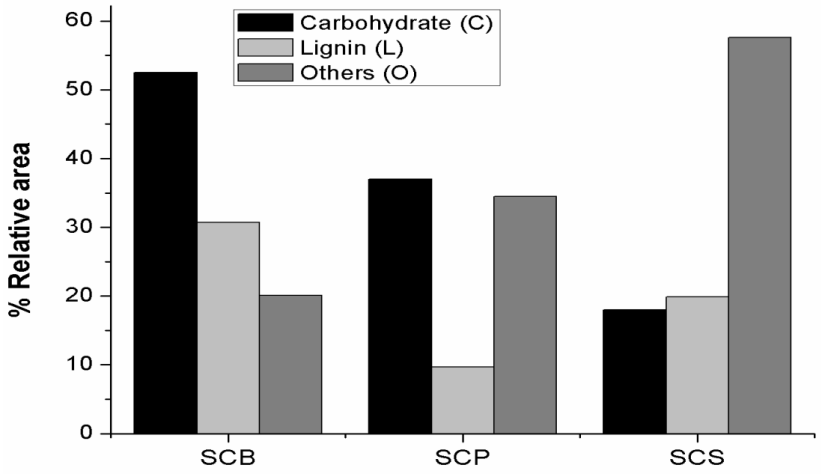

Figure 3. % Relative area total of carbohydrate, lignin and others components from ASL of SCB, SCP and SCS.

3.5 Identification of fragments of Klason lignin pyrolysis

The principle of the pyrolysis technique is the fragmentation of lignin into monomeric compounds (Lima et al., 2008). The pyrograms (Figure 4) for the Klason lignin samples of SCB, SCP and SCS showed the prevalence the lignin derivatives. It was identified 36, 33 and 25 compounds for SCB, SCP and SCS.

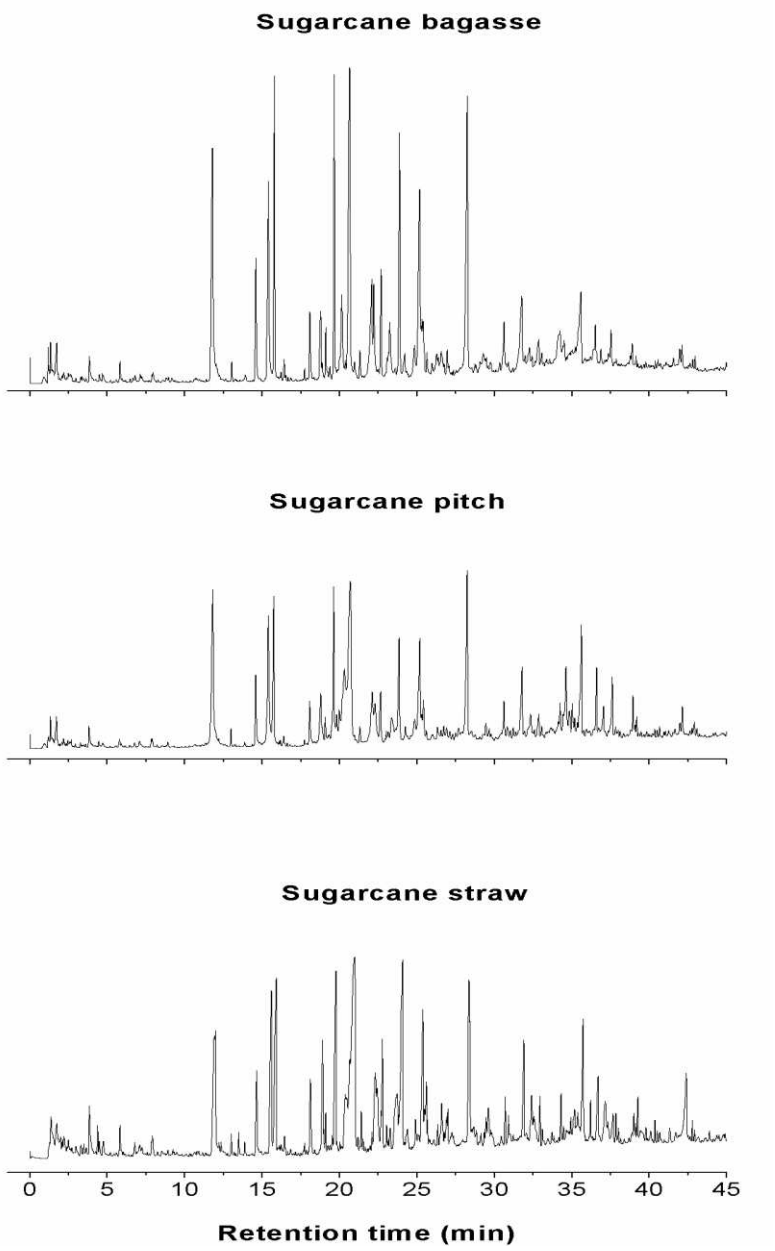

Figure 4. Pyrograms of Klason lignin of Sugarcane bagasse, Sugarcane pitch and Sugarcane straw. Peak identities for compounds are shown in Table 3.

Grass lignins are composed by monomeric phenylpropanoid units known as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) in different ratios, with H-units still comparatively minor. Moreover, grass are by presence of considerable amounts of the *p*-hydroxycinnamates (*p*-coumarates and ferulates) linked to lignin or/and polysaccharide (Del Rio et al., 2015). The pyrogram analysis showed the presence of lignin and *p*-hydroxycinnamate derivatives. The 4-vinylguaiacol and 4-vinylphenol were coming from *p*-hydroxycinnamate. The main pyrolysis products derived from lignin were phenol, 4-cresol, homoguaiacol, syringol, 4-methoxy-3-(methoxymethyl)phenol.

By pyrogram analysis, it is possible to realize that the Klason lignin for sugarcane residues is mainly composed by derivatives from guaiacyl lignin (guaicol, 5-methylguaiacol, homoguaiacol, guaiacylpropane, 2-methoxy-4-(methoxymethyl)phenol, for example). It was expected since the Klason lignin is acid insoluble lignin, therefore the lignin more condensed.

Among the carbohydrate derivatives, the only compounds identified were furfural and acetic acid, indicating that the lignin derived by hydrolysis acid from method Klason is effective for isolation of lignin. The others compounds identified are showing relative areas above 5.0% of total peaks.

Table 3. Main compounds identified by Py-GC-MS in Klason lignin of parts Sugarcane (SCB, SCP and SCS).

Retention time (min)	Compound	% Relative area			Origin
		SCB	SCP	SCS	
1.169	Chloracetic acid	0.25	-	-	C,L
1.322	Pent-3-en-1-ol	0.45	0.65	1.00	L
1.727	Acetic acid	1.13	1.54	0.66	C,L
3.829	Toluene	0.64	0.97	1.15	L
5.804	Furfural	0.16	0.25	0.42	C
11.781	Phenol	9.59	11.40	6.81	L
13.026	4-Methoxytoluene	0.28	0.45	0.28	L
14.584	2-Cresol	3.02	2.70	2.15	L
15.392	4-Cresol	6.70	6.76	6.52	L
15.785	Guaiacol	6.99	5.93	6.94	L
16.414	2,6-Dimethylphenol	0.38	0.34	-	L
17.726	2-Ethylphenol	0.18	0.10	0.22	L
18.070	2,4-Dimethylphenol	1.62	1.60	1.87	L
18.766	4-Ethylphenol	1.92	1.94	2.36	L
19.099	5-Methylguaiacol	0.77	0.61	0.47	L
19.366	NI	0.21	0.25	-	-
19.649	Homoguaiacol	6.63	5.34	6.07	L
20.131	Catechol	1.90	-	1.92	L
20.637	4-vinylphenol	13.03	12.74	14.52	*
21.299	5-Ethyl-2-methylphenol	0.62	0.54	0.61	L
22.067	3-Methoxycatechol	2.23	1.28	1.40	L
22.200	3-Methylcatechol	1.52	0.49	0.54	L
22.674	4-Ethylguaiacol	2.58	1.66	2.58	L
23.232	Homocatechol	0.86	1.13	2.41	L
24.064	4-Vinylguaiacol	5.89	4.17	7.35	*
24.193	3-Methoxy-5-methylphenol	0.47	0.27	-	L
24.823	NI	0.66	0.61	0.40	-
25.163	Syringol	5.87	4.16	3.91	L
25.627	Guaiacylpropane	0.32	0.89	0.72	L

26.940	(*E*)-Isoeugenol	0.38	0.32	0.16	L
28.233	2-Methoxy-4-(methoxymethyl)phenol	10.70	7.49	6.35	L
30.617	1-(4-hydroxy-3,5-dimethoxyphenyl)ethanone	1.28	1.32	0.91	O
31.757	2,5-Dimethoxy-4-methylbenzaldehyde	2.74	3.07	2.69	L
35.575	Methoxyeugenol	3.76	2.54	-	L
36.503	Acetosyringone	0.28	2.30	1.95	L
36.868	Methyl p-coumarate	0.25	-	3.57	L
37.513	NI	0.58	0.73	0.64	-
41.945	Oleic acid	0.24	0.79	0.58	O
42.106	Palmitic acid	0.18	0.68	0.79	O

**p*-Hydroxycinnamates derivatives

4. CONCLUSION

The present work constitutes, to our knowledge, the structural characterization of Klason and acid-soluble lignins of sugarcane straw, picth, and bagasse. Fifty two, fifty seven, forty nine primary pyrolysis products of acid-soluble lignin from SCB, SCP and SCS were identified, respectively. Analytical pyrolysis of acid-soluble lignin from SCB, SCP and SCP showed a prevalence of peaks related to products derived from carbohydrate. Thirty six, thirty three and twenty five primary pyrolysis products of Klason lignin from SCB, SCP and SCS were identified. Only furfural and acetic acid were identified as derivatives compounds of carbohydrate during pyrolysis of Klason lignin. Lignin derivatives peaks showing relative areas above 90% of total peaks, indicated that the lignin derived by hydrolysis acid from method Klason is effective for isolation of lignin. The Py-GC/MS technique allowed the identification of the presence of residual lignin in Klason and acid-soluble lignins from sugarcane residues.

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Capítulo 3: Production of fermentable sugars from sugarcane bagasse by enzymatic hydrolysis after autohydrolysis and mechanical refining

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ABSTRACT

The autohydrolysis process has been considered a simple, low-cost and environmental friendly technology for generation of sugars from biomass. In order to improve accessibility of enzymes during enzymatic hydrolysis as well as to allow the recovery of hemicellulose in the filtrate, the sugarcane bagasse was pretreated using autohydrolysis followed by a mechanical refining process. The autohydrolysis was carried out in three different conditions. Autohydrolysis at 190 ^{0}C for 10 min provided the highest overall sugar (19.2/100 g raw bagasse) in prehydrolyzate. The enzymatic hydrolysis step was performed for all the post-treated solids with and without refining at enzyme loadings of 5 and 10 FPU/g for 96 h. A total of 84.4% of sugar can be recovered from sugarcane bagasse at 180 ^{0}C for 20 min with 5 FPU/g enzyme charge. The economic analysis for the proposed method showed that the bioethanol production can have a financial return larger than 12%.

Keywords: sugarcane bagasse; autohydrolysis; refining; enzymatic hydrolysis; fermentable sugar

1. Introduction

The negative impact of fossil fuels on the environment as well as the increased concern for the security of the oil supply has stimulated the search for renewable fuel alternatives. At present, the most common renewable fuel is ethanol produced from sugar or grain (starch); however, this raw material base by itself may not be sustainable to meet the worldwide needs for ethanol in the future. Consequently, future large-scale use of ethanol will greatly be based on production of ethanol from lignocellulosic materials. In addition, the bioethanol produced from lignocellulosic biomass is an attractive alternative since lignocellulosic raw materials do not compete with food crops and they are less expensive than conventional agricultural feedstocks (Rabelo et al., 2011).

Lignocellulosic biomass, mainly composed of cellulose, hemicellulose, and lignin, is the most abundant renewable resource available for the industrial production of fuel ethanol.

Generally, the production of bioethanol from lignocellulosic biomass via biological process involves the following steps: (1) pretreatment either to remove lignin or hemicellulose to make the cellulose more accessible to enzymatic attack, (2) depolymerization of carbohydrate polymers to produce free sugars by cellulase mediated action, (3) fermentation of hexose and/or pentose sugars to produce ethanol, and (4) distillation of ethanol (Canilha et al., 2012).

The biomass pretreatment is the most important processing challenge in the production of biofuel. Pretreatment is required for removing the lignin and/or hemicelluloses, reducing cellulose crystallinity, and increasing the porosity of the material (Sarkar et al., 2012) to enhance the enzymatic susceptibility of the carbohydrates (Mosier et al., 2005). An effective pretreatment must preserve the utility of the hemicelluloses and avoid the formation of inhibitors (Margeot et al., 2009). An economical pretreatment should use inexpensive chemicals and require simple equipment and procedures (Margeot et al., 2009). In general, pretreatment technologies can be divided into different categories: physical (milling and grinding), physicochemical (steam pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), biological, electrical, or a combination of these. The following pretreatment technologies have promise for cost-effective pretreatment of lignocellulosic biomass for biological conversion to fuels and chemicals (Kumar et al., 2009).

