DANILA MORAIS DE CARVALHO

SOME FACTORS AFFECTING THE PRODUCTION OF SECOND GENERATION ETHANOL FROM EUCALYPTUS, SUGARCANE BAGASSE AND SUGARCANE STRAW

Thesis presented to the Federal University of Viçosa, as part of the requirements of the Post-Graduation Program in Forest Science, for obtaining the title of *Doctor Scientiae*.

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SUMMARY

ABSTRACT

CARVALHO, Danila Morais de, D. Sc., Universidade Federal de Viçosa, February, 2016. **Some factors affecting the production of second generation ethanol from eucalyptus, sugarcane bagasse and sugarcane straw**. Adviser: Jorge Luiz Colodette. Co-adviser: José Humberto de Queiroz.

The ethanol has been considered a promising biofuel to replace fossil-based fuels. The strategic use of eucalyptus, sugarcane bagasse and sugarcane straw in second generation technology to ethanol production was investigated in this work, by performing various pretreatment processes followed by simultaneous saccharification and fermentation (SSF). In article I it is presented the chemical characterization of eucalyptus, sugarcane bagasse and straw before and after hydrothermal (H_2O), diluted acid (4.5% H_2SO_4) and alkaline (15% NaOH) pretreatments. It was determined that the significant amount of silica present in sugarcane bagasse and straw led to overestimation of Klason lignin of these biomasses. A novel approach to report the chemical composition of biomasses, based on the complete mass balance, was suggested and proved to be useful to assess both, raw materials and pretreated biomasses. The formation of pseudo-extractives in eucalyptus wood and pseudo-lignin in bagasse and straw as result of pretreatments was observed. Article II presents the chemical and structural characterization of xylans isolated from eucalyptus, bagasse and straw *via* two different methods, namely: peracetic acid delignification followed by dimethyl sulfoxide extraction and sodium chlorite delignification followed by dimethyl sulfoxide extraction. The xylan obtained from eucalyptus was identified as an O-acetyl-4-O-methylglucuronoxylan type, containing 39 acetyl groups units and 11 4-O-methylglucuronic acids per 100 units of xylose on the backbone. In addition, one 4-O-methylglucuronic acid was also substituted by one terminal galactosyl unit. The xylan obtained from bagasse and straw was an arabinoxylan type, which contained 100 xylose units: 29 acetyl groups units: 5 arabinofuranosyl units for bagasse, proporcionally, and 100 xylose units: 8 acetyl groups units: 6 arabinofuranosyl units for straw, proporcionally. Article III describes the effect of hydrothermal and diluted acid (1.5, 3.0 and 4.5% H_2SO_4) pretreatments on the chemical composition of biomasses and their subsequent conversion into ethanol. It was observed that lowering pretreatment pH resulted in improved lignin and carbohydrates removal. The eucalyptus presented the highest ethanol production after hydrothermal

pretreatment, but with relative low yield. After acid pretreatments, bagasse and straw showed higher ethanol productions then eucalyptus. The pretreatment performed at 4.5% H_2SO_4 was the most efficient. Article IV assesses the effect of alkaline charge during alkaline (5, 10 and 15% NaOH) pretreatments on the chemical composition of biomasses and their subsequent conversion into ethanol. It was observed that higher alkaline charge provided the highest lignin and carbohydrates removal. For the alkaline pretreatments, the bagasse proved to be the most promising biomass for ethanol production. The pretreatment with 15% NaOH was the most efficient. Article V presents an optimization of the cold alkaline extraction (CAE) pretreatment regarding temperature (20ºC, 30ºC and 40ºC), reaction time (10, 35 and 60 min) and NaOH concentration (70, 90 and 110 g L^{-1}), focusing on xylan removal from biomasses and subsequent conversion of the xylan-depleted biomasses into ethanol. The optimal conditions for xylan removal from eucalyptus wood, sugarcane bagasse and sugarcane straw were, respectively: 40ºC, 60 min and 70 g L^{-1} NaOH; 33ºC, 60 min and 110 g L^{-1} NaOH; and 31ºC, 55 min and 110 g L^{-1} NaOH. Under these pretreatments conditions, substantial amounts of lignin were also removed from the biomasses. For the eucalyptus wood, the formation of pseudo-extractives was observed during the CAE pretreatments. The sugarcane straw pretreated with CAE was the most promising biomass for production of second generation ethanol. For the CAE pretreatments, higher ethanol yields were achieved with sugarcane bagasse and straw in relation to eucalyptus wood. In summary, the results accumulated from this doctoral thesis suggested that bagasse and straw are suitable biomasses for production of second generation ethanol. The use of these lignocellulosic biomasses creates the possibility of integrating first and second platforms for ethanol production, which turns residues into main product.

RESUMO

CARVALHO, Danila Morais de, D. Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Alguns fatores que afetam a produção de etanol de segunda geração a partir de eucalipto, bagaço e palha de cana-de-açúcar**. Orientador: Jorge Luiz Colodette. Coorientador: José Humberto de Queiroz.

O etanol tem sido considerado um promissor biocombustível para substituir combustíveis fósseis. O uso estratégico de eucalipto, bagaço e palha de cana-de-açúcar em tecnologias de segunda geração para a produção de etanol foi estudada neste trabalho, usando vários processos de pré-tratamentos seguidos por sacarificação e fermentação simultâneas (SFS). No artigo I é apresentada a caracterização química de eucalipto, bagaço e palha de cana-de-açúcar antes e após os pré-tratamentos hidrotérmico (H_2O), ácido diluído (4,5% H_2SO_4) e alcalino (15% NaOH). Foi determinado que o significativo teor de sílica presente em bagaço e palha da cana-de-açúcar causaram superestimação da lignina Klason nessas biomassas. O novo método para reportar a composição química das biomassas, baseado no completo balanço de massas, foi sugerido e provou ser útil para avaliar ambas, matéria-prima e biomassa pré-tratada. A formação de pseudo-extrativos na madeira de eucalipto e pseudo-lignina no bagaço e na palha foi observada como resultado dos pré-tratamentos. O Artigo II apresenta a caracterização química e estrutural das xilanas isoladas a partir de eucalipto, bagaço e palha usando dois métodos, sendo eles: deslignificação com ácido peracético seguida por extração com dimetilsulfóxido e deslignificação com clorito de sódio seguida por extração com dimetilsulfóxido. A xilana obtida a partir do eucalipto foi identificada como do tipo O-acetil-4-O-metilglucuronoxilana, contendo 39 unidades de grupos acetilas e 11 ácidos 4-O-metilglucurônicos para cada 100 unidades de xilose na cadeia principal. Além disso, um ácido 4-O-metilglucurônico foi também substituído por uma unidade de galactosil terminal. A xilana obtida a partir de bagaço e palha foi do tipo arabinoxilana, que apresentou proporcionalmente 100 unidades de xiloses: 29 unidades de grupos acetilas: 5 unidades de arabinofuranosil para o bagaço e proporcionalmente 100 unidades de xiloses: 8 unidades de grupos acetilas: 6 unidades de arabinofuranosil para a palha. O Artigo III descreve o efeito dos pré-tratamentos hidrotérmico e ácido diluído (1,5%, 3,0% e 4,5% de H_2SO_4) na composição química das biomassas e sua subsequente conversão em etanol. Observou-se que a redução no pH

dos pré-tratamentos favoreceu a remoção de lignina e carboidratos. O eucalipto apresentou a maior produção de etanol após o pré-tratamento hidrotérmico, mas com rendimento relativamente baixo. Após os pré-tratamentos ácidos, bagaço e palha mostraram maiores produções de etanol que o eucalipto. O pré-tratamentos realizados com 4,5% de H_2SO_4 foi o mais eficiente. O Artigo IV avalia o efeito da carga alcalina durante os pré-tratamentos alcalinos (5%, 10% e 15% NaOH) na composição química das biomassas e sua subsequente conversão em etanol. Observou-se que as maiores cargas alcalinas propiciaram as maiores remoções de lignina e carboidratos. Para pré-tratamentos alcalinos, o bagaço provou ser a biomassas mais promissora para produção de etanol. O pré-tratamento com 15% de NaOH foi o mais eficiente. O Artigo V apresenta a otimização do pré-tratamento de extração alcalina a frio (EAF) referente à temperatura (20ºC, 30ºC e 40ºC), tempo de reação (10, 35 e 60 min.) e concentração de NaOH (70, 90 e 110 g L^{-1}) com foco na remoção de xilanas das biomassas e subsequente conversão das biomassas deficientes em xilanas em etanol. As condições ótimas para a remoção de xilanas de madeira de eucalipto, bagaço e palha da cana-de-açúcar foram respectivamente: 40ºC, 60 min. e 70 g L^{-1} de NaOH; 33ºC, 60 min. e 110 g L^{-1} de NaOH; e 31ºC, 55 min. e 110 g L^{-1} de NaOH. Nessas condições de pré-tratamentos, considerável quantidade de lignina também foi removida das biomassas. Para a madeira de eucalipto, a formação de pseudo-extrativos foi observada durante os pré-tratamentos de EAF. A palha da cana-de-açúcar pré-tratada por EAF foi a biomassa mais promissora para a produção de etanol de segunda geração. Para os pré-tratamentos de EAF, os maiores rendimentos em etanol foram obtidos para bagaço e palha da cana-de-açúcar que para a madeira de eucalipto. Em resumo, os resultados acumulados por essa tese de doutorado sugeriram que bagaço e palha são biomassas aplicáveis à produção de etanol de segunda geração. O uso dessas biomassas lignocelulósicas cria a possibilidade de integrar primeira e segunda plataformas para a produção de etanol, transformando resíduo em produto principal.