Autohydrolysis is one method often used for pretreatment of lignocellulosic materials. This method has been considered a simple, low-cost, and environmentally friendly pretreatment technology for generation of sugars from lignocellulosic materials (Garrote et al., 1999). The autohydrolysis process uses water and lignocellulosic feedstocks as the only reagents (Ertas et al., 2014) and occurs over a wide range of temperatures (130–230 ^{o}C) and pretreatment times (from a few seconds to several hours) (Saska and Ozer, 1995). The autohydrolysis pretreatment promotes hemicellulose depolymerization (mainly converted into soluble oligomers as a major reaction product) and lignin transformation due to the high temperature (Lee et al., 2009), which enhances the accessibility of enzymes to solid substrates during the subsequent enzymatic hydrolysis to mono-sugars (Mosier et al., 2005). In spite of the simplicity of autohydrolysis, it has not received much attention due to the low sugar recoveries at economical enzyme dosages.

Mechanical refining has been commonly used in the pulp and paper industry. The main effects of refining in fibers are internal and external fibrillation, fines formation, fiber shortening or cutting, and fiber curling or straightening (Gil et al., 2009). Those effects

improve the enzyme accessibility of cellulose through the increase of surface area and reduction of particle size. It has been reported that the refining can significantly improve the enzymatic conversion of pretreated ligonocellulosics at low enzyme dosages (Koo et al., 2011, Han et al., 2014; Ertas et al., 2014). In this study autohydrolysis was combined with refining to improve the overall pretreatment step.

Lignocellulosic materials from different crop residues have been used for conversion to ethanol; among them, sugarcane bagasse is the most abundant lignocellulosic material in tropical countries (Rabelo et al., 2011). Brazil is the biggest producer of sugarcane in the world. It has been estimated that production will be around 652 million tons for sugarcane for 2014/2015. The sugarcane basically consists of stem and straw. The residual fraction from the sugarcane stem after juice extraction is named bagasse (Canilha et al., 2012). In general, 1 ton of sugarcane generates 280 kg of bagasse (Rabelo et al., 2011). Normally, sugarcane bagasse is used as the main source of the energy required in sugar mills and ethanol distilleries and also for generating electricity to be sold to the grids. Nevertheless, a significant portion of the produced bagasse is underexplored. It has been reported that upon the technological improvements made to the boilers it is possible to satisfy the energy requirements of the plants with only half of the produced bagasse. Due to the large capacity of this biomass as an industrial waste, there is a growing interest in developing biorefinery concepts and methods for the production of fuels and chemicals that offer economic, environmental, and strategic advantages (Rabelo et al., 2011).

The present paper deals with pretreatment and biological transformation of sugarcane bagasse into an added value product, emphasizing on fuel ethanol production. A simple process that consists of autohydrolysis followed by refining was proposed. The aim of this study was to assess the efficiency of autohydrolysis and refining pretreatments in the production of fermentable sugars from sugarcane bagasse.

2. Materials and Methods

2.1. Raw material

Sugarcane bagasse chips were provided by a sugarcane manufacturer located in the Brazilian Southeastern region. A fraction of the sugarcane bagasse chips were converted into

sawdust, classified according to TAPPI T257-cm85 standard procedure, dried to 20% moisture, and stored in glass jars for compositional analysis.

2.2. Compositional analysis

The total extractives and ash content of original and pretreated raw materials were measured according to TAPPI T264 cm-97 and TAPPI T211 om-93. The moisture, ash, Klason lignin (acid–insoluble lignin), acid–soluble lignin, and acetyl group contents of original and pretreated raw materials were determined by National Renewable Energy Laboratory's (NREL) Laboratory Analytical Procedures (Ehrman, 1994, 1996; Templeton and Ehrman, 1994). For sugar analysis, 0.3 g samples were hydrolyzed with 3.0 mL of 72% (w/w) H_2SO_4 for 2 h at room temperature. Hydrolysates were diluted to 4% (w/w) H_2SO_4 with 84 mL deionized water (DI) and autoclaved for 1 h at 121 ^{o}C. Mono-sugars were analyzed by a HPLC system (Agilent 1200, Agilent, Santa Clara, CA), including a Shodex SP0810 column (8 x 300 mm, Showa Denko, Tokyo, Japan). All samples were eluted at 80 ^{o}C with Mili-Q water at a flow rate of 0.5 mL/min with peak detection using a refractive index detector, set at 35 ^{0}C. Before analysis, all the samples were filtered through a 0.20 µm nylon syringe filter (Millipore, Billerica, MA). Sugar contents were quantitatively determined by comparison with standard sugars. The 4-O-methylglucuronic acid was measured according to Scott 1979. All experiments were duplicated.

2.3. Autohydrolysis pretreatment

Three different autohydrolysis conditions were studied: 180 ^{0}C for 20 min, 180 ^{o}C for 40 min, and 190 ^{o}C for 10 min. The pretreatments were carried out in a 1.0 L alloy C-276 reactor (Parr Instruments Company, Moline, IL). For each batch cook, 50 g of oven dry sugarcane bagasse samples were placed in the reactor and supplemented with the proper amount of deionized water in order to set water to solid ratio of 10:1. The autohydrolysis process was quantify by severity factor. This factor was calculated by equation below (Overend and Chornet, 1987):

$$\text{severity factor} = \log_{10}[t_1 \times \exp(T_1 - 100)/14.75]$$

where t_1 and T_1 are the pretreatment time (min) and temperature (^oC), respectively. The value of 14.75 is an empirical parameter related to temperature and activation energy. The values of severity factor are shown in Table 2. This express the influence of temperature and time on autohydrolysis process.

After the autohydrolysis stage was completed, the reactor was cooled to room temperature with running tap water, and pretreated samples were filtered through cheese cloth. After the filtration, filtrate was collected in a plastic vial and stored in a refrigerator at 4^oC for pH, sugar, and byproduct analyses. Mono-sugars of the separated filtrate were analyzed by the HPLC system after acid hydrolysis of samples by using 4% (w/w) H_2SO_4 for 1 h at 121 ^oC. The filtrates were filtered through a 0.20 µm nylon syringe filter prior to analysis. All autohydrolysis pretreatments were performed in duplicate. The remaining solid residues were washed, approximately, to neutral pH. After that, the solid residues were centrifuged to achieve relatively uniform moisture content. The moisture contents of solid residues were tested using (NREL) Laboratory Analytical Procedures (Ehrman, 1994). Pretreated solid residues were disintegrated using a refiner with a plate opening of 0.005 inches. Obtained pulps were centrifuged and their moisture contents were tested. Approximately 24 g of oven dried pulps with 10% consistency were subsequently subjected to a PFI mill refining post-treatment at 6000 revolutions. The post-treated pulp was centrifuged and fluffed in order to determine the consistency. The refined and post-treated samples were stored in a refrigerator at 4 ^oC for enzymatic hydrolysis.

2.4. Byproduct analysis

Acetic acid, formic acid, hydroxymethylfurfural (HMF), and furfural were analyzed by a HPLC system (Dionex UltiMate 3000, Sunnyvale, CA) equipped with a Bio-Rad Micro-Guard column, a refractive index detector, and a multi wavelength ultraviolet detector (UVD170U). Compounds were successfully separated by using a 300 x 7.8 mm BioRad Aminex HPX-87H column, eluted at 65 ^oC with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Acetic acid and formic acid contents of samples were analyzed at 210 nm and HMF and furfural at 277 nm. Standard curves were made for each byproduct compound using a 5-point calibration. All the samples were filtered through a 0.20 µm filter before HPLC analysis.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of refined and post-treated substrates was performed with a mixture of Cellic CTec2 and Cellic HTec2 (Novozymes, Franklinton, NC). The activity of CTec2 was tested to be 139 FPU/g (filter paper unit, described as l mol of glucose produced per minute with filter paper as a substrate) (Ghose, 1987). Enzyme dosages of 5 and 10 FPU/g oven dry substrate were applied. Two grams of oven dry substrate were supplemented with enzyme mixture and then added into 50 mM acetate buffer (pH = 4.8) to achieve a 5% (w/w) the consistency of solution. Sodium azide (0.1%, w/w) was used in the media to inhibit microbial contamination (Lee et al., 2010). All samples were incubated at 50 ^{o}C in an air incubator shaker (Series 25, New Brunswick Scientific Co., NJ) at 180 rpm for 96 h.

After the enzymatic hydrolysis, samples were immediately placed into boiled water for 3 min and then centrifuged. Supernatants were filtered through a 0.20 µm filter to be recovered for sugar analysis. The solid residues were filtered through a pre-weighed filter paper (Whatman No.1). The filter papers along with solid residues were dried in an oven at 105 ^{0}C to determine the weight losses of the substrates. All the experiments were performed in duplicate. Sugar concentrations of enzymatic hydrolysates were determined by a HPLC system (Agilent 1200, Agilent, Santa Clara, CA), including a Shodex SP0810 column (8 x 300 mm, Showa Denko, Tokyo, Japan). All samples eluted at 80 ^{o}C with Mili-Q water at a flow rate of 0.5 mL/min with peak detection using a refractive index detector, set at 35 ^{o}C.

2.6 Economic modeling

In order to evaluate the financial feasibility of the proposed conversion process, economic modeling was carried out based on the optimum condition developed in this study in terms of highest total sugar recovery at affordable enzyme price. The mass and energy balance were acquired by using WinGEMS V.5.3, which is widely used in pulp and paper industry for process simulation. The integrated bioethanol production was illustrated in Fig.1, where 500,000 OD metric tons of biomass were feed into the reactor at the autohydrolysis condition of 180 °C for 20 min. The resulting slurry was mechanically refined and sent to a screw press for liquor and solids separation. The filtrate was subjected to hydrolyzate cleanup unit for a control of degradation products concentration which may hurt the downstream fermentation process. In addition, the filtrate was heat exchanged with cold water to extract the excess heat for a suitable enzymatic hydrolysis environment. After enzymatic hydrolysis,

a sugar steam at 18% concentration which was derived from 84.4% total sugar recovery was delivered to fermentation system, where fermentation efficiencies of 95% for hexoses and 80% for pentoses were assumed according to the NREL ethanol report (Humbird et al., 2011). The resulting crude ethanol stream was further purified through distillation and dehydration processes. The solid waste were dewatered to 60% consistency through a high efficiency pressure filter and then sent to the biomass boiler for heat recovery.

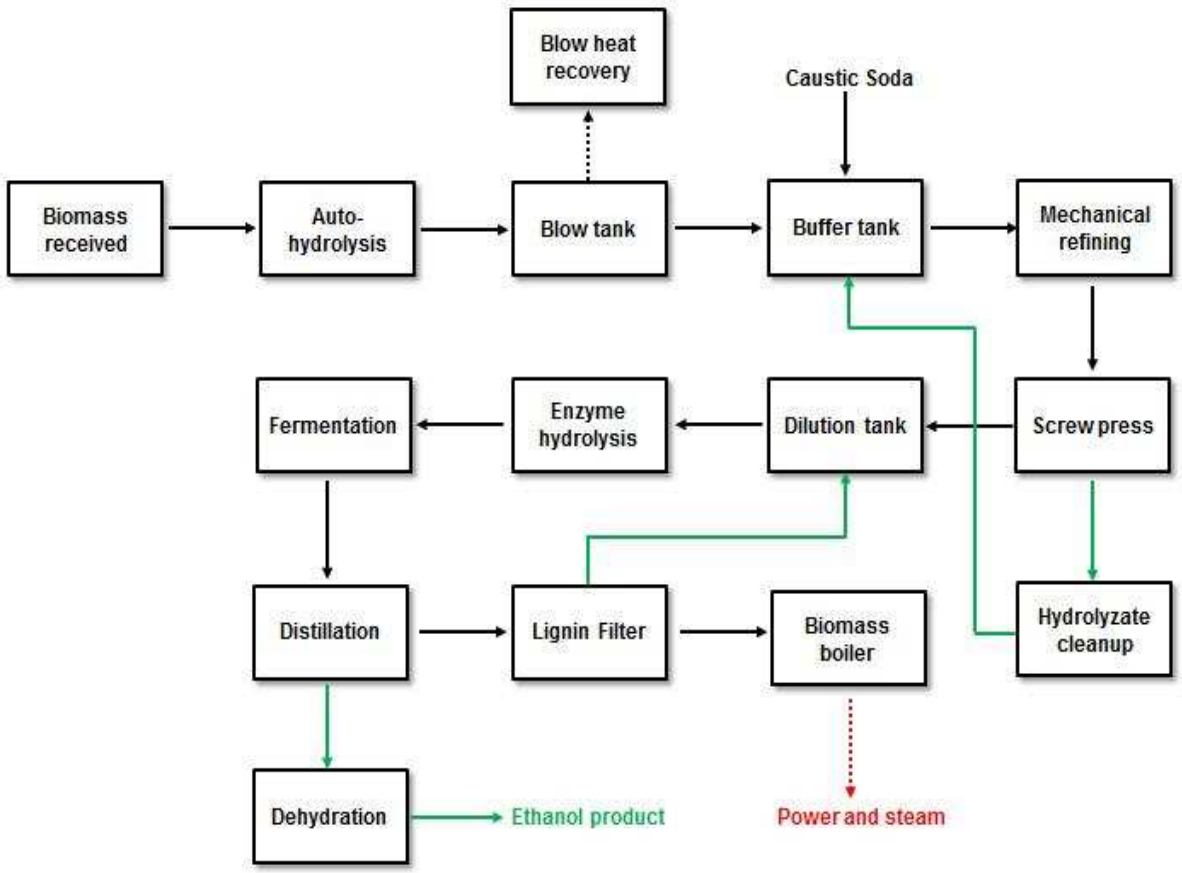


Fig. 1. Schematic of integrated bioethanol production from autohydrolysis of sugarcane bagasse.