GENERAL INTRODUCTION

The ethanol is a strategic biofuel for Brazilian economy and it has been recognized as the most promising biofuel to replace fossil-based fuels. Bioethanol is a versatile fuel, suitable to be used in neat form or blended with gasoline to gasoline engine, due its high octane number (108). In addition, the low cetane number (8) and the high heat of vaporization (0.91 MJ/kg) of bioethanol avoids its self-ignition into diesel engine (Balat and Balat, 2009; Dermibas, 2009; Balat et al., 2008; Demirbaş et al., 2005). Bioethanol is a primary alcohol (C_2H_5OH) and the oxygen present in its chemical structure improves the combustion process, reducing hydrocarbon, carbon monoxide and particulate emission during the burning, however with the possibility to increase the nitrogen oxide emission (Balat et al., 2008). In Brazil the ethanol is usually produced from sugarcane juice, but in recent years the production of second generation ethanol from non-food lignocellulosic biomass has showed up as one sustainable alternative, as it is generated from renewable resources and reduces greenhouse gas emissions (Demirbaş et al., 2005).

Lignocellulosic biomasses such as sugarcane bagasse (Souza et al., 2012; Santos et al., 2010), sugarcane straw (Santos et al., 2014; Oliveira et al., 2013; Hari Krishna et al, 2001) and eucalyptus (Ballesteros et al., 2004) has been studied as promising feedstocks for the production of second generation ethanol and, in this scenario, Brazil presents a great potential as a supplier of such renewable resources. Sugarcane bagasse and straw are both lignocellulosic residues generated from sugarcane industry in approximately amount of 92 million tons/year each (2015/16 harvest) (Conab, 2015; Oliveira et al., 2013). In addition to a wide sugarcane production, Brazil is also an important producer of fast-growing woods, such as eucalyptus, which is cultivated in the country for many industrial purposes (González-García et at, 2012).

The production of second generation ethanol, however, faces a number of challenges. One is to improve the efficiency of bioconversion processes of biomass into ethanol. The bioconversion process to ethanol production has few steps and the pretreatment is considered as a key one. During pretreatments the accessibility and digestibility of cellulose is improved by removal of chemical components from biomasses (e.g. lignin and hemicelluloses) and increased cellulose swelling (Meng and Ragauskas, 2014; Foston and Ragauskas, 2012). Although different pretreatment conditions have been currently investigated, the pretreatments are not completely

efficient so far (Pu et al., 2013; Yang e Wyman, 2008; Chandra et al., 2007). Another challenge is the large number of methodologies for chemical characterization of biomasses before and after pretreatment, which can generate a large number of data that, without standardization, harms comparison (Canilha et al., 2012). In addition, the lack of reliable data on chemical composition of certain biomasses is problematic, particularly for those non-traditional ones such as agricultural wastes. One example is the relative little information about chemical and structural features of xylan (main hemicelluloses in eucalyptus and sugarcane residues) (Magaton et al., 2008; Ebringerová et al., 2005; Evtuguin et al., 2003). Xylans are the most reactive components from biomasses and a natural source of organic acids (Vegas et al., 2008; Garrote et al., 2007). They play important role, especially during hydrothermal pretreatment, in which xylan is the only source of acids acting as catalyst in the chemical reactions. Unlike the xylan from eucalyptus, which have been recently studied (Magaton et al., 2008; Evtuguin et al., 2003), little information about xylan from sugarcane bagasse and straw is available. In addition to have important role during chemical processes the xylan itself has also been considered versatile alternatives for the production of novel materials (Ebringerová et al., 2005), with a promising future. The proper characterization of xylans is paramount for their rational use.

The growing interest in the use of lignocellulosic biomasses for ethanol production together with a few gaps in the second generation ethanol technology were the chief motivations for this thesis. The work aimed at studing the production of second generation ethanol from eucalyptus, sugarcane bagasse and sugarcane straw and investigate the effect of the different pretreatments: hydrothermal, diluted acid, alkaline and cold alkaline extraction on this process. In addition, it was proposed a novel protocol to report the chemical composition of untreated and pretreated biomasses, based on the complete mass balance. Furthermore, the chemical structure of xylan from these biomasses was investigated, and the information gathered was used to assess the effect of various pretreatments on chemical composition of biomasses.

This thesis was structured in the following five articles:

- **Article I**: Assessment of chemical transformations in eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid and alkaline pretreatments (Published in the Industrial Crops and Products Journal – Carvalho et al., 2015);

- **Article II**: Comparative characterization of acetylated heteroxylan from eucalyptus, sugarcane bagasse and sugarcane straw (Submitted to the Carbohydrate Polymers Journal);

- **Article III**: Assessment of hydrothermal and acid pretreatments for bioethanol production from eucalyptus, sugarcane bagasse and straw (Submitted to the Industrial Crops and Products Journal);

- **Article IV**: Assessment of alkaline pretreatment for bioethanol production from eucalyptus, sugarcane bagasse and straw (Submitted to the Industrial Crops and Products Journal); and

- **Article V**: Cold alkaline extraction applied as pretreatment for bioethanol production from eucalyptus, sugarcane bagasse and sugarcane straw (Submitted to the Energy Conversion and Management Journal).

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Article I

Assessment of chemical transformations in eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid and alkaline pretreatments

ABSTRACT - The impact of hydrothermal, dilute acid and alkaline pretreatments on the chemical structure of eucalyptus wood, sugarcane bagasse and straw were compared with a view to their subsequent bioconversion into ethanol. Sugarcane bagasse and straw contain high amounts of extractives (15.0% and 12.2%, respectively), ash (2.3% and 7.9%, respectively) and silica (1.4% and 5.8%, respectively). If not properly corrected, the presence of silica would lead to the overestimation of the lignin, while high amounts of extractives would cause the overestimation of the content of sugars in biomass. Applying a novel approach through the use of complete mass balance, bagasse and straw proved to contain lower amounts of lignin (18.0% and 13.9%, respectively) than previously reported for these raw materials, and certainly a much lower amount of lignin than eucalyptus (27.4%). The syringyl to guaiacyl units ratio (S/G) for lignin in bagasse and straw (1.1 and 0.5, respectively) was lower than that for eucalyptus (2.7), suggesting a different reactivity during chemical pretreatments. The xylan content in sugarcane bagasse and straw was much higher than that in eucalyptus, with a significantly lower degree of substitution for uronic acids and acetyl groups. The sugarcane straw showed the highest mass loss during the investigated pretreatments, especially under alkaline conditions, with a total biomass yield of only 37.3%. This result was obtained most likely due to the high amount of free phenolic groups traditionally observed in grass lignin, in addition to the high frequency of ester bonds between lignin and hemicelluloses. During the hydrothermal and dilute acid treatments, mostly hemicelluloses were removed, followed by the formation a significant amount of pseudo-lignin structures for bagasse and straw. The alkaline pretreatment affected the lignin content. For eucalyptus, the formation of structures similar in their behavior to extractives (i.e., soluble in toluene and ethanol, subsequently referred to as “pseudo-extractives”) was observed during all three pretreatments, with formation of 12.4% for hydrothermal, 18.9% for dilute acid and 8.7% for alkaline pretreatment. This information, combined with actual yields, should be taken into account when assessing the impact of pretreatments on the chemical composition and structure of biomass.

Keywords: Complete mass balance, pretreatments, pseudo-extractives, pseudo-lignin, silica content, sugarcane.

RESUMO - O efeito dos pré-tratamentos hidrotérmico, ácido diluído e alcalino na estrutura química da madeira de eucalipto e de bagaço e palha da cana-de-açúcar foi comparado visando seus subsequentes usos para a bioconversão em etanol. Bagaço e palha da cana-de-açúcar possuem elevados teores de extrativos (15,0% e 12,2%, respectivamente), cinzas (2,3% e 7,9%, respectivamente) e sílica (1,4% e 5,8%, respectivamente). Quando não corretamente corrigido, o teor de sílica causa

superestimação do teor de lignina, enquanto que elevados teores de extrativos causam superestimação do teor açúcares na biomassa. Utilizando a nova abordagem baseada no completo balanço de massas, bagaço e palha apresentaram mais baixos teores de lignina (18,0% e 13,9%, respectivamente) que os previamente reportados para estas matérias-primas e consideravelmente inferior ao teor de lignina do eucalipto (27,4%). A relação siringil/guaiacil (S/G) para a lignina de bagaço e palha (1,1 e 0,5, respectivamente) foi menor que a apresentada para o eucalipto (2,7), sugerindo reatividades diferentes durante pré-tratamentos químicos. Os teores de xilanas para bagaço e palha da cana-de-açúcar foram muito maiores que o do eucalipto, com grau de substituição por ácidos urônicos e grupos acetila consideravelmente menor. A palha da cana-de-açúcar apresentou a maior perda de massa durante os pré-tratamentos investigados, sobretudo sob condição alcalina, com rendimento sólido total de apenas 37,3%. Este resultado foi obtido muito provavelmente devido aos elevados teores de fenóis livres tradicionalmente observado em lignina de gramíneas, além da elevada quantidade de ligações éster entre lignina e hemiceluloses. Durante os pré-tratamentos hidrotérmico e ácido diluído, principalmente as hemiceluloses foram removidas e, como consequência, uma grande quantidade de estruturas de pseudo-lignina foram formadas do bagaço e da palha. Durante o pré-tratamento alcalino foram removidas quantidades significativas de lignina. Para o eucalipto, a formação de estruturas com comportamento similar ao de extrativos (i.e., solúveis em tolueno e etanol, posteriormente requeridas como "pseudo-extrativos") foi observada durante todos os três pré-tratamentos, com formação de 12,4% no pré-tratamento hidrotérmico, 18,9% no pré-tratamento ácido diluído e 8,7% no pré-tratamento alcalino. Esta informação, combinada com o rendimento total, deve ser considerada na avaliação do efeito dos pré-tratamentos na composição química e estrutural da biomassa.

Palavras-chave: Completo balanço de massa, pré-tratamentos, pseudo-extrativos, pseudo-lignina, teor de sílica, cana-de-açúcar.

1. Introduction

There is a growing interest worldwide in the development of new methodologies to produce products, chemicals, energy, and fuels from renewable sources (Ragauskas et al., 2006). The forecast for the period 2010–2040 suggests that the global demand for oil consumption will increase by 26.2%. At least part of these energy requirements could be supplied by renewable sources (Kralova and Sjöblom, 2010).

Sugarcane is one of the Brazil's main agricultural crops. The country's production for 2015/16 is 659 million tons of sugarcane cultivated on 9.0 million ha of land, with an average productivity of 73 tons/ha. Sugarcane is used mainly to produce sugar and ethanol (1st generation) from sucrose (Conab, 2015). Bagasse (sugarcane stalks) and straw (sugarcane tips and leaves) are the main wastes generated by the

sugarcane industry. After being processed, sugarcane forms 14% bagasse and 14% straw on a dry weight basis (Oliveira et al., 2013). As a result, the generation of 92 million tons of bagasse and 92 million tons of straw is expected during the 2015/16 harvest.

Sugarcane bagasse and straw are lignocellulosic biomasses that both have a great potential for their bioconversion to 2nd generation biofuels (Santos et al., 2014; Oliveira et al., 2013; Cardona et al.,2010). According to several authors, bagasse consists of 39-45% cellulose, 23-27% hemicelluloses, 19-32% lignin, 1-3% ashes and 5-7% extractives (Canilha et al., 2011; Rabelo et al., 2011; Rocha et al., 2011; da Silva et al., 2010), while straw has a typical chemical composition of 33-45% cellulose, 18-30% hemicelluloses, 17-41% lignin, 2-12% ashes and 5-17% extractives (Santos et al., 2014; Costa et al., 2013; da Silva et al., 2010; Saad et al., 2008). Wood biomass is another possible raw material for bioethanol production. Eucalyptus is the most cultivated gender in Brazil and is used for different industrial processes (González-García et al., 2012). Eucalyptus has relatively low extractives and ash content, and more than 90% of its chemical composition is formed by cellulose, hemicelluloses and lignin. The typical chemical composition of eucalyptus is 46-49% cellulose, 18-23% hemicelluloses, 29-33% lignin, 0.1-0.2% ash and 2-5% extractives (Pereira et al., 2013; Zanuncio et al., 2013).

The biological conversion of lignocellulosic biomass to biofuels includes the following main steps: pretreatment, enzymatic hydrolysis, fermentation, distillation/dehydration to meet fuel specification and effluent treatment (Rubin, 2008). Pretreatment is one of the key steps for the efficient and cost-competitive conversion of lignocellulosic materials into bioethanol. The main role of pretreatment technologies is to reduce biomass recalcitrance (protection made by the complex organization of lignocellulosic components in the cell wall which avoid carbohydrates degradation by enzymes) and, thereby, to enable higher sugar yield through enzymatic hydrolysis (Pu et al., 2013; Foston and Ragauskas, 2012; Zhao et al., 2012; Chandra et al., 2007). Numerous methods, including dilute acid, hydrothermal, alkaline, organic solvents, ionic liquids (IL's), and ammonia fiber expansion (AFEX), have been developed to overcome biomass recalcitrance. The impact of these commonly applied pretreatment technologies on lignocellulosic structure has been recently reviewed by Hu and Ragauskas (2012). Despite the different mechanism of each pretreatment, the goal for each of them is to increase cellulose accessibility (Meng and Ragauskas, 2014).

Dilute acid pretreatment is considered to be the most promising pretreatment technology for enhancing sugar release by enzymatic hydrolysis (Pu et al., 2013; Yang and Wyman, 2008; Chandra et al.,2007). During the dilute acid pretreatment, biomass is subjected to the combined action of an acid pH, heat and pressure, with a residence times from 1 min to 1 hour. Typically, sulfuric acid with a concentration of 0.4-2.0% (w/w) and at a temperature of 140-200ºC is used. Hydrothermal pretreatment, also called autohydrolysis or hot water pretreatments just uses water as a reaction medium without additional chemicals and, therefore, has a low recycling and environmental cost. Usually performed in high temperature (140-220ºC) and pressure, the mildly acid condition of this treatment is result from the release of organic acids from biomass (Pu et al., 2013; Garrote et al., 1999). These acid pretreatments, dilute acid and hydrothermal, promote structural changes in lignin and cellulose, as well as solubilization of hemicelluloses (Santos et al., 2014; Lee et al., 2010), resulting in the increased specific surface area of fibres and increased plant cell wall pore size and, thus, reduced biomass recalcitrance. However, increasing dilute acid and hydrothermal pretreatments severity may cause the degradation of xylan to furfural, which is an inhibitor for the formation of ethanol during fermentation.

In the alkaline pretreatment, cellulose accessibility and hydrolysis are increased due the removal of lignin, some acetyl groups, and various uronic acid substitutions on hemicellulose (Chen et al., 2012). Alkaline treatment also results in swelling of cellulose, which leads to an increase in the internal surface area (Alvira et al., 2010). Sodium hydroxide (NaOH) is the alkaline source most often used. For sugarcane bagasse, this pretreatment has been shown to extract most of the lignin and significantly improve cellulose accessibility during the enzymatic hydrolysis (Pandey et al., 2000). Similar results were obtained for sugarcane straw (Hari Krishnaet al., 1998).

Despite the existence of a significant amount of data regarding the chemical composition of various biomasses, the methodologies used to obtain these values differ in various studies, resulting in different numbers for the same materials. These discrepancies create a problem when comparing the results of various investigations, and there is an obvious need for the standardization of the methodologies used for the determination and reporting of the chemical composition of the new types of industrial lignocellulosic biomasses such as agricultural wastes (Canilha et al., 2012).

A correct evaluation of the chemical composition and its changes during the various pretreatments for novel types of lignocellulosic material (such as sugarcane

bagasse and straw) is necessary for the assessment of the impact of various pretreatments on the chemical composition and structure of biomass.

The objectives of the present work were: (i) to perform a comprehensive chemical characterization of original and pretreated eucalyptus, sugarcane bagasse and sugarcane straw biomasses using a novel approach that takes into consideration each biomass constituent, including extractives and silica contents; and (ii) to evaluate three types of pretreatments - hydrothermal, dilute acid, and alkaline - with respect to their impact on the chemical composition and structure of main biomass constituents, such as cellulose, lignin and hemicelluloses. It is hoped that such information will contribute to a better understanding of the biomass recalcitrance phenomenon and provide useful guidance for researchers when deciding on the most suitable pretreatment technology for future bioconversion of these biomasses.

2. Experimental

2.1 Materials

A 7-year old clonal hybrid of eucalyptus (*Eucalyptus urophylla* × *Eucalyptus grandis*) was supplied by a pulp company in the form of chips. Chips were screened and those with dimensions smaller than 0.5 cm x 3 cm x 3 cm were collected for chemical analyses and pretreatments. Five-months old sugarcane (cultivar RB867515) bagasse and straw (leaves and tips) were supplied by Center Sugarcane Experimentation (Oratórios, Minas Gerais State, Brazil) at the Federal University of Viçosa, in the form of small pieces (10 mm diameter). The sugarcane bagasse was obtained after chipping and pressing the sugarcane stalk. The sugarcane straw was collected in the field and fragmented in a shredder. All materials were stored in airtight plastic bags at room temperature prior to use. The chemicals used were sulfuric acid 95-97% and sodium hydroxide lentils (analytical grade), both purchased from Merck Milipore, Germany.

2.2 Methods

Samples of eucalyptus, bagasse and straw (100 g each) were subjected to various pretreatments: (i) hydrothermal; (ii) acid – 4.5% w/w H_2SO_4; and (iii) alkaline – 15% NaOH (on a dry biomass basis). The liquor:biomass ratio used was 2:1 for eucalyptus and 7:1 for bagasse and straw (dry weight basis), due their structure more porous. All the pretreatments (i, ii and iii) were performed in a Regmed reactor (2 L capacity) with constant agitation for 90 min to reach the set temperature (175ºC) and an

additional 15 min for the pretreatment itself. After the treatment, the reactor was cooled, and the treated biomass was washed with an excess of water and centrifuged at 800 rpm for 4 min. Before washing, a sample of liquor was collected for pH mensurement. The treated biomass was conditioned for 24 hours at 23 ± 1ºC and 50 ± 2% relative humidity and then stored at room temperature in airtight containers.

2.3 Analyses

The untreated and treated biomasses were converted to sawdust at a 40/60 mesh by using a Wiley mill bench model. The sawdust was conditioned for 24 hours at 23 ± 1ºC and 50 ± 2% relative humidity and then stored in airtight containers. The sawdust moisture content was determined according to TAPPI T 264 cm-07.

The ash content in the samples was measured according to TAPPI 211 om-02 standard method. The silica content (acid insoluble part of the ash) was determined using acid hydrolysis of the ashes, followed by gravimetric determination according to TAPPI 244 cm-11 standard method. The total extractive contents in the eucalyptus, sugarcane bagasse and straw were obtained by the sequential extraction with 1:2 ethanol-toluene (5 h) and 95% ethanol (4 hours) in a Soxhlet extractor, followed by hot water extraction (1 hour) according to TAPPI T 264 cm-07 method. Ash silica, and total extractive contents were determined in triplicate.

The lignin and sugar contents were determined based on extractive-free biomass by acid hydrolyses: 12 mol L^{-1} (72%) sulfuric acid was added to 0.3 g of sawdust in a glass beaker, thoroughly mixed and kept at 30ºC for 1 hour (in a water bath). Then the mixture was diluted to 0.41 mol L^{-1}(~3%) with distilled water and placed into an autoclave at 121ºC for 1 hour. Klason lignin content was determined gravimetrically as insoluble residue according to Gomide and Demuner (1986). Acid-soluble lignin was measured by UV-spectroscopy (Cary 50 Probe, Varian) at 215 and 280 nm wavelengths according to Goldschimid (1971), and the concentration was calculated according to Eq. (1). Klason and acid-soluble lignin contents were determined in triplicate.

$$Acid-soluble\ lignin = \left[4.538 \times \left(read\ (215\ nm) - read\ (280\ nm)\right) \times 1.1\right] \quad (1)$$

Sugar content was analyzed according to Wallis et al. (1996). The sugar content was determined in an acid hydrolyzate by ion chromatography (IC) with a Dionex ICS3000, using a pulsed amperometric detector, a CarboPac PA1 column (Thermo Scientific, USA), an injection

volume of 25 µL and a flow rate of 1 mL min^{-1}. External sugar standards used for calibration were glucose (Merck, Germany), xylose (Merck, Germany), galactose (Sigma–Aldrich, Germany), mannose (Merck, Germany) and arabinose (Sigma, USA). Fucose (Sigma, Slovakia) was used as an internal standard. Sugar content was determined in triplicates and the results reported as anhydrosugar.