The financial assumptions and major simulation results are listed in Table 1. Briefly, the project was sized at 500,000 OD metric tons of biomass per year starting from 2016 with a project life of 15 years. The tax rate was set at 34% and a 10-year straight line depreciation schedule was used. A discounted free cash flow analysis was employed to evaluate the financial return at 12% discount rate. A 5 multiples of year-15 EBITDA was set to account for the remaining asset value after the project life. All the indirect costs including maintenance, capital reinvestment, and other mill fixed costs were set proportional to the Replacement Asset Value. The enzyme price was assumed at $1 per kg enzyme product which equals to

$5.7 per kg enzyme protein according to methodology developed by Phillips (Phillips et al., 2013). The labor cost was derived based on a staffing plan, including typical salary, wage, and benefit costs. All the other cost information was based on the latest Brazilian market price.

Table 1. Operative and financial assumption and key simulation results used in the economic analysis.

Description	Value	Description	Value
Startup year	2016	Hours per year	8400
Terminal year	2031	Excess spending in startup year,% of direct cost	15%
Feedstock supply, dry tons per year	500,000	Ethanol yield, liter per dry ton biomass	370
% of CAPEX spending in year -2	10%	Power cost, $ per MWH	103.6
% of CAPEX spending in year -1	50%	Gasoline denaturant, $ per liter	1.22
% of CAPEX spending in year -0	40%	Natural gas cost, $ per MMBTU	11.0
% of Nominal capacity, project year 1	50%	Enzyme cost, $ per kg enzyme product	1.0
% of Nominal capacity, project year 2	85%	Yeast cost, $ per liter ethanol	0.004
Working capital on materials, % of direct cost	15%	Labor cost, $ per Year	1,197,578
Working capital on product, % of revenue	5%	Maintenance expense, % of RAV[c]	2%
Years depreciation schedule	10-S/L[a]	Capital reinvestment, % of RAV[c]	1%
Tax rate, with tax loss carryforward	34%	Other fixed costs, % of RAV[c]	3%
Discount rate	12%	Sales and other overhead, % of sales	2%
Terminal value, year 15 EBITDA[b] multiple	5.0		

Note: All prices are expressed as US dollars and based on Brazilian market.
[a]10-S/L = 10 years straight line depreciation schedule
[b] EBITDA = Earnings Before Interest, Taxes, Depreciation and Amortization
[c] RAV = Replacement Asset Value

3. Results and discussion

3.1 Chemical composition of sugarcane bagasse

The chemical composition of sugarcane bagasse is shown in Table 2. It was noticed that sugarcane bagasse mostly consists of glucan and xylan, 40.6% and 20.9%, respectively for a total carbohydrate content of 72.1%. These values are important since, for biorefinery concept, raw materials with high glucan and xylan contents are preferable candidates for use in the production of bioethanol (Krishnan et al., 2010). The glucan fraction was similar to that reported in the reported literature (Krishnan et al., 2010, Amores et al., 2013), but the xylan and lignin fractions were lower (Amores et al., 2013).

Table 2. Yield and chemical composition of raw material and autohydrolyzed sugarcane bagasse. Results based on 100 g of oven dry original raw material. The range and average of duplicate measurements is reported.

	Raw Material	180 °C - 20 min	180 °C - 40 min	190 °C - 10 min
Severity factor		3.66	3.95	3.65
Glucan	40.6 ± 0.4	40.4 ± 0.1	36.0 ± 1.0	34.0 ± 0.7
Xylan	20.9 ± 0.6	7.2 ± 0.6	3.8 ± 0.1	4.5 ± 0.1
Galactan	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Arabinan + Mannan	4.0 ± 0.4	2.5 ± 0.1	1.8 ± 0.1	2.0 ± 0.0
4-O methylglucuronic acid	2.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Acetyl groups	3.3 ± 0.2	1.1 ± 0.4	0.4 ± 0.1	1.0 ± 0.1
Total Lignin	19.6 ± 0.0	13.4 ± 0.2	9.7 ± 0.0	10.9 ± 0.3
Klason Lignin	17.4 ± 0.0	12.6 ± 0.2	9.3 ± 0.1	10.3 ± 0.3
ASL[a]	2.2 ± 0.0	0.8 ± 0.1	0.4 ± 0.1	0.6 ± 0.0
Total Extractives	5.9	6.4	6.3	6.0
Ash	4.0 ± 0.1	1.7 ± 0.0	1.9 ± 0.0	1.9 ± 0.0
Others[b]	1.6	0.3	0.4	0.4
Yield[c]	100	73.5 ± 5.5	59.0 ± 4.9	61.4 ± 3.8

a Acid soluble lignin.
b By difference.
c Solid recovery yield, (calculated by gram of solid residue recovered after autohydrolysis treatment/100 g oven dry raw material).

The ash content (4.0%) was higher than other values found in the literature (Krishnan et al., 2010, Amores et al., 2013). However, the bagasse had a lower ash content when compared to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash, respectively (Canilha et al., 2011). Because of its lower ash content, bagasse offers numerous advantages for usage in bioconversion processes using microbial cultures (Pandey et al., 2000). Also, sugarcane bagasse consists of 3.3% acetyl groups, 2.4% 4-O-methylglucuronic acid, and 1.6% other components such as protein and lipids. The composition of sugarcane bagasse may vary depending on the growing season, harvesting procedure, processing method, growing location, as well as analytical procedures (Amores et al., 2013).

3.2 Effect of autohydrolysis conditions on the composition of sugarcane bagasse

The effect of autohydrolysis conditions on the composition of the sugarcane bagasse are also shown in Table 2. During autohydrolysis, the hemicelluloses were depolymerized and converted into soluble oligomers (the major reaction products) and mono-sugars. At the same time, other side-processes that occurred include the partial dissolution of lignin (mainly acid-

soluble lignin) and ashes, solubilization of proteins, and generation of by-products (furfural, 5-hydroxy-2-methylfurfural (HMF), acetic acid, formic acid) from sugars (Lee et al., 2009). This explains the reduction of the yield of solid residue during the autohydrolysis process. The low yield value of about 59.0% occurred at autohydrolysis conditions of 180 ^{o}C and 40 min. In the same conditions, as a result of hydrolysis of hemicelluloses, 81.8% of original xylan and 87.9% of original acetyl groups were solubilized, whereas 4-O-methylglucuronic acid in the original biomass was completely solubilized. This removal indicates the potential of the autohydrolysis as method for activation of bagasse for enzymatic hydrolysis, since hemicelluloses form a physical barrier that surrounds cellulose fibers protecting them from enzymatic attack (Taherzadeh and Karimi, 2008). The yield loss was significantly lower at 180 ^{o}C and 20 min. It will be important to evaluate the conditions that result in increased enzymatic hydrolysis. The overall sugar recovery was dependent on the autohydrolysis yield and the enzymatic hydrolysis efficiency.

Autohydrolysis also reduced the lignin content. In the acidic condition of the autohydrolysis process, the lignin depolymerization occurred predominantly through homolytic cleavage of α-O-4 and β-O-4 bonds, generated lignin fragments which are solubilized in liquor. Also, the lignin was released to filtrate as part of lignin-carbohydrate complexes (Trajano et al., 2013). The delignification process during the autohydrolysis is mainly driven by lignin structure. Under acidic condition, lignin can go both depolymerization and condensation. In non-woody biomass, like sugarcane bagasse, a significant amount of p-coumaric and ferulic acid presented as part of lignin can be easily hydrolyzed into the prehydrolyzate. In addition, the higher amount of LCC in the non-woody biomass may contributes to a higher lignin removal when hemicellulose was hydrolyzed during hydrothermal treatment. A lignin reduction of around 50% at 180 ^{0}C and 40 min was observed. However, the autohydrolysis process showed slight effect on cellulose content. This may be explained by the resistance of cellulose to thermal treatment due to the highly packed crystalline structures.

3.3 Effect of autohydrolysis on pH (acidity)

During the pre-hydrolysis in water, autohydrolysis, acetyl groups were cleaved from the β-(1–4)-linked xylan backbone and the acetic acid released acted as a catalyst for the hydrolysis of glycosidic bonds. The resulting pH in the prehydrolyzate ranged between 3 and

4 (Sixta, 2006). Also, hydronium ions generated from water autoionization and from the ionization of acidic species (uronic acids and formic acid) further catalyzed a series of autohydrolysis reactions (Garrote et al., 1999; Lee et al., 2009, 2010). The pH of the autohydrolysis filtrate for 20 and 40 min at 180^0C decreased from around 6.0 to 3.7 and 3.4, respectively. For 10 min at 190 ^{o}C, the pH decreased from around 6.0 to 3.6. Therefore, the lowest pH measured was 3.4 at the autohydrolysis condition of 40 min at 180 ^{o}C. Examination of the chemical composition of the autohydrolyzed residue in Table 1 showed that the lower pH led to less acetyl groups and 4-O-methylglucuronic acid contents. This indicates that during autohydrolysis treatment higher severity promoted the cleavage of acetyl groups into acetic acid. Similar tendencies have been supported by previous study (Lee et al., 2009; Ertas et al., 2014). Also, higher severity promotes the ionization of uronic acid.

3.4 Effect of autohydrolysis on byproducts

Some of the goals of an effective pretreatment process are (1) to avoid degradation of sugars formed and (2) to limit formation of inhibitory products (Sarkar et al., 2012). Inhibitors can potentially block the formation of ethanol during the fermentation process (Canilha et al., 2012) and also decrease the overall sugar yield (Ertas et al., 2014). The formation of inhibitory products on pretreatment of lignocellulose depends on both the biomass and the pretreatment conditions such as temperature, time, pressure, pH, redox conditions, and addition of catalysts (Klinke et al., 2004). During autohydrolysis, the pentoses and hexoses can be degraded to furfural and 5-hydroxymethylfurfural (5-HMF), respectively (Carvalheiro et al., 2004). The 5-HMF can be further degraded, forming levulinic acid and formic acid (Clark and Mackie, 1984). Also, the formic acid can be formed from furfural under acidic conditions at elevated temperatures (Dunlop, 1948). Acetic acid can be formed from acetyl groups linked to xylan (Carvalheiro et al., 2004). The effects of autohydrolysis conditions on the byproducts formation including furfural, HMF, acetic acid, and formic acid are shown in Fig. 2. The generation of byproducts increased with residence time at the same temperature (180 ^{o}C). Acetic acid and furfural were the major byproducts generated during the autohydrolysis. The highest concentrations of by-products were obtained from autohydrolysis condition of 40 min at 180 ^{0}C, which was in agreement with previous results reporting increasing concentrations with increasing severity of pretreatment (Amores et al., 2013; Ertas et al., 2014). It should be noted that the increase of the concentrations of byproducts also results

in a lower filtrate pH. In the meantime, the increased solubilization of hemicellulose led to increased acid generation, which may indicate a mutual effect between acid generation and hemicellulose removal (Lee et al., 2009; Ertas et al., 2014).

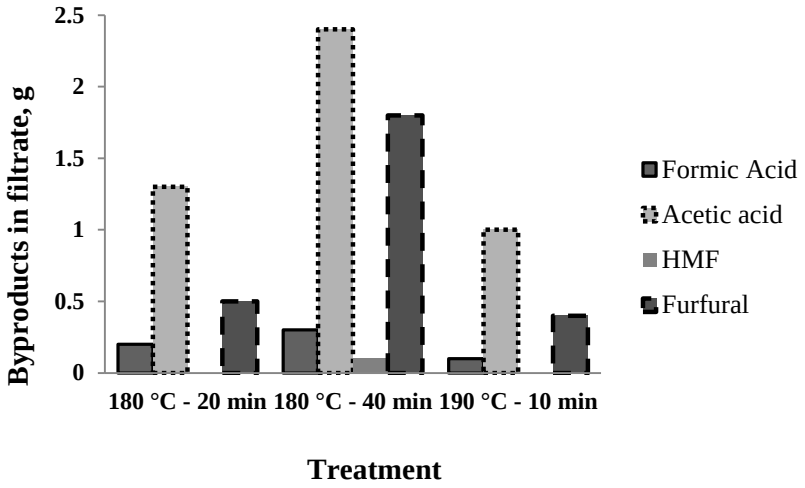


Fig. 2. Effect of autohydrolysis conditions on the production of byproducts in the autohydrolysis filtrate.