The uronic acids were measured after hydrolysis with sulfuric acid to 5-formyl-2-furoic acid, which was calorimetrically determined after reaction with 3,5-dimethylphenol by UV spectroscopy at 450 nm wavelength (Scott, 1979). The uronic acid content was calculated according to Eq. (2). The acetyl group contents were determined in the extractive-free sawdust after hydrolysis with oxalic acid at 120ºC for 80 min using high performance liquid chromatography (HPLC) with UV detection, according to Solar et al. (1987). The HPLC instrument used was from Shimadzu with an SCL-10A system controller, LC-18.5 mm column, 25 cm (18.5 mm x 25 cm) (Shimadzu LC Shim-pack CLC-ODS, octadecyl). The temperature was 40ºC, the injection volume was 20 µL, and the flow rate was 0.6 mL min^{-1}. The mobile phase was H_3PO_4, 37 mmol L^{-1}, pH 2.2 adjusted with NaOH. Uronic acids and acetyl group were determined in triplicate.

$$Uronic\ acids = \left[10.16 \times \left(read\ (450\ nm) - read\ (400\ nm)\right) + 0.13\right] \quad (2)$$

The syringyl/guaiacyl ratio of lignin was determined in the extractive-free sawdust after oxidation with nitrobenzene and sodium hydroxide at 170ºC for 2.5 h. After oxidation, the lignin was extracted with chloroform and diluted in 1:1 acetonitrile/water. The solution obtained was analyzed by an HPLC method with UV detection, according to Lin and Dence (1992). The HPLC instrument used was the same as described previously. The temperature was 40ºC, the injection volume was 20 µL, and the flow rate was 1.0 mL min^{-1}. The mobile phase was acetonitrile/water (1:6 v/v). Syringyl/guaiacyl ratio was determined in duplicate.

The crystallinity index of the cellulose was obtained by an X-ray method using the diffractometer PANalytical, XPERT Promodel. Scans were collected from the raw material sawdust before extractive removal at 40 kW and 40 mA using a cobalt tube. The crystallinity index (*CrI*%) (Eq. (3)) was calculated by dividing the difference between the maximum intensity in the crystalline part (I_{002}) and the minimum intensity in the amorphous part (I_{am}) of the peaks (arbitrary unit) by the maximum intensity in the crystalline part (I_{002}) (Segal et al., 1959). Crystallinity was determinedin duplicate.

$$CrI = \frac{(I_{002} - I_{am})}{I_{002}} * 100 \quad (3)$$

2.4 Determination of mass balance

The sum of the components (cellulose, hemicelluloses, lignin, extractives and ash) in biomass can be adjusted to 100% using hydrolysis loss factors, generating a complete mass balance. However, these factors must be estimated for each species individually (Pettersen, 1984). A low content of proteins can also be present in both eucalyptus (Parajó et al., 2004) and sugarcane bagasse (Cordova et al., 1998). As previous results indicated, proteins get solubilized during acid pretreatment (Lee et al., 2009; Parajó et al.,2004), we did not take proteins content into consideration in this work.

In present work we applied a novel approach to obtain a complete mass balance for each sample. This approach takes into consideration the presence of silica and extractives in the biomasses and consists of the following two steps:

Step 1: The total lignin ($Lig_{ext\text{-}free}$) and anhydrosugars content ($Sug_{ext\text{-}free}$) were determined in the extractive-free biomass (following classical methodologies). The silica content (Si_{wb}) was measured first in the original biomass (whole biomass), including extractives, and then the average value was divided by extractive-free biomass (100 - Ext_{wb}) to obtain the silica content for the extractive-free biomass ($Si_{ext\text{-}free}$), according to Eq. (4).

$$Si_{ext-free}(\%) = [Si_{wb} / (100 - Ext_{wb})] \times 100 \quad (4)$$

The sum of the contents of lignin ($Lig_{ext\text{-}free}$), anhydrosugars($Sug_{ext\text{-}free}$) and silica ($Si_{ext\text{-}free}$) was assumed to correspond to100% of the extractive-free material. The discrepancy in the sum of all components was assigned to the losses of the sugar components as a result of the acid hydrolysis and was proportionally re-distributed among various sugar components:

Step 2: The values obtained for anhydrosugars ($Sug_{ext\text{-}free}$) and the lignin ($Lig_{ext\text{-}free}$) in extractive-free biomass (obtained in Step 1) were converted to the corrected numbers following a similar calculation methodology (Eqs. (5) and (6)).

$$Sug_{wb}(\%) = Sug_{ext-free} \times \{[\,100 - (Ash_{wb} + Ext_{wb})]/(100 - Si_{ext-free})\} \quad (5)$$

$$Lig_{wb}(\%) = Lig_{ext-free} \times \{[\,100 - (Ash_{wb} + Ext_{wb})]/(100 - Si_{ext-free})\} \quad (6)$$

where Sug_{wb} is the average of anhydrosugar content in the whole biomass, $Sug_{ext\text{-}free}$ is the anhydrosugar content in the extractive-free biomass, Lig_{wb} is the average of lignin content in the whole biomass, $Lig_{ext\text{-}free}$ is the lignin content in the extractive-free biomass, Ash_{wb} is the average of ash content in the whole biomass, Ext_{wb} is the average of extractive content in the whole biomass, and $Si_{ext\text{-}free}$ is the silica content obtained in Step 1.

After these steps, the sum of all constituents corresponds to 100%.

3. Results and discussion

3.1 Biomass characterization

3.1.1 Impact of extractives and ashes on the mass balance

When determining the chemical composition of biomass, prior to lignin and sugar analyses, extractives are generally removed from the plant materials (TAPPI 264 cm-07) and the contents of the main constituents are reported relative to the extractive-free biomass. In general, wood contains a small amount of extractives, which results in a low impact of extractives on the mass balance. Sugarcane bagasse and straw, on the other hand, contain a much higher amount of extractives and ashes, and a careful analysis of their contributions to the mass balance is necessary. For eucalyptus, the extractive values in the range of 2.1-5% have been reported (Pereira et al., 2013; Zanuncio et al., 2013). In the present work, the extractive content for eucalyptus was 2.3%; values of 15.0% and 12.2% were found, respectively, for extractives in sugarcane bagasse and straw. Using the same sequential extraction as in the present work (1:2 ethanol-toluene → 95% ethanol → hot water), Andrade and Colodette (2014) reported an extractive content of 8.5% for bagasse and Santos et al. (2014) reported an extractive content between 14-16.8% for straw.

Figure 1 shows the total chemical composition of eucalyptus, bagasse and straw including the contents of extractives and ashes (i.e., complete mass balance). Together, extractives and ashes accounted for 17.3% and 20.1% of the total mass of bagasse and

straw, respectively (Fig. 1B and C). The corresponding number for eucalyptus wood was only 2.5% (Fig. 1A).

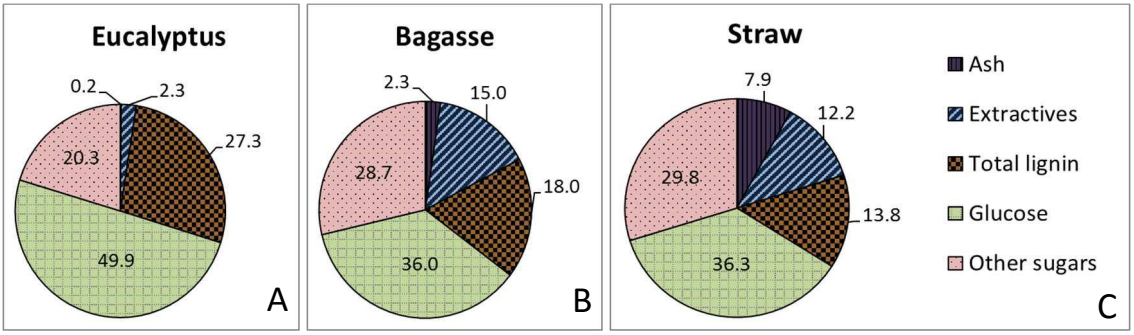

Figure 1 - Chemical composition of eucalyptus wood (**A**), bagasse (**B**) and straw (**C**), taking into consideration extractives (complete mass balance).

It is optimal to report the chemical composition of various constituents based on the whole biomass, accounting for all constituents. Consequently, the differences in the reported chemical composition when comparing extractive-free biomass and whole biomass were 3.4% and 2.4% for lignin in bagasse and straw, respectively, and 10.5% and 9.4% for sugars, respectively. For eucalyptus, such differences were only 0.6% and 0.5% for lignin and sugars, respectively (Table 1).

Table 1 - Lignin and anhydrosugar content present in eucalyptus, bagasse and straw based on extractive-free biomass and whole biomass[a].

Biomasses	Klason lignin %	Soluble lignin%	Glucose %	Xylose %	Galactose %	Mannose %	Arabinose %	Uronic acids %	Acetyl groups%
				Extractive-free biomass					
Eucalyptus	24.0 (0.1)	3.97 (0.02)	49.4 (0.7)	12.0 (0.2)	1.20 (0.10)	0.90 (0.05)	0.30 (0.10)	4.00 (0.05)	1.90 (0.04)
Bagasse	19.5 (0.1)	1.87 (0.06)	41.8 (0.8)	24.8 (0.2)	0.87 (0.12)	0.93 (0.12)	2.27 (0.21)	1.48 (0.05)	3.04 (0.02)
Straw	14.0 (0.3)	2.17 (0.08)	41.4 (1.2)	26.0 (0.2)	0.93 (0.06)	0.30 (0.10)	3.90 (0.05)	1.30 (0.02)	1.65 (0.01)
				Whole biomass[a]					
Eucalyptus	23.5	3.87	49.9	12.1	1.21	0.91	0.30	3.90	1.85
Bagasse	16.4	1.57	36.0	21.4	0.75	0.80	1.96	1.24	2.56
Straw	12.0	1.86	36.3	22.8	0.81	0.26	3.42	1.11	1.41

(…) Standard deviation.

[a]Calculated from average of chemical components.

3.1.2 Impact of silica on the determination of lignin content

As it is typical for grasses, sugarcane bagasse and straw have a particularly high content of ash and silica. More than 1.5% ashes for sugarcane bagasse and more than 4% ashes for sugarcane straw have been reported previously (Andrade and Colodette, 2014; Santos et al., 2014; Oliveira et al., 2013; Alves et al., 2010).

A value of 7.9% was obtained for the total ash content of the straw, of which 5.8% corresponds to the silica content (73% of total ash content). For the bagasse, 2.3% ash, including 1.4% (62%) from the silica, was detected. Eucalyptus presented extremely low levels of ash (0.16%), while no silica was detected (Fig. 2). These low values for the eucalyptus confirmed the results reported by other authors (Pereira et al., 2013; Alves et al., 2010).

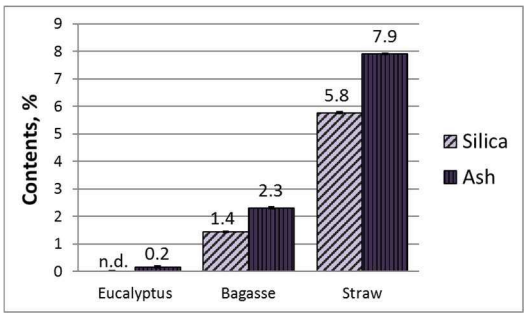

Figure 2 - Total ash and silica content in the raw materials.

The presence of the high content of silica in bagasse and straw leads to the overestimation of the acid-insoluble lignin (Klason lignin) as it co-precipitates with lignin during the acid hydrolysis. In the present work, the presence of crystalline fragments of silica was detected in the insoluble lignin residue after the acid hydrolyses. In order to report the lignin content correctly, the contributions from the silica contents, 1.7% and 6.6% for bagasse and straw, respectively, were deducted from the Klason lignin values. Lignin correction due to the presence of silica is especially important for grasses, as seen for the sugarcane bagasse and straw. For the eucalyptus, the silica contribution to the insoluble lignin was insignificant (Fig. 3A). The sum of the insoluble lignin (excluding the silica content) and the soluble fragments generated by the acid hydrolysis treatment provided the total amount of lignin. For the eucalyptus, the total amount of lignin (sum of Klason lignin and soluble lignin) was shown to be 27.97% (Table 1), which is similar to the data reported by other authors (Pereira et al., 2013; Zanuncio et al., 2013; Alves et al., 2010).

Figure 3B evidences the importance of reporting lignin content based on the whole biomass by comparing such values with those for extractive-free biomass. For the total lignin, the difference in values between the extractive-free biomass and whole biomass was 0.6%, 3.4% and 2.4% for eucalyptus, bagasse and straw, respectively. For

bagasse and straw, the difference between the values was quite significant, which confirms the need for the value corrections.

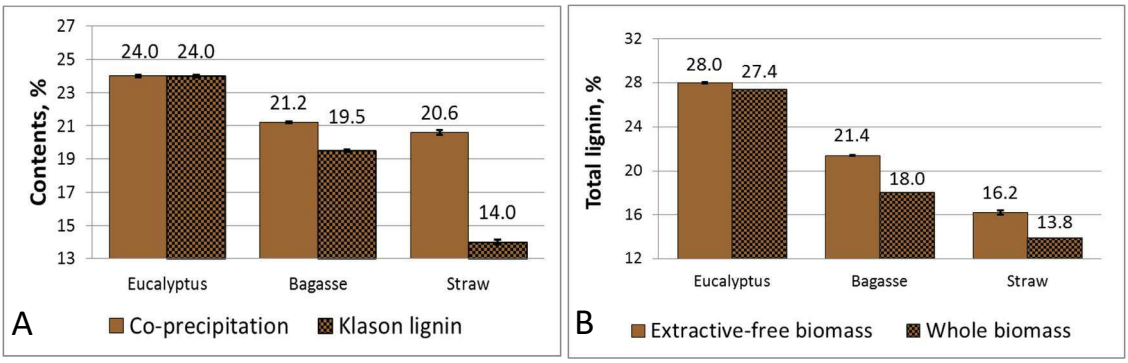

Figure 3 - The Klason lignin containing the silica ("co-precipitation") and Klason lignin content (silica content was deducted) (**A**) and the difference between the content of total lignin (**B**) determined considering extractive-free biomass and whole biomass.

3.1.3 Content and chemical structure of lignin

Lignin is considered to be the main contributor to biomass recalcitrance. In general, lower lignin content is associated with higher cellulose accessibility and digestibility. Lignin composition and chemical structure, as well as lignin-carbohydrate complexes (LCC) linkages, also play a role in the enzymatic hydrolysis of cellulose.

Lignin is a polyphenolic polymer corresponding to approximately 15–30% of plant biomass. It is formed by phenyl propane rings with different degrees of hydroxylation and methylation. Lignin from hardwood is formed by guaiacyl (G) and syringyl (S) units; in grasses, lignin also contains p-hydroxyphenyl (H) unit, in addition to the G and S units (Brandt et al., 2013). The lignin macromolecule is connected primarily *via* carbon-carbon (C-C) and carbon-oxygen (C-O) bonds, with the aryl-ether bond (β-*O*-4) being the most important inter-unit linkage when it comes to the behavior during the chemical pretreatment. Syringyl lignin in hardwoods contains methoxy groups in C3 and C5 positions, which prevents condensation reactions during chemical processing and, as result, this type of lignin is more susceptible than others to degradation and removal during the pretreatments used for subsequent ethanol production (Santos et al., 2011).

Eucalyptus used in the present work contains a larger amount of lignin than bagasse and straw. However, the S/G value is an important parameter to consider when evaluating the degradation of lignin. As shown in Fig. 4, the S/G content for the

eucalyptus lignin was much higher than that for bagasse and straw lignin, suggesting a higher reactivity during the chemical pretreatments.

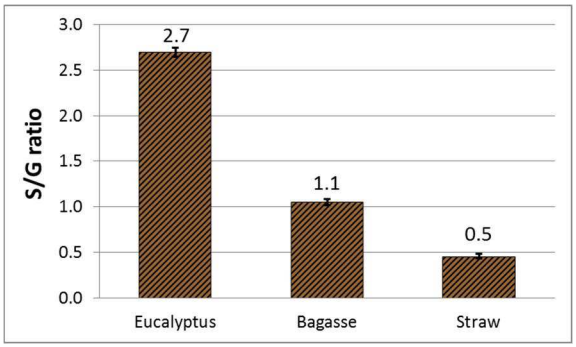

Figure 4 - Lignin S/G ratio for eucalyptus, sugarcane bagasse and sugarcane straw.

3.1.4 Anhydrosugar content

For ethanol production, the higher content of C6-sugars (glucose, galactose and mannose) in the material is considered to be beneficial for ethanol yield. The accurate estimation of the sugar content is very important for the evaluation of the biomass raw material. Considering, for example, the glucose content, the discrepancy between the values obtained when comparing the extractive-free biomass and the whole biomass was 0.5%, 5.8% and 5.1% for eucalyptus, bagasse and straw, respectively (Fig. 5).

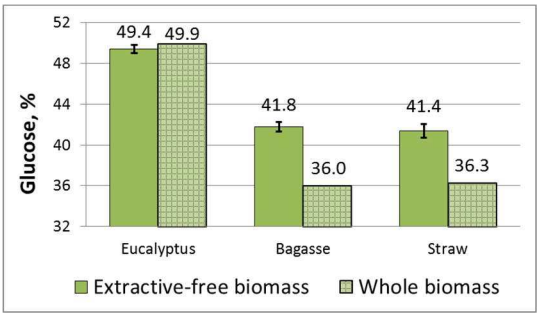

Figure 5 - The difference between the content of glucose determined considering extractive-free biomass and whole biomass.

Xylan is the main hemicellulose for hardwood such as eucalyptus (Shatalov et al., 1999) and for grasses such as bagasse (Brienzo et al., 2009) and straw. Studies show that hemicelluloses contribute to biomass recalcitrance. Also, hemicelluloses and lignin due the cellulose fibrils covering decrease their accessibility by enzymes (Pu et al., 2013). By removing high percentage of hemicellulose the cellulose digestibility improves and it has been showed in several studies (Kumar et al., 2012; Ishizawa et al.,

2007). Also, the high content of acetyl groups in xylan chains increases lignocellulosic recalcitrance (Chen et al., 2012). Acetyl groups and uronic acid are released during pretreatments, such as hydrothermal and dilute acid, and catalyze the hydrolysis of hemicelluloses and oligosaccharides by generation of hydronium ion in the medium, in addition to increase the hydrolytic activity of this pretreatment (Parajó et al., 2004).

The pentose content (xylose and arabinose) in the sugarcane bagasse (23.4%) and straw (26.2%) was higher than in the eucalyptus (12.4%). Based on the xylose content, the xylan content in sugarcane bagasse and straw was almost twice as high as that in eucalyptus, with a significantly lower degree of substitution for uronic acids and acetyl groups (Table 1). The different content of uronic acids and acetyl groups in the biomasses reflects the differences in the chemical structure of the xylan of eucalyptus, bagasse and straw, and may affect the response of these materials during hydrothermal pretreatment. In other study, xylose content of 23.8% has been found (Alves et al., 2010) in sugarcane bagasse, which represents more than 10% higher content than that reported for the eucalyptus wood (Zanuncio et al., 2013).