3.5 Sugar in the autohydrolysis filtrate

During the autohydrolysis process, hemicellulose and cellulose are removed from grass materials via solubilization of the generated oligo-sugars and mono-sugars (Lee et al, 2009). In this study, a solution of sulfuric acid (4% w/w) was used to hydrolyze all the oligo-sugars present in the autohydrolysis filtrate into mono-sugars. It was assumed that with this treatment all of the oligo-sugars were converted to their mono-sugars. Xylan has the highest concentration in autohydrolysis filtrate (Fig. 3), representing more than 80 % of all the sugars. In addition, xylan showed the highest variation with changes in the autohydrolysis condition. The highest amount of total sugars generated was at 190 ^{o}C for 10 min (19.2g), followed by 180 ^{o}C for 40 min (18.9 g) and 180 ^{o}C for 20 min (17.3g). It was expected that autohydrolysis at 180 ^{o}C for 40 min would generate more sugars, since this condition removed more sugars (Table 1). However, this was not the case and may be explained by the degradation of mono-sugars to byproducts such as furfural, HMF, and others, at the highest severity conditions as shown in Fig.1.

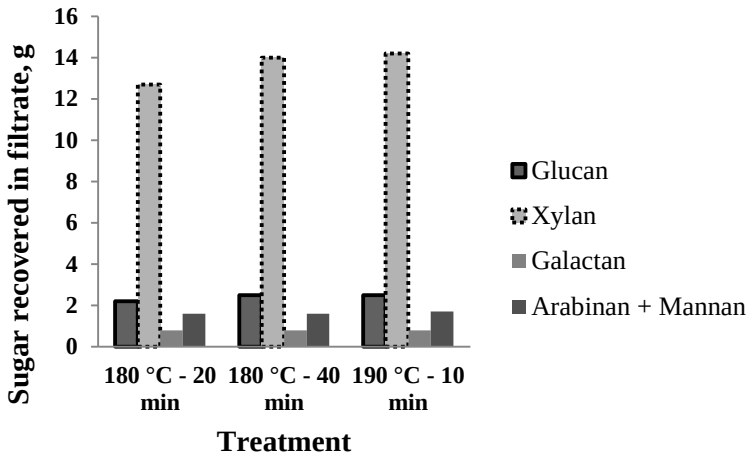


Fig. 3. Sugars in autohydrolyzate filtrate after acid-treated hydrolysis with 4% sulfuric acid treatment. Results based on 100 g of oven dry original raw material.

3.6 Effect of autohydrolysis conditions and refining on enzymatic hydrolysis

The enzymatic hydrolysis step was performed using the solid residues from each pretreatment. Two enzyme loadings were used in this study, 5 and 10 FPU/g oven dry substrate. The 5 FPU/g enzyme dosage was tested because for a process of enzymatic hydrolysis to be economically viable enzyme dosages need to be about 4-5 FPU/g of substrate (Ertas et al., 2014). The 10 FPU/g enzyme dose was used to evaluate the sugar recovery potential of the substrate if enzymes become cheaper in the future (Phillips et al., 2013).

According to Technical Association of the Pulp and Paper Industry (TAPPI) method T248, PFI refining is defined as the mechanical action applied to pulp between two parallel surfaces, under constant loading, moving in different peripheral speeds relative to one another, promoting a mechanical shearing action on the fibers. PFI refining has been reported as a interesting biomass pretreatment since can significantly improve the enzymatic conversion of pretreated lignocellulosics at low enzyme dosages (Koo et al., 2011, Han et al., 2014; Ertas et al., 2014). A common method to simulate refining in the lab is to use a PFI type lab refiner developed at the Norwegian Pulp and Paper Research Institute. The amount of refining action is measure using the number of revolutions in the PFI refiner. Previous study investigated the action of mechanical refining on enzymatic conversion and reduction of enzyme dosage. Four different revolution counts were applied (2,000, 4,000, 6,000, and 8,000). It was observed than at 6,000 PFI revolutions after green liquor pretreatment of hardwood allowed about a 50% decrease in enzyme charge at the same hydrolysis conversion of a non-refined sample, highlighting the ability of refining to reduce the required enzyme

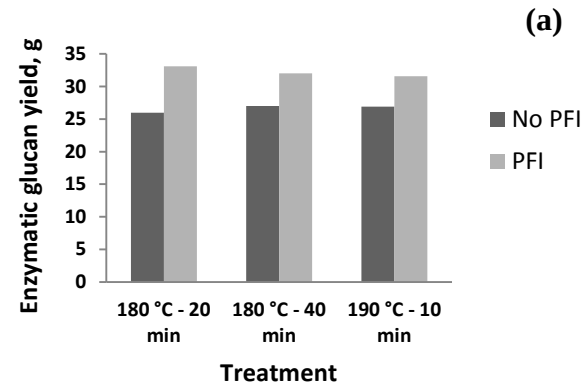
charge Also, the highest enzymatic conversion, 78%, was obtained when 6,000 PFI revolutions were applied (Koo et al., 2011). Based on this results, refining intensity of 6,000 revolution for PFI mill was tested.

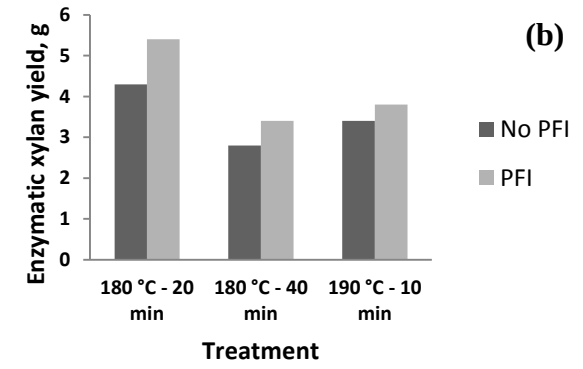
Fig.4 and Fig.5 show the enzymatic hydrolysis of the solid residue for the refined and unrefined samples for each autohydrolysis condition at 5 and 10 FPU/g, respectively. It was noted that for the two enzyme loadings studied, the treatment with PFI refining after the autohydrolysis increased the total sugar recovery, indicating that refining improves enzymatic conversion. Thus, the use of refining treatment before enzymatic hydrolysis is a critical step for the commercialization of autohydrolysis, since it promotes high sugar recovery at low enzyme dosages (Ertas et al., 2014).

For enzyme loading of 5 FPU/g, the enzymatic glucan yield increased with PFI refining post-treatment at each autohydrolysis condition (Fig. 4a). The highest glucan yield after enzymatic hydrolysis for substrate treated with PFI was 33.1 g per 100 g of starting raw material at autohydrolysis condition of 180 ^{o}C for 20 min, where more than 82% of cellulose in the solid residue was recovered. For the same autohydrolysis condition, but the substrate without PFI, the glucan yield was 26.0 g which is equivalent at 64.4% of cellulose in the solid residue. In this case, the PFI refining step increased glucan recovery by around 30%.

Also, the highest xylose yield (5.4g) was achieved after enzymatic hydrolysis for substrate treated with PFI refining from 180 ^{o}C for 20 min, corresponding to 75% of xylan recovered the solid residue. For the same autohydrolysis condition but the substrate without PFI, the xylan yield was 4.3 g. As seen in Fig. 4b, the enzymatic xylan yield decreased with increased time and temperature. Basically, this is due to the degradation of xylan to byproducts at higher severity conditions.

As shown in Fig. 4c, with enzyme loading of 5 FPU/g, 84.4% of carbohydrate in the raw sugar cane bagasse material was successfully recovered by enzymatic hydrolysis of substrate treated with PFI refining from 180 ^{o}C for 20 min, 17.3 g (26.0%) recovered in autohydrolysis filtrate and 38.8 g (58.4%) in the enzyme hydrolyzate.





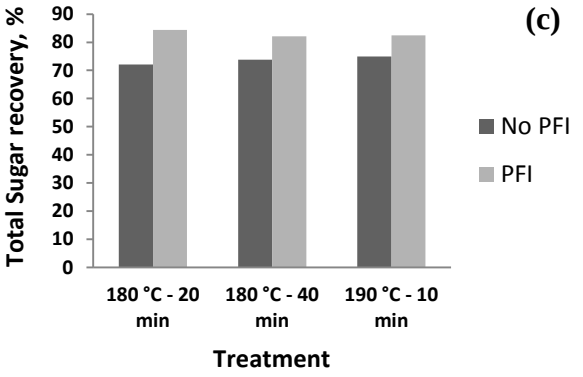
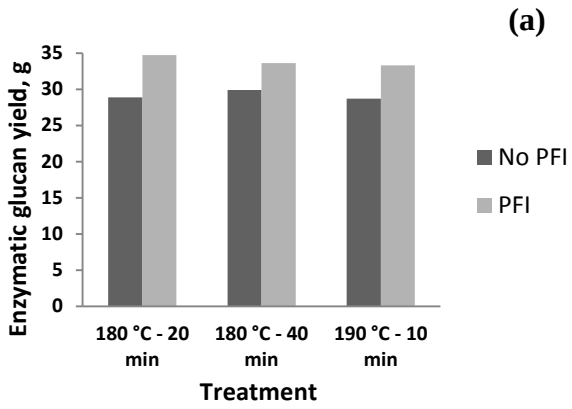
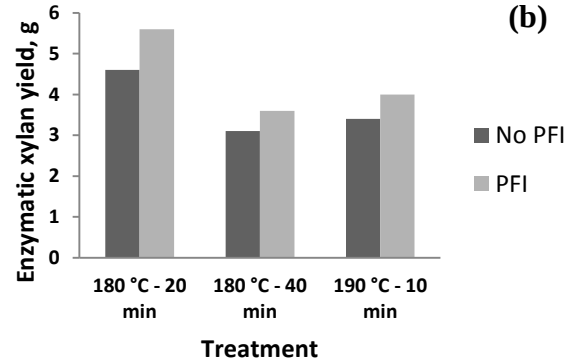


Fig. 4. Enzymatic hydrolysis of unrefined and refined solid residue from each pretreatment condition at 5FPU/g substrate enzyme loading for 96 h: (a) enzymatic glucan yield; (b) enzymatic xylan yield; (c) total sugar recovery.





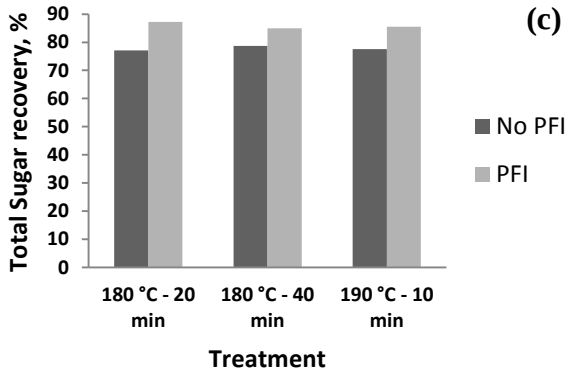


Fig. 5. Enzymatic hydrolysis of unrefined and refined solid residue from each pretreatment condition at 10FPU/g substrate enzyme loading for 96 h: (a) enzymatic glucan yield; (b) enzymatic xylan yield; (c) total sugar recovery.

The enzymatic hydrolysis step was performed using the solid residues from each pretreatment. Two enzyme loadings were used in this study, 5 and 10 FPU/g oven dry substrate. The 5 FPU/g enzyme dosage was tested because for a process of enzymatic

hydrolysis to be economically viable enzyme dosages need to be about 4-5 FPU/g of substrate (Ertas et al., 2014). The 10 FPU/g enzyme dose was used to evaluate the sugar recovery potential of the substrate if enzymes become cheaper in the future (Phillips et al., 2013). Fig.4 and Fig.5 show the enzymatic hydrolysis of the solid residue for the refined and unrefined samples for each autohydrolysis condition at 5 and 10 FPU/g, respectively. It was noted that for the two enzyme loadings studied, the treatment with PFI refining after the autohydrolysis increased the total sugar recovery, indicating that refining improves enzymatic conversion. Thus, the use of refining treatment before enzymatic hydrolysis is a critical step for the commercialization of autohydrolysis, since it promotes high sugar recovery at low enzyme dosages (Ertas et al., 2014).