3.2. Impact of the pretreatments on the chemical composition of biomasses

The impact of the hydrothermal, dilute acid and alkaline pretreatments on the chemical composition of the biomasses was analyzed and reported in the form of complete mass balance, as shown in Table 2.

Table 2 - Chemical composition (lignin, anhydrosugar, ashes and extractives/pseudo-extractives) of the pretreated biomasses reported based on the complete mass balance.

Biomasses	Klason lignin %	Soluble lignin%	Glucose %	Xylose %	Galactose %	Mannose %	Arabinose %	Ash %	Extractives[b] %
					Hydrothermal[a]				
Eucalyptus	22.12	3.06	54.02	6.87	0.48	0.87	-	0.17	12.40
Bagasse	24.37	1.10	42.60	24.30	0.19	0.19	0.68	0.98	5.59
Straw	21.17	0.95	40.37	25.45	0.32	0.62	2.07	5.24	3.81
					Acid[a] (4.5% w/w H_2SO_4)				
Eucalyptus	21.85	1.04	55.76	0.51	0.74	0.08	-	1.12	18.90
Bagasse	25.30	0.40	58.74	0.31	0.03	0.56	0.10	1.54	13.02
Straw	27.33	0.45	52.40	2.80	0.02	-	0.11	5.43	11.46
					Alkaline[a] (15% w/w NaOH)				
Eucalyptus	14.67	2.76	58.59	9.67	0.37	0.09	0.09	5.05	8.70
Bagasse	3.46	0.13	62.92	28.48	0.12	0.42	1.79	1.17	1.50
Straw	0.18	0.09	62.53	24.20	0.16	0.19	2.29	4.53	5.82

[a] Calculated from average of chemical components.

[b] Structures which were removed by extraction with toluene, ethanol and hot water according to TAPPI T 264 cm-07 method (see Experimental).

The pretreatments resulted in significant losses of the materials. As a result, the correct evaluation of the impact of each pretreatment on the chemical composition of biomasses requires to take into consideration the actual yields. The amount of the chemical components (ashes, extractives, total lignin, glucose and other sugars), in grams, in the biomasses before and after the pretreatments, was evaluated using the novel approach (see Section 2.4) and accounting for actual yields of pretreated samples (Fig. 6).

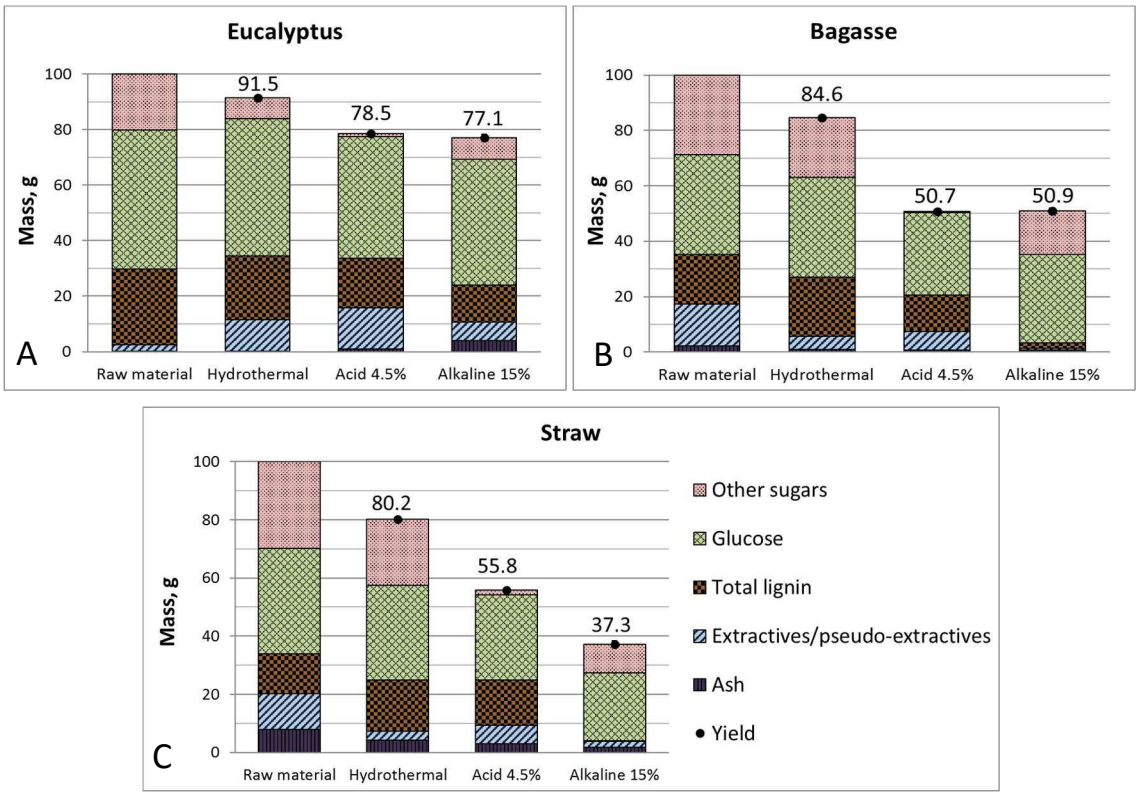

Figure 6 - Chemical composition of eucalyptus wood (**A**), bagasse (**B**) and straw (**C**) before and after the pretreatments (obtained by the combination of mass balance and actual yield of each biomass).

Crystallinity and degree of polymerization of cellulose are considered to be major parameters responsible for biomass recalcitrance and, thus, for affecting cellulose hydrozability by enzymes. Cellulose crystallinity indexes before and after hydrothermal, dilute acid and alkaline pretreatments for each biomass are shown in Table 3 and demonstrates the increase in the crystallinity index for all biomasses irrespective of the type of pretreatment. This can be related to the experimental conditions for the pretreatments. The increase in crystallinity is primarily associated with removal of cellulose from amorphous regions in fibers. Yu and Wu (2010), for

example, observed that the minimum temperature required to rupture the glycosidic bonds in chain segments within the crystalline portion of cellulose during the hydrothermal pretreatment was 180°C. Due to temperature of 175°C for all pretreatments in present work, it is likely that most cellulose in amorphous regions was removed. Also glucans removal helps to explain the crystallinity index increasing in alkaline treatments (Lisboa et al., 2007). However, the crystallinity index alone cannot adequately explain the differences in hydrozability of cellulose, and other parameters, including lignin/hemicellulose contents and distribution, should be taken into consideration.

Table 3 - Crystallinity index of raw material and pretreated biomasses.

Crystallinity index,%	Eucalyptus	Bagasse	Straw
Raw material	72.2 (0.2)	69.8 (0.8)	70.6 (0.4)
Hydrothermal pretreatment	76.0 (0.2)	71.7 (0.3)	77.9 (0.3)
Acid pretreatment	86.3 (1.8)	80.5 (0.6)	78.7 (0.3)
Alkaline pretreatment	85.2 (0.4)	80.9 (0.7)	84.6 (0.4)

(…) Standard deviation.

3.2.1 Hydrothermal and dilute acid pretreatments

The main effect of hydrothermal and dilute acid pretreatment methods is the increase of cellulose accessibility through the removal of hemicellulose. Some amount of lignin can also be removed (Alvira et al., 2010) or converted into condensed lignin structures (Lora and Wayman, 1978). During these pretreatments, the hydronium ions released by the acid or water promote the depolymerization of hemicellulose. Also, acetyl and uronic acid groups are released, which catalyzes the hydrolysis of hemicelluloses and oligosaccharides, especially in hydrothermal pretreatment (Vegas et al., 2008; Garrote et al., 2007). Compared to dilute acid, hydrothermal pretreatment has milder acid conditions as hydrolysis is catalyzed by organic acids released from biomass xylan. This means that, from a chemical point of view, the types of reactions of biomass components that occur during these pretreatments are similar, although they proceed to a lesser extent during the hydrothermal pretreatment. After the hydrothermal pretreatment of eucalyptus, bagasse and straw in the present work, the final pH values were 3.4, 4.5 and 5.3, respectively, which was close to the recommended pH interval of 4-7 (Hendriks and Zeeman, 2009). These values did not correlate with amounts of acetyl groups alone, but showed a good consistency with the total amount of acid moieties, i.e., acetyl and uronic acid groups (Table 1). Eucalyptus had 5.8% of acid

groups and bagasse and straw had 3.8% and 2.5%, respectively, considering the whole biomass (Table 1). After the acid treatment, the final pH values were 1.5, 1.2 and 1.5 for eucalyptus, bagasse and straw, respectively.

A yield loss took place during the hydrothermal and dilute acid pretreatments: 9.5-21.5% for eucalyptus, 15.4-49.3% for bagasse and 19.8-44.2% for straw, respectively, depending on the process and, as the result, on the acidity level.

Generally, the increased severity of the pretreatments (i.e., temperature, residence time and acidity) has an impact on the amount of xylan removed from the material. By increasing the severity the possibility of furfural (fermentation inhibitor) generation from xylan also increases. In addition, the degradation of hemicelluloses during dilute acid pretreatment can contribute to the formation of pseudo-lignin, which can damage much more the enzymatic hydrolysis that the remaining lignin (Hu et al., 2012a; Hu et al., 2012b; Sannigrahi et al., 2011).

In addition to the expected xylan removal during the hydrothermal and dilute acid treatments (Table 2), another two very important observations were made during this study. Firstly, the formation of significant amount of pseudo-extractives, structures, similar in their behavior to extractives (i.e., soluble in the solvents that are used during the standard TAPPI T 264 cm-07 method), was observed for the eucalyptus. The values were 12.4% and 18.9% after hydrothermal and dilute acid pretreatments, respectively, in comparison to the original 2.3% extractives content in eucalyptus. The quantities increasing (in gram) can be observed also in the Figure 6A. For the bagasse and straw, the contents of pseudo-extractives after acid pretreatment were lower than that for the original materials. However, observing the increase in values from hydrothermal to dilute acid pretreatments (Fig. 6B and C), it is reasonable to conclude that the origin of pseudo-extractives is similar to those from eucalyptus. Most likely, these structures are fragments of lignin and polysaccharides with lower molecular weights that re-precipitated on fibers, but which could be removed by extraction with organic solvents.