For enzyme loading of 5 FPU/g, the enzymatic glucan yield increased with PFI refining post-treatment at each autohydrolysis condition (Fig. 4a). The highest glucan yield after enzymatic hydrolysis for substrate treated with PFI was 33.1 g per 100 g of starting raw material at autohydrolysis condition of 180 ^{o}C for 20 min, where more than 82% of cellulose in the solid residue was recovered. For the same autohydrolysis condition, but the substrate without PFI, the glucan yield was 26.0 g which is equivalent at 64.4% of cellulose in the solid residue. In this case, the PFI refining step increased glucan recovery by around 30%.

Also, the highest xylose yield (5.4g) was achieved after enzymatic hydrolysis for substrate treated with PFI refining from 180 ^{o}C for 20 min, corresponding to 75% of xylan recovered the solid residue. For the same autohydrolysis condition but the substrate without PFI, the xylan yield was 4.3 g. As seen in Fig. 4b, the enzymatic xylan yield decreased with increased time and temperature. Basically, this is due to the degradation of xylan to byproducts at higher severity conditions.

As shown in Fig. 4c, with enzyme loading of 5 FPU/g, 84.4% of carbohydrate in the raw sugar cane bagasse material was successfully recovered by enzymatic hydrolysis of substrate treated with PFI refining from 180 ^{o}C for 20 min, 17.3 g (26.0%) recovered in autohydrolysis filtrate and 38.8 g (58.4%) in the enzyme hydrolyzate.

Similar to loading of 5 FPU/g, the glucan yield for enzyme loading of 10 FPU/g increased with PFI refining post-treatment at each autohydrolysis condition (Fig. 5a). Also, the maximum glucan yield was found to be 34.7 g per 100 g of starting biomass (86% of cellulose in the solid residue from this condition) after enzymatic hydrolysis of the substrate with PFI treatment from 180 ^{o}C for 20 min pretreated residue. With increasing enzyme loading from 5 to 10 FPU/g, the glucan yield of the substrate with PFI refining at 180 ^{o}C for

20 min increased from 33.1 to 34.7g. An increase of only 2 g rendered the high enzyme charge unnecessary when the sugar cane bagasse was treated with autohydrolysis and PFI refining.

As seen in Fig. 5b, the highest xylose yield (5.6g) was achieved after enzymatic hydrolysis of substrate treated with PFI refining from 180 ^{o}C for 20 min pretreated residue. Therefore, 78% of xylan total in the solid residue was recovered with treatment. At an enzyme loading of 5 FPU/g, the enzymatic xylan yield decreased with increase of residence time and temperature in the pretreatment stage because of byproducts formation. Increasing the enzyme charge from 5 to 10 FPU/g did not increase the xylan yield significantly. Fig. 5c shows that 87% of carbohydrate in the raw material was successfully recovered by enzymatic hydrolysis at 10 FPU/g of substrate for PFI refined residues from 180 ^{o}C for 20 min.

For the enzyme loading of 5 and 10 FPU/g, the best total sugar recovery and enzymatic hydrolysis performance was achieved by substrate produced from autohydrolysis pretreatment at 180 ^{0}C for 20 min combined with PFI refining post-treatment. In addition, depending on the autohydrolysis conditions and enzyme charge, the total sugar recovery can be increased by 12% when PFI refining is used as a post-autohydrolysis treatment.

3.7 Material balance

The material balances for autohydrolysis pretreatments with and without PFI treatment followed by enzymatic hydrolysis are shown in Table 2 and 3 respectively. The maximum and minimum solid recoveries were 73.5 and 59.0% due to the severity of the treatment. The minimum solid recovery was produced at the highest severity which indicates that higher severity conditions promote higher formation of byproducts including acetic acid, formic acid, furfural, and HMF. Similar tendencies have been supported by previous studies (Lee et al., 2009; Lee et al., 2010; Ertas et al., 2014).

The increase of time at the same temperature for autohydrolysis conditions promoted an increase in the total amount of sugar in the filtrate (Table 2 and 3). However, this amount was not proportional to loss of hemicellulose from raw material (Table 1) because of further sugar degradation at high severity. The sugar degradation led to the low total sugar recovery from autohydrolysis filtrate.

The material balance for autohydrolysis at 180 ^{o}C for 20 min with PFI refining treatment followed by enzymatic hydrolysis with dosage of 10 FPU/g showed the highest total sugar recovery, in which, 57.9g of sugar from prehydrolyzate and enzyme hydrolyzate were

generated from 100g bagasse. The same treatment, but with enzyme loading of 5 FPU/g, allowed for the recovery of 38.8 g of sugars in enzymatic hydrolysis, resulting in an overall sugar recovery of 84.4%. On the other hand, pretreatment without PFI refining at 180 ^{o}C for 20 with a 5 and 10 FPU/g enzyme charge showed a total sugar recovery of 72.1 and 77%. As seen in Table 3 and 4, the PFI refining post-treatment of the pulp from each pretreatment solid residue improved the enzymatic hydrolysis performance, which is very desirable.

Table 2. Material balances from autohydrolysis pretreatments with PFI refining treatment followed by enzymatic hydrolysis.

Temperature (^{0}C)	Time (min)	Solid recovery (%)	Sugars in filtrate (g)			FPU	Enzyme hydrolyzate (g)			Sugar recovery	
			G[a]	H[b]	T[c]		G[a]	H[b]	T[c]	(g)[d]	(%)[e]
180	20	73.5	2.2	15.1	17.3	5	33.1	5.7	38.8	56.1	84.4
						10	34.7	5.9	40.6	57.9	87.2
	40	59.0	2.5	16.4	18.9	5	32	3.6	35.6	54.5	82.1
						10	33.6	3.9	37.5	56.4	84.9
190	10	61.4	2.5	16.7	19.2	5	31.6	4	35.6	54.8	82.5
						10	33.3	4.3	37.6	56.8	85.5

[a] G: released glucan
[b] H: released xylan and other sugars.
[c] T: total sugars.
[d] Sum of sugars in filtrate + enzyme hydrolyzate.
[e] Percentage of sugar recovery, (calculated by sugar recovery (g)/ 66.4 (g)).

Table 3. Material balances from autohydrolysis pretreatments without PFI refining treatment followed by enzymatic hydrolysis.

Temperature (^{0}C)	Time (min)	Solid recovery (%)	Sugars in filtrate (g)			FPU	Enzyme hydrolyzate (g)			Sugar recovery	
			G[a]	H[b]	T[c]		G[a]	H[b]	T[c]	(g)[d]	(%)[e]
180	20	73.5	2.2	15.1	17.3	5	26.0	4.6	30.6	47.9	72.1
						10	28.9	5.0	33.9	51.2	77.1
	40	59.0	2.5	16.4	18.9	5	27.0	3.1	30.1	49.0	73.8
						10	29.9	3.4	33.3	52.2	78.7
190	10	61.4	2.5	16.7	19.2	5	26.9	3.6	30.5	49.7	75.0
						10	28.7	3.7	32.4	51.6	77.6

[a] G: released glucan
[b] H: released xylan and other sugars.
[c] T: total sugars.
[d] Sum of sugars in filtrate + enzyme hydrolyzate.
[e] Percentage of sugar recovery, (calculated by sugar recovery (g)/ 66.4 (g)).

3.8 Economic aspects

The capital investment, the utilization efficiency of enzyme relative to sugar yield, the cost of feedstock, and the ethanol sales price are vital in determining the economic feasibility

of the conversion process. Autohydrolysis is a capital-efficient process compared to many other technologies such as dilute acid pretreatment, lime pretreatment, and ammonium fiber expansion due to the low cost of constructions materials and no requirement on chemical recovery units (Wyman, 2013). The capital investment cost is around $1.4 per liter ethanol product which is lower than $1.8 per liter for the dilute acid process (Humbird et al., 2011). The high total sugar recovery, 84.4% at 5 FPU/g enzyme charge, obtained from autohydrolysis combined with mechanical refining is very competitive compared to many other complex technologies such as dilute acid pretreatment and ammonium fiber expansion (Wyman et al., 2005). Due to the large capacity of the sugarcane related business in the Brazil, a significant portion of the bagasse is underexplored. Such excess volume yields the great opportunity for the bagasse to become a cost-effective feedstock for bioethanol production. The impact of feedstock price on the minimum ethanol revenue for a 12% internal rate of return is displayed in Fig. 6. It is noted that the current fuel ethanol wholesales price in Brazil is between $0.5 and $0.6 per liter. In order to acquire a 12% return of the project, the sugarcane bagasse has to be below $50 per OD metric ton. If the bagasse price was determined by its heating value for electricity production, where 1 ton mill-run bagasse (50% moisture) can be replaced by 0.263 ton of bituminous coal worth $60/ton, it can be estimated that the bagasse is worth around $40 per OD ton. In this regard, the production of bioethanol from autohydrolysis of bagasse can have a financial return larger than 12%.

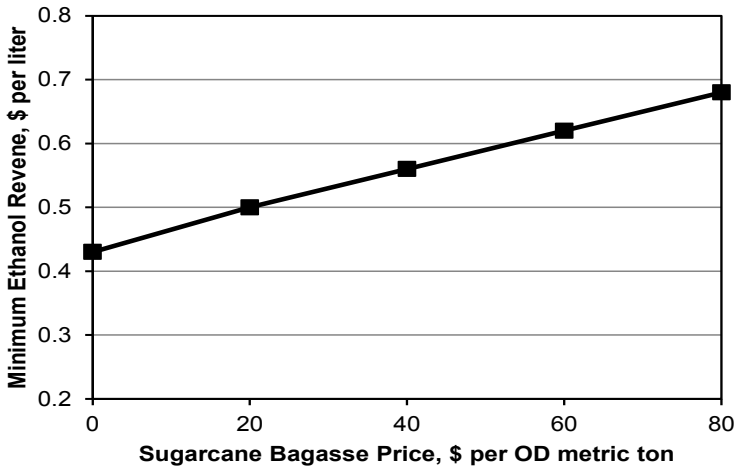


Fig.6 Minimum ethanol prices at different feedstock prices.

4. Conclusions

The use of autohydrolysis pretreatment followed by refining showed to be an effective method for developing a process with high sugar recovery resulting in an efficient process for

ethanol production from sugar cane bagasse. The highest total sugar released into the prehydrolyzate was 28.9%, which was achieved at 190 ^{o}C for 10 min. Nevertheless, a maximum of 84.4% of total sugar recovery was found at 180 ^{o}C for 20 min with refining at 5 FPU/g enzyme dosages. High sugar recoveries can be obtained at low enzyme dosages using this simple method consisting of only autohydrolysis combined with refining, which makes it a very environmentally friendly process since it uses no chemicals. The economic analysis showed than the production of bioethanol from autohydrolysis of bagasse can have a financial return larger than 12%.

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Capítulo 4: Autohydrolysis pretreatment followed by refining as method to enhance sugar recovery from sugarcane straw for bioethanol production.

ABSTRACT

Sugarcane is becoming one of the most important sources of lignocellulosic raw materials for producing second generation ethanol, and the integral utilization of this raw material is still a challenge for the industry. For a second generation bioethanol plant to be successful it will be necessary to utilize the straw in addition to the bagasse. The autohydrolysis pretreatment has been considered a simple, low-cost and environmental friendly technology for generation of sugars from biomass to improve accessibility of enzymes during enzymatic hydrolysis as well as to allow the recovery of hemicellulose in the filtrate. In this study, the sugarcane straw was pretreated using autohydrolysis followed by a mechanical refining. Two different autohydrolysis conditions were studied: 180 ^{o}C for 40 min, and 190 ^{o}C for 10 min. Autohydrolysis at 180 °C for 40 min provided the highest overall sugar (83.1%) with low enzyme application (10 FPU/g substrate) in subsequent hydrolysis process. The ethanol production from autohydrolysis of sugarcane straw seems to be a financial viable approach if a cost-efficient straw collection system is established. The resulting minimum ethanol revenue to achieve 12% internal rate of return can be in a range from $0.49 to $0.56 per liter ethanol product based on straw cost that varies from $18.3 to $40.2. Considering the current anhydrous ethanol wholesale price in Brazil ($0.5 to $0.6 per liter), the bioethanol production from autohydrolysis of sugarcane straw can be a financial viable approach if a cost-efficient straw collection system is established.

Keywords: sugarcane straw; autohydrolysis; refining; enzymatic hydrolysis; fermentable sugar

1. Introduction

A great challenge for the society is to become more sustainable, based on more realistic needs and rational utilization of the natural resources. In this context, energy is one of the most important issues, resulting in the development of biofuels with the potential for replacing fossil fuels. The benefits of biofuels have stimulated the increased worldwide interest for the production of ethanol from lignocellulosic biomass [1, 2]. The bioethanol

produced from lignocellulosic biomass is attractive since lignocellulosic raw materials do not compete with food crops and they are less expensive than conventional agricultural feedstocks [3]. Also, bioethanol reduces air pollution and also contributes to reduced greenhouse gas emissions [4].

Brazil is the biggest producer of sugarcane in the world. It has been estimated for 2014/2015 that 652 million tons for sugarcane will be produced [5]. The sugarcane industry generates considerable amounts of bagasse and straw, which are low-cost lignocellulosic residues [6]. The sugarcane straw (or trash or leaves) is the material left in the field and is divided into three principal components, fresh leaves, dry leaves, and tops [4].

In average, 1 ton of sugarcane generates 140 kg of straw [7, 8], considering the Brazilian sugarcane productivity which reach 85 tons per hectare, 12 tons of sugar cane straw are produced per hectare. Generally, the sugarcane straw is handled three different ways: (1) a small fraction is transported to the mills for heat/power generation, (2) the largest part is left in the fields where it serves as nutrient source, but this causes all sorts of difficulties to the subsequent crop, or (3) it is simply burnt in the fields to enable manual harvesting in places where mechanical harvesting is not possible [9]. Due to the large quantities of this biomass as an industrial waste, there is great interest in developing methods for the production of fuels and chemicals from this important raw material in a sustainable way [10].

Ethanol production from sugarcane residues could be an alternative for partial replacements of fossil fuels because it provides energy that is renewable and less carbon intensive than gasoline [4]. In fact, the sugarcane straw is an interesting raw material for ethanol production due to its suitable chemical composition, comprised of cellulose, hemicelluloses, lignin, and small amounts of extractives and mineral salts [11].

In general, the production of bioethanol from lignocellulosic biomass by biological process involves the following steps: (1) pretreatment for partial removal of lignin and/or hemicelluloses making the cellulose more accessible; (2) depolymerization of carbohydrate polymers to produce free sugars through enzymatic mediated reactions; (3) fermentation of sugars (hexose and/or pentoses) into ethanol; and (4) distillation of ethanol [4].

In bioethanol production, the hydrolysis step is critical for the conversion of straw polysaccharides in fermentable sugars. The biomass pretreatment is the most important processing challenge in the production of biofuel. Many pretreatments methods have been envisioned to facilitate the enzymatic hydrolysis [4, 11]. Pretreatment technologies can be divided into different categories: physical (milling and grinding), physicochemical (steam

pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), biological, electrical, or a combination of these [12]. Pretreatment is required for removing the lignin and hemicelluloses, reducing cellulose crystallinity and increasing the porosity of the material [13], to enhance the enzymatic susceptibility of the carbohydrates [14]. An effective pretreatment should make the hemicelluloses available for conversion to products (bioethanol) and avoid the formation of inhibitors [15, 16]. An economical pretreatment should use inexpensive chemicals and require simple equipment and procedures [16, 17]. The following pretreatment technologies are promising for cost-effective pretreatment of lignocellulosic biomass for biological conversion to fuels and chemicals [12].

The autohydrolysis is an environmentally friendly process in which biomass is pretreated with hot water and used to extract the hemicelluloses into the water phase [18]. A wide range of temperatures (130–230 ^{o}C) and pretreatment times (from a few seconds to several hours) may be used in this process [19]. During the autohydrolysis process the acidic groups bounded to the hemicelluloses are released by high temperatures. These acids, mainly acetic acid and hydronium ions, which come from water autoionization, participate in the hydrolysis of a fraction of the polysaccharides to fermentable sugars [18]. Besides hemicellulose depolymerization, autohydrolysis process promotes lignin dissolution and transformation due to the high temperature, thus increasing the potential of cellulose hydrolysis [20,21], which also enhances the accessibility of enzymes to solid substrates during the subsequent enzymatic hydrolysis to mono-sugars [22]. Despite the simplicity of autohydrolysis, it has not received much attention due to the low sugar recoveries at economical enzyme dosages.

Mechanical refining has been largely used in pulp and paper industry. During the refining process, cellulosic fibres are mechanically treated in water, resulting in morphological and structural changes. Internal and external fibrillation, fines formation, fiber shortening or cutting and fibre curling or straightening are the main effects of refining on cellulosic fibres [23]. These effects improve the enzyme accessibility to cellulose through the increase in surface area available to enzymes, and particle size reduction [24, 25]. It has been reported that refining can significantly improve the enzymatic conversion of pretreated lignocellulosic material at low enzyme doses [26, 27, 28, 29, 30]. The objective of this study was combining autohydrolysis with refining to improve the pretreatment step for increasing the production of fermentable sugars from sugarcane straw.

2. Materials and Methods

2.1. Raw material

The sugarcane bagasse straw was collected at the approximate age of 6 month from high-performance sugarcane hybrid plantations, largely comprised of the UFV/RIDESA RB867515 variety, obtained from the *Saccharum* spp genus. The samples were supplied by a mid-sized ethanol mill located in the neighborhood of the São Pedro dos Ferros city, in the County with similar name, in Minas Gerais State, Brazil (geographical coordinates are 20^{o} 10' 0" South, 42^{o} 31' 0" West, Altitude of 373 meters). A fraction of the sugarcane straw were converted into sawdust, classified according to TAPPI T257-cm85 standard procedure [31], dried to 20% moisture and stored in glass jars, for composition analysis.

2.2. Compositional analysis

The total extractives and ash content of original and pretreated raw materials were measured according to TAPPI T264 cm-97 [31] and TAPPI T211 om-93 [31]. The moisture, ash, Klason lignin (acid–insoluble lignin), acid–soluble lignin and acetyl groups contents of original and pretreated raw materials were determined according to National Renewable Energy Laboratory's (NREL) Laboratory Analytical Procedures [32, 33, 34]. For sugar analysis of 300 mg samples was hydrolyzed with 3.0 mL of 72% (w/w) H_2SO_4 for 2 h at room temperature. Hydrolysates were diluted to 4% (w/w) H_2SO_4 with 84 mL deionized water (DI) and autoclaved for 1 h at 121 °C. Mono-sugars were analyzed by a HPLC system (Agilent 1200, Agilent, Santa Clara, CA), including a Shodex SP0810 column (8 x 300 mm, Showa Denko, Tokyo, Japan). All samples eluted at 80 ^{o}C with Mili-Q water at a flow rate of 0.5 mL/min with peak detection using a refractive index detector, set at 35 ^{o}C. Before analysis, all the samples were filtered through a 0.20 µm nylon syringe filter (Millipore, Billerica, MA). Sugar contents were quantitatively determined by comparison with standard sugars. The 4-O-methylglucuronic acid was measured according to Scott [35]**.** All experiments were duplicated.

2.3. Autohydrolysis pretreatment

Two different autohydrolysis conditions were studied: 180 ^{o}C for 40 min and 190 ^{o}C for 10 min. The pretreatments were carried out in a 1.0 L alloy C-276 reactor (Parr

Instruments Company, Moline, IL). For each batch of cook, 50 g of oven dry sugarcane straw samples were placed in the reactor and supplemented with the proper amount of deionized water in order to set water to solid ratio of 10:1. The autohydrolysis process was quantify by severity factor. This factor was calculated by equation below [36]:

$$\text{severity factor} = \log_{10}[t_1 \times \exp(T_1 - 100)/14.75]$$

where t_1 and T_1 are the pretreatment time (min) and temperature (^{o}C), respectively. The value of 14.75 is an empirical parameter related to temperature and activation energy. The values of severity factor are shown in Table 2. This express the influence of temperature and time on autohydrolysis process. After the autohydrolysis stage was completed, the reactor was cooled to room temperature with running tap water, and pretreated samples were filtered through cheese cloth. After the filtration, filtrate was collected in a plastic vial and stored in a refrigerator at 4 $^{\circ}$C for pH measurement, sugar, and byproduct analyses. Mono-sugars of the separated filtrate were analyzed by the HPLC system after acid hydrolysis of samples by using 4% (w/w) H_2SO_4 for 1 h at 121 °C. The filtrates were filtered through a 0.20 µm nylon syringe filter prior to analysis. All autohydrolysis pretreatments were performed in duplicate.

The remaining solid residues were washed, approximately, at neutral pH. After that, the solid residues were centrifuged to achieve relatively uniform moisture content. The moisture contents of solid residues were tested by (NREL) Laboratory Analytical Procedures [32]. Pretreated solid residues were disintegrated using a refiner having a plate opening of 0.005 inches. The obtained pulps were centrifuged and their moisture contents were tested. Approximately 24 g of oven dried pulps with the consistency of 10% were subsequently subjected to a PFI mill refining post-treatment at 6000 revolutions. The post-treated pulp was centrifuged and fluffed in order to determine the consistency. The refined and post-treated samples were stored in a refrigerator at 4 °C for enzymatic hydrolysis.

2.4. Byproduct analysis

Acetic acid, formic acid, hydroxymethylfurfural (HMF), and furfural were analyzed by a HPLC system (Dionex UltiMate 3000, Sunnyvale, CA) equipped with a Bio-Rad Micro-Guard column, a refractive index detector and a multi wavelength ultraviolet detector (UVD170U). Compounds were successfully separated by using a 300 x 7.8 mm BioRad Aminex HPX-87H column, eluted at 65 ^{o}C with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

Acetic acid and formic acid contents of samples were analyzed at 210 nm and HMF and furfural at 277 nm. Standard curves were made for each byproduct compound using a 5-point calibration. All the samples were filtered through a 0.20 µm filter before HPLC analysis.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of refined and post-treated substrates was performed with a mixture of Cellic CTec2 and Cellic HTec2 (Novozymes, Franklinton, NC). The activity of CTec2 was tested to be 139 FPU/g (filter paper unit, described as l mol of glucose produced per minute with filter paper as a substrate) [37]. Enzyme dosages of 5, 10 FPU/g oven dry substrate were applied. Two grams of oven dry substrate was supplemented with enzyme mixture and then added into 50 mM acetate buffer (pH = 4.8) to achieve a 5% (w/w) the consistency of solution. Sodium azide (0.1%, w/w) was used in the media to inhibit microbial contamination [38]. All samples were incubated at 50 ^{o}C in an air incubator shaker (Series 25, New Brunswick Scientific Co., NJ) at 180 rpm for 96 h.

After the enzymatic hydrolysis, samples were immediately placed into boiled water for 3 min and then centrifuged. Supernatants were filtered through a 0.20 µm filter to be recovered for sugar analysis. The solid residues were filtered through a preweighed filter paper (Whatman No.1). The filter papers along with solid residues were dried in an oven at 105 °C to determine the weight losses of the substrates. All the experiments were performed in duplicate. Sugar concentrations of enzymatic hydrolysates were determined by a HPLC system (Agilent 1200, Agilent, Santa Clara, CA), including a Shodex SP0810 column (8 x 300 mm, Showa Denko, Tokyo, Japan). All samples eluted at 80 °C with Mili-Q water at a flow rate of 0.5 mL/min with peak detection using a refractive index detector, set at 35 ^{o}C.

2.6 Financial Analysis

The process simulation and economic modeling were carried out to evaluate the financial feasibility of the overall process for the production of bioethnaol. The integrated bioethanol production process was described specifically in previous study [39].

The process simulation was completed in the WinGEMS V.5.3 which is a powerful tool to achieve mass and energy balance in pulp and paper industry. The results of the simulation were imported into Excel spreadsheet for economic modeling, which includes

fixed capital investment estimate, operational cost estimate, and discounted free cash flow analysis. The scope of the analysis is 500,000 dry metric tons per year feedstock input. The total fixed capital investment (around $1.4 per liter ethanol product) is relatively lower compared to dilute acid process in the NREL ethanol report which can be attributed to the simplicity of the process and the lower construction cost in Brazil [40,41]. The operational cost estimate comprises directing costs (feedstock, enzymes, chemicals, natural gas, and power) and indirect costs (labor, maintenance, overhead, depreciation, and other mill fixed costs) with annual inflation and improvement indexes. The discounted free cash flow analysis was carried out at a target internal rate of return of 12% to deduce the minimum ethanol revenue (MER). The main assumptions for the economic modeling and the cost of material were summarized in Table 1.

Table 1. Main assumptions and cost information used in the economic analysis.

Description	Value	Description	Value
Project life, years	15	Feedstock supply, dry tons per year	500,000
Tax rate, with tax loss carryforward	34%	Ethanol yield, liter per dry ton biomass	362
Years depreciation schedule	10-S/L[a]	Power cost, $ per MWH	103.6
Discount rate	12%	Gasoline denaturant, $ per liter	1.22
Terminal value, year 15 EBITDA[b] multiple	5.0	Natural gas cost, $ per MMBTU	11.0
Working capital on materials, % of direct cost	15%	Caustic soda, $ per ton	420
Working capital on product, % of revenue	5%	Enzyme cost, $ per kg enzyme product	1.0
Maintenance expense, % of RAV[c]	2%	Corn steep liquor, $ per ton	51.5
Capital reinvestment, % of RAV[c]	1%	Diammonium phosphate, $ per ton	900
Other fixed costs, % of RAV[c]	3%	Sorbitol, $ per ton	1050
Sales and other overhead, % of sales	2%	Labor cost, $ per Year	1,197,578

Note: All prices are expressed as US dollars and based on Brazilian market.