Secondly, a significant increase in the Klason lignin values for bagasse and straw was observed as a result of the acid pretreatments (Fig. 6B and C), probably due to the formation of pseudo-lignin on xylan degradation and re-condensation, as well as due to the condensation reaction within the lignin molecule itself on low acidity (Lora and Wayman, 1978). Lignin in bagasse and straw had a significantly lower S/G ratio than lignin in eucalyptus, which means that a larger number of guaiacyl units (which more readily participate in the formation of C-C linkages,which are resistant towards acids and bases) was present in these materials. For eucalyptus, no increasing in the

Klason lignin values when comparing with the raw material was verified as a result of hydrothermal and dilute acid pretreatments. For the bagasse, and especially for the straw, however, the increasing in the Klason lignin values comparing with the raw material were in the range of 49-54% and 76-128%, respectively (based on the whole biomass). These numbers confirm previous results for hardwoods (Kumar et al., 2013; Studer et al., 2011). Giving the inhibiting effect of pseudo-lignin on cellulose enzymatic hydrolysis (Hu et al., 2012b), more mild pretreatment conditions for these two types of biomasses are recommended to suppress this process.

Taking into consideration a higher cost for the dilute acid process equipment and the need for acid neutralization (considering the subsequent enzymatic hydrolysis) and more complicate liquor recovery after pretreatment, hydrothermal pretreatment seems to be a more feasible pretreatment alternative for the sugarcane residues.

3.2.3 Alkaline pretreatment (NaOH)

Alkaline pretreatment increases the cellulose accessibility by removing lignin and increasing the surface area of cellulose fibres. In addition, alkaline pretreatment removes some acetyl and uronic acid groups, which have a negative effect on enzymatic hydrolysis, from hemicelluloses (Alvira et al., 2010). A swelling of cellulose fibers under the alkaline conditions may also occur (Cardona et al.,2010).

In the present work, the pH values after the alkaline treatment were 14.0, 12.7 and 12.4 for eucalyptus, sugarcane bagasse and straw, respectively, which indicates the need for the neutralization step prior to the subsequent saccharification and fermentation. The yield after the alkaline pretreatment was very different for the various biomasses. For the eucalyptus, a higher yield was observed (77.1%), and the main components removed were lignin and hemicelluloses (Fig. 6A). The bagasse had a 50.9% yield, with a more significant mass loss due to the lignin removal. The yield for the straw was the lowest among the biomasses studied and only 37.3% of material was recovered left after the pretreatment.

There is an obvious difference in the impact of alkaline treatment on the delignification of eucalyptus wood and in the sugarcane residues. For eucalyptus, approximately 40% of lignin was removed together with approximately 20% of xylan. The formation of pseudo-extractives during the alkaline treatment of eucalyptus also occurred, but in 1.4-2.2 times fewer quantities as compared to hydrothermal and acid treatments (Table 2). Higher content of ash in pretreated eucalyptus was probably due to

the residual amount of chemicals in the material. For bagasse, 80% of lignin was removed, while in straw only traces of lignin were left after the pretreatment. Xylan content in bagasse and straw, on the other hand, seemed to be less affected during the alkaline treatment. Similar results for the more efficient delignification of agricultural wastes by alkaline treatment were reported previously (Taherzadeh and Karimi, 2008), which can be assigned to at least two structural characteristics of grass lignin. The first aspect is the higher amount of free phenolic hydroxyl groups in grass lignin compared to wood lignin that, when ionized in alkaline conditions, contribute to improve the lignin solubility (Granata and Argyropoulos, 1995; Nimz et al., 1981; Grabber et al., 2004). And the second aspect it the presence of significant amount of ester bonds between the hemicelluloses and hydroxycinnamic acid residues (namely *p*-coumaric acid and ferulic acid) in grasses cell wall. The delignification of grasses during alkaline conditions is most likely favoured by the hydrolysis of ferulate esters, which contributes to decrease the cross-link between lignin and arabinoxylans (Grabber et al., 2004; Hammel, 1997). The quantities of extractives were significantly reduced, especially in the case of bagasse. Ash content was reduced by approximately 50% for bagasse and straw.

4. Conclusions

- Novel approach based on the complete mass balance (where the contents of extractives and silica are taken into account) was suggested as an appropriate way to compare various biomasses and to assess the impact of chemical pretreatments;
- Sugarcane bagasse and straw contain lower amounts of lignin (18.0% and 13.9%, respectively) than previously reported for these raw materials, much lower amount of lignin than that found in eucalyptus (27.4%). The lignin S/G ratio for bagasse and straw lignin (1.1 and 0.5, respectively) was lower than that for eucalyptus lignin (2.7). The xylan content in bagasse and straw was much higher than that in eucalyptus, with a lower degree of substitution for uronic acids and acetyl groups;
- Along with the removal of xylan during the hydrothermal and dilute acid pretreatments, the formation of significant quantities of pseudo-lignin was observed for the sugarcane bagasse and straw. In eucalyptus, the formation of pseudo-extractives (structures, soluble in neutral organic solvents) was observed during all three pretreatments, with a content of 12.4% for hydrothermal,18.9% for dilute acid and 8.7% for alkaline pretreatment; and

- The eucalyptus wood demonstrated a lowest mass loss during the investigated pretreatments. The sugarcane straw showed the highest mass loss, especially under alkaline conditions, with a total biomass yield of only 37.3%.

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Article II

Comparative characterization of acetylated heteroxylan from eucalyptus, sugarcane bagasse and sugarcane straw

ABSTRACT - This study compared the chemical and structural properties of heteroxylan isolated from sugarcane bagasse, sugarcane straw and eucalyptus (*Eucalyptus urophylla* × *Eucalyptus grandis*). The xylan samples were isolated by extraction from peracetic acid (PAA) or sodium chlorite ($NaClO_2$) delignified holocellulose with dimethyl sulfoxide (DMSO). It was observed that the xylan samples from the sugarcane bagasse and straw differed slightly from each other in their chemical and structural composition and substantially when compared with eucalyptus xylan. Consequently, different properties and behavior can be expected during chemical processing. The PAA as a delignification method was more efficient than $NaClO_2$ for xylan extraction for all biomasses, resulting in more pure xylans and higher extraction yields in the subsequent treatment with DMSO. The structure of the isolated acetylated xylans was confirmed by FTIR (sharp band at 1730 cm^{-1}) and ^1H NMR (typical signal of acetyl group at 2.08 ppm) analysis. The O-acetyl-4-O-methylglucuronoxylan was identified in the eucalyptus, with a molar ratio of xylose units to branches of 4-O-methylglucuronic acid of 10:1.1 and a degree of acetylation of 0.39. All 4-O-methylglucuronic acid units occurred in the acetylated xylose. The molecular weight and polydispersity index of the eucalyptus xylan were 42 kDa and 1.4, respectively. The xylan in the bagasse and straw was an arabinoxylan type and had a much lower degree of substitution than the xylan extracted from the eucalyptus. The molar ratio of xylose units to arabinosyl substitutions in the xylan from bagasse and straw was 10:0.5 and 10:0.6, respectively. In addition, the xylans from bagasse and straw had a lower degree of acetylation (0.29 and 0.08, respectively). In the xylans from bagasse and straw, arabinosyl substitutions occurred more frequently at position O-3 (Xyl-3Ara) than at position O-2 (Xyl-2Ara). The xylans from bagasse and straw had a molecular weight of 38 kDa and 30 kDa, respectively, and a polydispersity index of 1.5 and 1.7, respectively.

Keywords: ^1H NMR spectroscopy, 4-O-methylglucuronic xylan, acetylated xylan, arabinoxylan, linkage analysis, sugarcane.

RESUMO - Este estudo comparou as propriedades químicas e estruturais de heteroxilanas isoladas de bagaço de cana-de-açúcar, palha de cana-de-açúcar e eucalipto (*Eucalyptus urophylla* × *Eucalyptus grandis*). As amostras de xilanas foram isoladas por extração com dimetilsulfóxido (DMSO) da holocelulose preparada pela deslignificação da biomassa bruta com ácido peracético (PAA) ou clorito de sódio ($NaClO_2$). Foi observado que a composição química e estrutural das xilanas de bagaço e palha de cana-de-açúcar diferiram ligeiramente entre si mas significativamente quando comparadas às xilanas de eucalipto. Consequentemente, diferentes propriedades e comportamentos para essas hemicelulose são esperados durante processos químicos. Para todas as biomassas, o método de deslignficação com PAA mostrou-se mais efetivo

que o $NaClO_2$, resultando em xilanas mais puras e com maiores rendimentos de extração no tratamento subsequente com DMSO. A estrutura das xilanas acetiladas isoladas foi confirmada pelas análises de IVTF (banda aguda a 1730 cm^{-1}) e de RMN de hidrogênio (sinal típico de grupo acetila a 2.08 ppm). A O-acetil-(4-O-metilglicurono)xilana foi identificada no eucalipto com razão molar de unidades de xilose para ramificações de ácido 4-O-metilglicurônico de 10:1,1 e grau de acetilação de 0,39. Todos os ácidos 4-O-metilglicurônicos ocorreram em xiloses acetiladas. A massa molecular e o índice de polidispersividade das xilanas de eucalipto foram 42 kDa e 1,4, respectivamente. A xilana no bagaço e palha foi do tipo arabinoxilana e apresentou consideravelmente menor grau de substituição que as xilanas extraídas do eucalipto. A razão molar das unidades de xilose para substituições de arabinose nas xilanas de bagaço e palha foi 10:0,5 e 10:0,6, respectivamente. Além disso, as xilanas do bagaço e da palha apresentaram menor grau de acetilação (0,29 e 0,08, respectivamente) em relação à do eucalipto. Nas xilanas de bagaço e palha, as substituições de arabinose ocorreram mais frequentemente na posição O-3 (Xil-3Ara) que na posição O-2 (Xil-2Ara). As xilanas de bagaço e palha apresentaram massa molecular de 38 kDa e 30 kDa, respectivamente e índice de polidispersividade de 1,5 e 1,7, respectivamente.