[a]10-S/L = 10 years straight line depreciation schedule

[b] EBITDA = Earnings Before Interest, Taxes, Depreciation and Amortization

[c] RAV = Replacement Asset Value

3. Results and discussion

3.1 Chemical composition of sugarcane straw

The chemical composition results for sugarcane straw are shown in Table 2. These results show that the sugarcane straw cellulose and hemicelluloses account for 65.6% of the weight which consists of glucan 34.0%, xylan 21.4%, 0.7 galactan, arabinan plus manan

5.3%, acetyl groups 2.5% and 4-O-methylglucuronic acid 1.7%. The sugarcane straw is an effective raw material for ethanol production due to its high glucan and xylan contents. Glucan and xylan fraction are in good agreement with values reported in the literature [42, 43]. Total lignin content (21.3%) was lower than reported by Ferreira-Leitão [4] and Canilha et al. [4]. Also, sugarcane straw showed lower lignin content when compared to other commonly investigated lignocellulosic feedstocks such wheat straw, 22.6% [30] and rice straw, 36% [44]. High lignin content is undesirable since it is a major barrier to enzyme access to the carbohydrate fraction which may increase the cost for conversion of biomass to ethanol [45]. The ash content (6.0%) is agreement with Krishnan et al. [42], and lower than many other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash, respectively [46]. Part of the ash content in the straw may have resulted from contact with the ground during harvesting procedures and might be avoided using different harvesting technologies and methods [42]. The chemical composition of sugarcane straw varies based on multiple factors, including crop variety, climate conditions, location and mode of growth, and physical and chemical composition of soil, harvesting procedures and storage system [47], as well as analytical procedures.

Table 2. Yield (%) and chemical composition (%) of raw material and autohydrolyzed sugarcane straw. Results based on 100 g of oven dry original raw material. The range and average of duplicate measurements is reported.

	Raw Material	180 °C - 40 min	190 °C - 10 min
Severity factor		3.95	3.65
Glucan	34.0 ± 0.5	30.3 ± 1.0	33.2 ± 0.7
Xylan	21.4 ± 0.0	3.8 ± 0.1	5.4 ± 0.1
Galactan	0.7 ± 0.1	0.3 ± 0.0	0.6 ± 0.0
Arabinan + Mannan	5.3 ± 0.4	1.1 ± 0.1	2.8 ± 0.0
4-O methylglucuronic acid	1.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Acetyl groups	2.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Klason Lignin	18.9 ± 0.0	12.2± 0.1	12.1 ± 0.3
ASL[a]	2.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0
Total Lignin	21.3 ± 0.1	12.6 ± 0.0	12.5 ± 0.4
Total Extractives	8.6 ± 0.0	8.7 ± 0.0	9.0 ± 0.1
Ash	6.0 ± 0.0	4.3 ± 0.0	4.2 ± 0.1
Others[b]	1.6	1.0	0.3
Yield[c]	100	62.1 ± 0.1	68.0 ± 0.4

a Acid soluble lignin.
b By difference.
c Solid recovery yield, calculated by (gram of solid residue recovered after autohydrolysis treatment/100 g oven dry raw material).

3.2 Effect of autohydrolysis conditions on the composition of sugarcane straw

The chemical composition and yield of autohydrolyzed sugarcane straw are shown in Table 2. As shown in Table 1, the yield of solid residue decreases from 68% to 62.1% with increasing severity factor from 3.65 (190 ^{o}C, 10 min) to 3.95 (180 ^{o}C, 40 min). The reason of decrease of solid yield is that during autohydrolysis process, water under high temperature and pressure penetrates into biomass causing a partial depolymerization of hemicellulose and cellulose, and partial dissolution of lignin [48]. This results in a yield loss of solid residue during the autohydrolysis process. As result of hydrolysis of hemicelluloses, 82.2% of original xylan is solubilized, while 4-O-methylglucuronic acid and acetyl groups contents are completely solubilized. Hemicelluloses can constitute a physical barrier protecting cellulose against enzymatic attack [49]. Hence, their removal during the autohydrolysis process enhances cellulose accessibility to enzymes. The xylan remaining in the autohydrolyzed material is likely to be strongly bound to the lignin, making it less susceptible to hydrolysis [50]. The effect of the autohydrolysis process on cellulose was much less pronounced then on the hemicelluloses. The maximum glucan degradation occurred at 180 ^{o}C and 40 min with 11% of the original glucan being solubilized. The cellulose resistance to degradation is likely due to its crystalline nature. During autohydrolysis the lignin depolymerization occurs predominantly through homolytic cleavage of α-O-4 and β-O-4 bonds under acidic condition, generating soluble lignin fragments which are solubilized in liquor [51,52]. Also, the some part of the lignin released in the autohydrolysis treatment may derive from lignin-carbohydrate complexes [52]. The results on Table 2 shows that about 42% of original lignin was dissolved under autohydrolysis conditions investigated. Similar results have been observed for wheat straw [30] and sugarcane bagasse [53]. The decreased of lignin content during autohydrolysis process enhance hydrolysis of cellulose since the removal of lignin increases the pores availability on the fiber surfaces, thus increasing cellulose accessibility to the enzymes [49]. During the autohydrolysis the system pH is lowered causing dissolution of metals salts, oxides, hydroxides and organically bound metals, thus reducing ash content by 30% [54]. This is beneficial since certain metals (K, Mg, Mn, Fe, and Cu) may decrease the enzyme activity [55]. The increase of total extractives content is probably caused by lignin breakdown during autohydrolysis producing small fragments that are extractable by solvents may to be degraded making it in the extractive analysis [56].

3.3 Effect of autohydrolysis on pH (acidity), on byproducts production and sugar released

Under the conditions employed in the autohydrolysis process, acetyl groups linked to xylans are cleaved generating acetic acid, which acts as a catalyst for the hydrolysis of glycosidic bonds of the biomass. The resulting pH in the prehydrolyzate ranged between 3 and 4 [57]. Also, hydronium ions generated from water autoionization and from the ionization of acidic species (uronic acids and formic acid) further catalyze a series of autohydrolysis reactions [20, 38, 58]. Therefore, the autohydrolysis filtrate pH varies as a function of total weaks acid generated during the treatment [59]. In this study, the autohydrolysis filtrate pH decreased to 3.8 and 3.6 for the 180 °C and 40 min and 190 °C and 10 min reaction conditions, respectively. Previous studies have shown that lower filtrate pH leads to more effective hemicelluloses removal from biomass [20, 30]. However, the results of Table 2 indicate that the condition that caused higher hemicellulose solubilization (180 °C and 40 min) was at a higher pH than that with the lower hemiceluloses solubilization (190 °C and 10 min). This is probably due to the differences in the severity conditions of autohydrolysis treatment.

In addition to deconstruct the biomass structure for easy accessibility of enzymes, an effective pretreatment must minimize the degradation of fermentable sugars and avoid degradation products that affect both enzymatic hydrolysis and fermentation [42]. During autohydrolysis the pentoses and hexoses can be degraded to furfural and 5-hydroxymethylfurfural (5-HMF), respectively [59]. The 5-HMF can be further degraded, forming levulinic acid and formic acid [60]. Also, the formic acid can be formed from furfural under acidic conditions at elevated temperatures [61]. Acetic acid is formed from acetyl groups linked to xylans [59]. The formation of these will depend on both the biomass and the pretreatment conditions such as temperature, time, pressure, pH, redox conditions, and addition of catalysts [62]. The effects of autohydrolysis conditions on the byproducts formation including furfural, HMF, acetic acid and formic acid are shown in Fig. 1. Their formation is related with reaction temperature and/or time; in the other words, with the treatment severity [43]. However, in this study the byproducts concentration was very similar for both the autohydrolysis treatments (Fig. 1). As seen in Fig.1 acetic acid and furfural were the main byproducts in the filtrate. It has been reported that acetic acid acts as inhibitor when its concentration is between 4 and 10 g/L [63]. In our experiments, the acetic acid concentrations were under 1.72 g/L. The concentrations of furfural found in our experiments (< 1.62 g/L), were below the limiting value (> 2g/L) that causes fermentation inhibition [64].

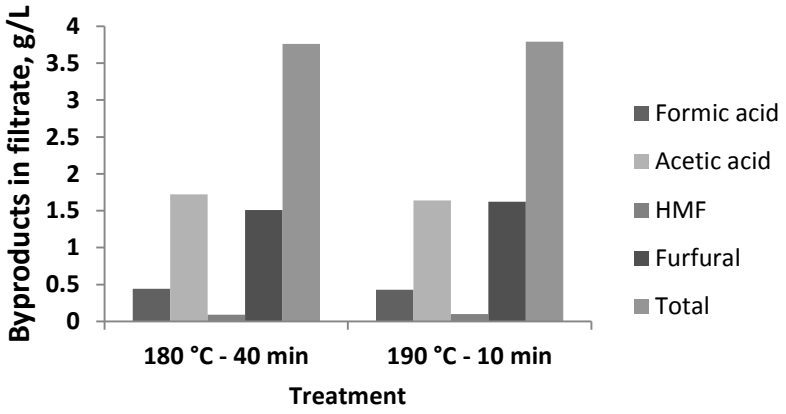

Figure 1. Effect of autohydrolysis conditions on the byproducts production in the autohydrolysis filtrate.

During the autohydrolysis process, hemicelluloses and cellulose are partially converted into oligo-sugars and mono-sugars [20]. In order to transform the oligo-sugars into monosugars so that they could be quantified, the autohydrolysis filtrates were further hydrolyzed using a solution of sulfuric acid (4% w/w). Figure 2 shows that the xylan is the main sugar component in the autohydrolysis filtrate, representing more than 70% of all the sugars. This result was anticipated since hemicelluloses are more susceptible to acid hydrolysis than cellulose [65]. The 180 °C for 40 min condition generated more sugars in the filtrates than the 190 °C for 10 min, and this result is consistent with those previously shown (Table 2) that also indicated more sugar removal from raw material at the 180 °C for 40 min condition. Assuming that total sugar recovery is the sum of sugars present in the autohydrolysis filtrate plus those in the pretreated solids, the 180 °C for 40 min condition recovered the maximum amount, i.e., 73.2% of the theoretical total sugar. It is worth noting that total sugar recovery is not proportional to loss of hemicelluloses from raw material (Table 1) because of further sugar degradation.

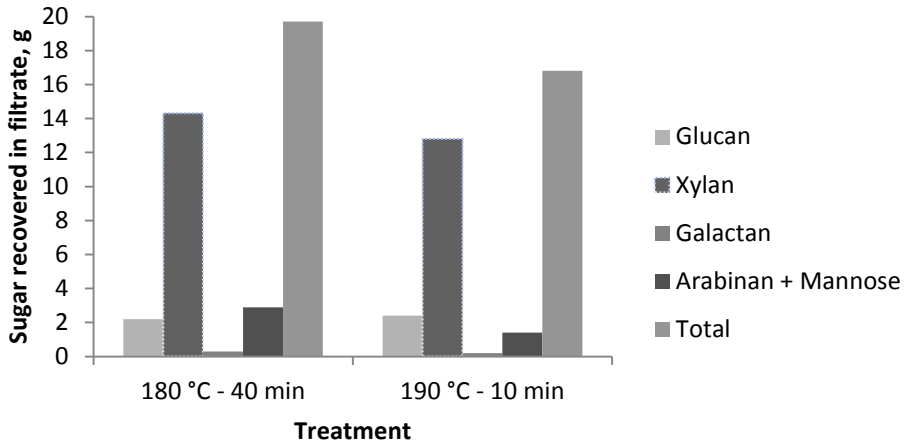

Figure 2. Sugars in autohydrolyzate filtrate after acid-treated hydrolysis with 4% sulfuric acid treatment. Results based on 100 g of oven dry original raw material.

3.6 Effect of autohydrolysis conditions and refining on enzymatic hydrolysis

Previous studies have indicated the potential of mechanical refining to improve sugar release during enzymatic hydrolysis of biomass, at low enzyme dosages [26, 27, 28, 29, 30, 39]. Such mechanical treatment is an alternative to make autohydrolysis pretreatment more attractive and commercially viable. In this study, the step of enzymatic hydrolysis was performed on autohydrolysed and PFI refined solid residues, with the enzyme loads of 5 and 10 FPU/g oven dry substrate. This range of enzyme dose was chosen considering that, as a rule of thumb, the enzyme load of 5 FPU/g of substrate has been established as the maximum value for an economically feasible operation on the basis of current enzyme prices [66]. The 10 FPU/g enzyme dosage was used to evaluated the sugar recovery potential of the substrate if enzymes become cheaper in the future.

As seen in Fig. 3a and 3b the glucan and xylan yield, respectively, were very similar for the two autohydrolysis conditions investigated. The highest glucan yields were observed for the pretreatment performed at 190 $^{\circ}$C for 10 min, 27.7 and 30.1g per 100g of starting raw material for enzyme loading of 5 and 10 FPU/g, respectively. More than 83% of the cellulose in the solid residue cellulose was recovered at 5 FPU, which is equivalent to 45.1% of carbohydrate based on starting raw material. For both autohydrolysis conditions used, an increasing enzyme loading from 5 to 10 FPU/g increased glucan yield by approximately 2.5g only.

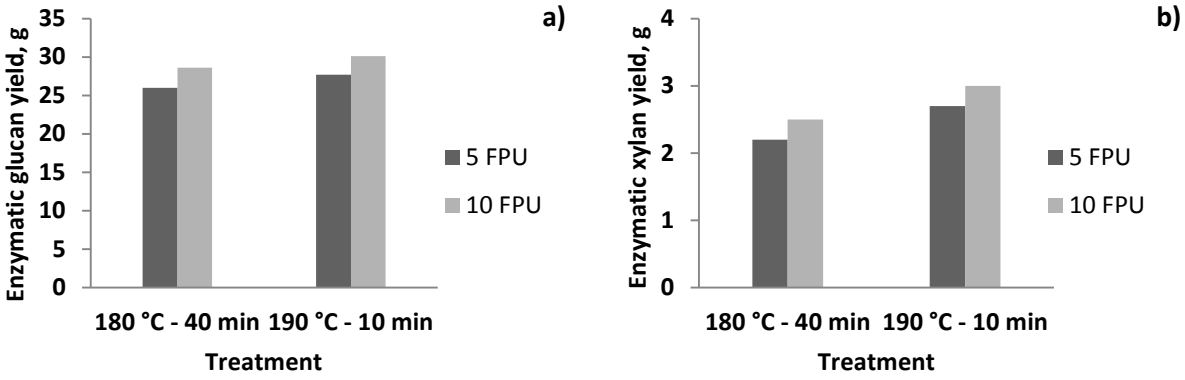

Fig. 3. Enzymatic hydrolysis of refined solid residue from each pretreatment condition at 5 and 10 FPU/g substrate enzyme loading for 96 h: (a) enzymatic glucan yield; (b) enzymatic xylan yield.

The highest xylose yield were observed for the pretreatment preformed at 190 °C for 10 min, 2.7 and 3.0 g per 100g of starting raw material for enzyme loading of 5 and 10 FPU/g, respectively. About 50% of the xylans were recovered from the solid residue regardless of enzyme dose, indicating that a 5 FPU/g charge is sufficient for processing autohydrolyzed and PFI refined straw.

Figure 4 shows that 78.3% of straw carbohydrate can be successfully recovered by enzymatic hydrolysis with an enzyme charge of 5 FPU/g of substrate considering the material pretreated at 180 °C for 40 min condition. Of the total recovered, 19.7g (32.1%) comes from the autohydrolysis filtrate and 28.4g (46.6%) from the enzyme hydrolyzate of the solid residues. If an enzyme charge of 10 FPU/g substrate is used instead, 83.1% of the straw carbohydrate is recovered. This gain may not justify the extra cost of the higher enzyme charge. The results obtained in this study regarding the sugar recovery is higher when compared to the others raw materials, e.g., the maximum total sugar recovery for wheat straw reported in previous study [30], in same autohydrolysis conditions, combined with refining post-treatment, and using 10 FPU enzyme charge, was 68.7%, which was lower than the value found in this study (83.1%), indicating the potential of sugar cane straw aiming bioethanol production. A sugar recovery of 70.4% for ball milled pretreated sugarcane straw under very optimized conditions [9] has been also reported in previous, which is lower than the values obtained in this study, using autohydrolysis combined with mechanical refining.

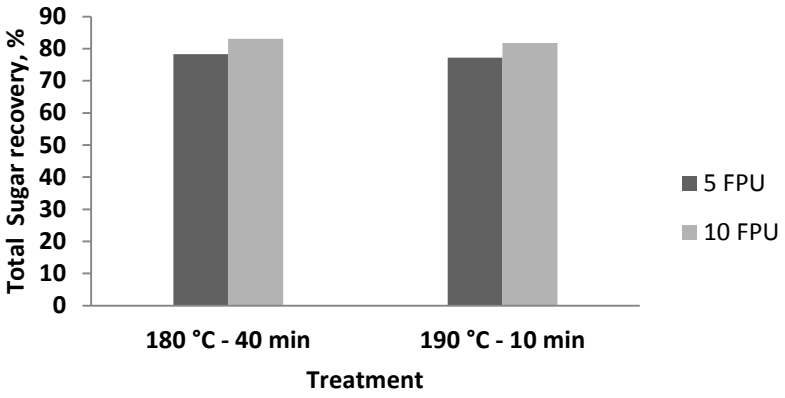

Fig. 4. Total sugar recovery of enzymatic hydrolysis of refined solid residue from each pretreatment condition at 5 and 10 FPU/g substrate enzyme loading for 96 h.

3.7 Material balance

The material balance for autohydrolysis pretreatments combined with PFI treatment followed by enzymatic hydrolysis is shown in Table 3. The solids recovery for the autohydrolysis conditions of 180 ^{o}C for 40 min and 190^{o}C for 10 min were 62.1% and 68.0%, respectively; the lowest solid recovery corresponds to the condition that removed most hemicelluloses.

Table 3. Material balances from autohydrolysis pretreatments with PFI refining treatment followed by enzymatic hydrolysis.

Temperature (°C)	Time (min)	Solid recovery (%)	Sugars in filtrate (g)			FPU	Enzyme hydrolyzate (g)			Sugar recovery	
			G[a]	H[b]	T[c]		G[a]	H[b]	T[c]	(g)[d]	(%)[e]
180	40	62.1	2.2	17.5	19.7	5	26.0	2.4	28.4	48.1	78.3
						10	28.6	2.8	31.4	51.1	83.2
190	10	68.0	2.4	14.4	16.8	5	27.7	2.9	30.6	47.4	77.2
						10	30.1	3.3	33.4	50.2	81.8

[a] G: released glucan
[b] H: released xylan and other sugars.
[c] T: total sugars.
[d] Sum of sugars in filtrate + enzyme hydrolyzate.
[e] Percentage of sugar recovery, calculated by (sugar recovery (g)/ 61.4 (g) (total sugars of raw material)

When analyzing the material balance for autohydrolysis performed at 180 ^{o}C for 40 min with PFI refining treatment followed by enzymatic hydrolysis with dosage of 10 FPU/g showed the highest total sugar recovery, in which 51.1g of sugar from prehydrolyzate plus

enzyme hydrolyzate were generated from 100g bagasse. The same treatment but with enzyme loading of 5 FPU/g, recovered 48.1g of sugars in prehydrolyzate and enzymatic hydrolysis resulting in an overall sugar recovery of 78.3%. However, the total recovery for two enzymes doses evaluated was slightly similar; being the increase off the enzyme charge from 5 to 10 FPU/g raised total sugar recovery around 5% only. The difference in the overall sugar recovery between the two pretreatment conditions are very comparable, and the choice will depend on the capital cost for the equipment. In the case of the higher temperature the retention time is lower and it is expected that this will result in a lower equipment cost.

3.8 Process economics

The economics of the integrated bioethanol production process is largely driven by the feedstock price, enzyme utilization efficiency, ethanol yield, capital investment cost, and the ethanol wholesales price. The autohydrolysis process combined with mechanical refining has considerably reduced the enzyme usage and improved the efficiency of sugar release which sustained a relatively high total fermentable sugar recovery. This process simplicity decreases capital investment cost significantly in relation to other processes [67, 68]. Only limited information is available on the sugarcane straw pricing because most attention has been towards the utilization of sugarcane bagasse. Leal has reported that the delivered sugarcane straw price can vary between $18.3 to $40.2 per dry metric per ton, depending on the straw collection routes and storage methods [69]. The resulting minimum ethanol revenue to achieve 12% internal rate of return can be in a range from $0.49 to $0.56 per liter ethanol product. Considering the current anhydrous ethanol wholesale price in Brazil ($0.5 to $0.6 per liter), the bioethanol production from autohydrolysis of sugarcane straw can be a financial viable approach if a cost-efficient straw collection system is established. It is noted that the economic performance of the process is also subject to the scope change of feedstock input, which is dictated by the local availability and the rationality on how much straw should be left in the field to optimize the sustainability of sugarcane cultivation. By the way, the production of bioethanol from autohydrolysis of sugarcane straw can have a financial return larger than 12%.

In previous study, the total cost of refining including capital and power cost (Power consumption was estimated as 150 kwh per dry ton loading) adding onto the minimum ethanol revenue is $0.03 per liter ethanol, relative to more than $0.1 per liter ethanol cost saving generated by refining through ethanol yield improvement. Therefore it is a very

attractive approach to combine autohydrolysis with mechanical refining to implement cellulosic ethanol commercialization [67].

4. Conclusions

- The high carbohydrates content (>60%) makes sugarcane straw an attractive feedstock for ethanol production;
- Autohydrolysis followed by mechanical refining proved effective technique for deconstruction of sugarcane straw aiming ethanol production;
- This method allowed for high sugar recovery (78.3%) with low enzyme application (5 FPU/g substrate) in subsequent hydrolysis process;
- When autohydrolysis pretreatment was performed at 180 ^{o}C for 40 min followed of PFI refining, the increase of the enzyme charge from 5 to 10 FPU/g raised total sugar recovery by only 5%;
- The difference in the overall sugar recovery between the two autohydrolysis conditions, 180 ^{o}C for 40 and 190 ^{o}C for 10, are very comparable. So, the choice will depend on the capital cost for the equipment. In the case of the higher temperature the retention time is lower and it is expected that this will result in a lower equipment cost;
- During autohydrolysis 19.7 and 16.8/100g raw straw sugar can be recovered from prehydrolyzate; and 3.76 and 3.79/100 g raw straw byproducts can be generated by 180 ^{o}C for 40 and 190 ^{o}C for 10 min autohydrolysis conditions, respectively;
- The bioethanol production from autohydrolysis of sugarcane straw seems to be a financial viable approach if a cost-efficient straw collection system is established. It reaching to have a financial return larger than 12%.

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CONCLUSÕES GERAIS

Através dos resultados obtidos nesse estudo foi possível a obtenção de dados para colaborar com a literatura sobre a composição dos extrativos do bagaço e da palha da cana de açúcar. Foram identificados 47 e 67 compostos para as amostras de bagaço e da palha da cana-de-açúcar, respectivamente. Foi observado que as maiores classes de compostos presentes são os ácidos graxos e carboidratos.

No que diz respeito à caracterização dos compostos presentes na lignina Klason e na lignina solúvel, foi possível a identificação de 52, 57 e 49% dos produtos da pirólise para o bagaço, medula e palha de cana de açúcar, respectivamente. Os produtos encontrados na lignina Klason não oriundos da lignina foram da ordem de 10% e eram constituídos principalmente de furfural e acido acético. Já para as amostras de lignina solúvel em ácido, houve uma predominância de produtos derivados de carboidratos. Foi possível identificar a presença de lignina na fração solúvel em ácido.

O uso de pré-tratamento de auto-hidrólise seguido de refino, se mostrou como uma tecnologia promissora para a obtenção de etanol de segunda geração a partir do bagaço de cana-de-açúcar. Foi possível a obtenção de 28,9% de açúcar hidrolisáveis oriundos do filtrado da pré-hidrolise. Para o processo de refino, o tratamento a 180°C durante 20 minutos possibilitou a maior obtenção de açúcares hidrolisáveis (84,4%). Os pré-tramentos possibilitaram que grandes quantidades de açúcares hidrolisáveis fossem obtidos, aplicando-se baixa dosagem de enzimas (5 FPU/grama de substrato), sendo que a análise econômica de um possível projeto utilizando as condições de estudo tem uma taxa de retorno estimada em 12% .

Do mesmo modo, quando se avaliou o uso da palha de cana-de-açúcar para a produção de etanol, o pré-tratamento de auto-hidrólise seguido do refino, também se mostrou como uma tecnologia promissora. Ainda no que tange a matéria prima, a palha apresentou elevado conteúdo de carboidratos (>60%). No melhor cenário de processamento (180°C por 40 minutos foi possível à obtenção de 78,3% de açúcares hidrolisáveis, onde também foram empregadas dosagens de enzimas em valores considerados baixos (5 FPU/grama de substrato). Quanto a viabilidade econômica, o estudo indicou que se for possível a coleta, estocagem e armazenamento da matéria prima com baixos custos, essa matéria prima pode prover taxas de retorno superiores a 12% .

Com os dados obtidos neste estudo foi possível verificar que os resíduos da cana de açúcar contribuem com o campo de biorrefinaria. Uma vez que o bagaço e a palha de cana de

açúcar podem ser fonte promissoras de bioquímicos valiosos os quais podem ser utilizados na indústria de cosméticos, alimentos ou farmacêutica, como também, na produção do etanol de 2G.