Palavras-chave: Espectroscopia H^1-RMN, 4-O-metilglicuronoxilana, xilanas acetiladas, arabinoxilana, análise de ligações, cana-de-açúcar.

1. Introduction

In recent years, renewable resources have been investigated intensively in connection with the manufacturing of advanced polymeric materials (Alekhina et al., 2014; Egües et al., 2014; Fall et al., 2014; Zhang et al., 2013; Zhang et al., 2012; Shaikh et al., 2009) and their bioconversion into platform chemicals and biofuels (Baeyens et al., 2015; Cai et al., 2014; Vincent et al., 2014; Cardoso et al, 2013; Oliveira et al., 2013; Souza et al., 2012; Cardona et al., 2010; Santos et al., 2010). Non-traditional lignocellulosic feedstock has attracted a growing interest for these applications and a number of researchers have performed in-depth studies of the chemical composition of these new potential feedstocks (Rowley et al., 2013; Bian et al., 2010; Sun et al., 2004). However, current knowledge regarding the chemical and structural properties of the macromolecular components from non-traditional biomasses is not sufficient to guide the new class of chemical transformation industries.

Lignocellulosic biomass feedstocks are an important source of chemical components, such as cellulose, lignin and hemicelluloses, which can be used for a variety of purposes in the chemical conversion industry. Hemicelluloses, in particular, can account for up to 50% of the chemical composition in annual and perennial plants,

and have a promising future in the chemical conversion industries as a versatile and more environmentally friendly alternative for the production of advanced materials, fuels and chemicals, and also foodstuffs (Ebringerová et al., 2005).

The hemicelluloses differ in chemical composition and structural pattern. In general, hemicelluloses are heteropolysaccharides from the plant cell wall formed by different sugars: D-glucose, D-xylose, L-arabinose, D-galactose, D-mannose, D-glucuronic acid, 4-O-methy-D-glucuronic acid and L-fucose. In addition to various sugars, acetyl groups are usually observed in hemicelluloses (Bian et al., 2010). Xylans are one of the most abundant hemicelluloses found in vegetal plants, including grasses and hardwood plants. The backbone in xylan is formed by β-(1→4)-D-xylopyranose units with possible branches in positions C-2 and/or C-3 and with certain number of acetyl groups. The main side groups in the xylan backbone are arabinofuranose (Ara*f*), typical for grass plants, and 4-O-methylglucuronic acid (4-O-MeGlcA), typical for hardwood. Other substitutions in xylan can also occur, but they are less abundant. (Magaton et al., 2008; Ebringerová et al., 2005; Evtuguin et al., 2003).

The amount of xylan, as well as its chemical composition, differs between the various plant biomasses. Our previous work showed that the amount of xylan found in sugarcane bagasse and sugarcane straw is at least twice the amount found in eucalyptus, although with a lower substitution for uronic acids and acetyl groups (Carvalho et al., 2015). (Arabino)glucuronoxylan (AGX) and arabinoxylan (AX) are typical for grasses, including sugarcane, in which branches of arabinose, 4-O-methylglucuronic acid and acetyl groups in the backbone of xylose are observed (Ebringerová et al., 2005). Also acetylated, the xylan from eucalyptus contains substitutions of 4-O-MeGlcA (Magaton et al., 2008; Ebringerová et al., 2005; Evtuguin et al., 2003). In addition, residues of glucan and rhamnoarabinogalactan have been described to be chemically linked to the 4-O-MeGlcA (Evtuguin et al., 2003).

Sugarcane bagasse and sugarcane straw, which are residues from the sugarcane industry, are generated in large amounts mainly in tropical countries, including Brazil, and thus might be an abundant potential source of xylan for the chemical conversion industries (Carvalho et al., 2015; Conab, 2015; Oliveira et al., 2013). In Brazil, eucalyptus is the most widely cultivated wood gender and is used by forest-based industries for a number of purposes. The xylan characteristics in sugarcane bagasse and straw differ from that present in eucalyptus. Certainly, a more in-depth evaluation of the chemical and structural features of xylans from sugarcane bagasse and straw may open new possibilities for better utilization of these agricultural residues and their xylan. To

the best of our knowledge, little information on the structural patterns of sugarcane bagasse and straw xylans is known.

In the present work, acetylated heteroxylan was isolated from sugarcane bagasse and sugarcane straw, as well as from eucalyptus, by dimethyl sulfoxide (DMSO) extraction of peracetic acid or sodium chlorite delignified holocelluloses. The isolated hemicelluloses were throughly characterized by their monosaccharide composition, methylation linkage analysis, size-exclusion chromatography (SEC), Fourier transform infrared spectrometry (FTIR) and ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR). Few studies have been done regarding the structure and use of xylan from bagasse and straw, and we believe that the reason for this is the insufficient knowledge about their chemical and structural characteristics. Information on the structure of xylan in these lignocellulosic biomasses will provide a better understanding of their behavior during chemical treatments and also create new possibilities for uses of xylan biopolymer, such as in novel materials.

2. Experimental

2.1 Materials

A representative 7-year old clonal hybrid of eucalyptus (*Eucalyptus urophylla* × *Eucalyptus grandis*) was supplied by a Brazilian pulp company. Five-months old sugarcane (cultivar RB867515) bagasse (stalks after fragmentation and pressing) and straw (leaves and tips) were supplied by Center of Sugarcane Experimentation (Oratórios, Minas Gerais State, Brazil). The eucalyptus, sugarcane bagasse and sugarcane straw were converted to sawdust (< 35 mesh) by using a Wiley mill bench model.

The chemicals used were ethanol 96% (VWR, France), toluene 99.8% (Sigma Aldrich, USA), ethylenediamine tetracetic acid (EDTA) 99% (Sigma Aldrich, USA), peracetic acid (PAA) 39% (Sigma Aldrich, USA), sodium hydroxide (NaOH) pellets analytical grade (Merck Milipore, Germany), acetone 99.5% (VWR, France), acetic acid 100% (VWR, France), sodium acetate 99% (Merck, USA), sodium chlorite ($NaClO_2$) 80% (Alfa Aesar, Germany), dimethyl sulfoxide (DMSO) 99% (VWR, France), formic acid 98/100% (VWR, England), and methanol HPLC grade (Fisher Chemicals, UK).

2.2 Methods

Figure 1 depicts the working plan for the present study. Eucalyptus, sugarcane bagasse and straw were converted to sawdust (< 35 mesh) and then used for xylan extraction. Hollocelluloses were prepared from the extractives-free sawdust by both delignification methods; $NaClO_2$ and PAA. Xylan was extracted from the hollocelluloses by DMSO, precipitated by ethanol, centrifuged, purified and dried. Acetylated heteroxylan was analyzed using the following procedures: sugar analysis, linkage analysis, size-exclusion chromatography (SEC), ^1H nuclear magnetic resonance spectroscopy (^1H NMR) and Fourier transform infrared spectrometry (FTIR).

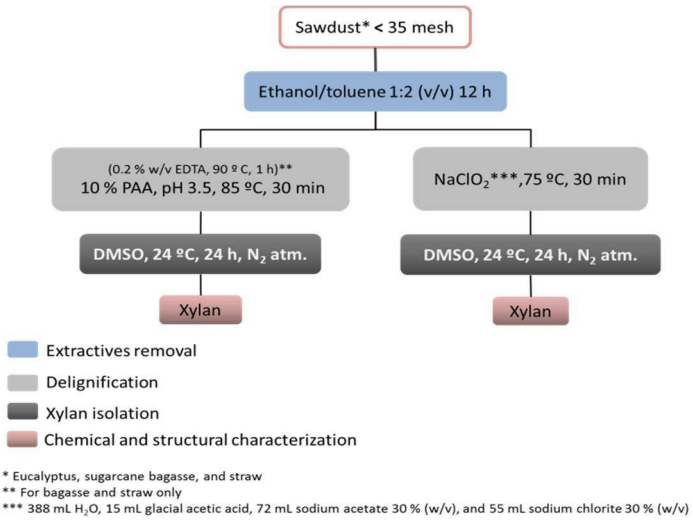

Figure 1 - Working plan for delignification, xylan isolation and chemical and structural characterization of xylan.

2.2.1 Extractives removal

Biomass sawdust (< 35 mesh) was extracted with ethanol/toluene 1:2 (v/v) for 12 hours in a Soxhlex extractor (Sun et al., 2004; Shatalov et al., 1999). Extractives-free sawdust was air-dried and stored in airtight plastic bags at room temperature prior to use. The moisture of the extractives-free sawdust was determined according to TAPPI T 264 cm-07.

2.2.2 Delignification

2.2.2.1 PAA delignification

Prior to delignification with PAA, the extractives-free bagasse and straw sawdust were treated with 0.2% (w/v) EDTA at 90ºC for 1 hour, with constant stirring (Brienzo et al., 2009), in order to remove the metal ions and prevent decomposition of the PAA. The delignification of bagasse, straw and eucalyptus (without previous EDTA treatment) was performed with 10 g of extractives-free sawdust treated with 500 mL of 10% peracetic acid (PAA) at pH 3.5 (adjusted with sodium hydroxide solution), at 85ºC for 30 minutes, with constant stirring. After the treatment, the solution was cooled in an ice bath and diluted twice with water. The holocellulose was collected on a porous glass filter P2 (porosity 100), washed with 5 L of warm distilled water and, soon thereafter, with 50 mL of acetone/ethanol 1:1 (v/v) (adapted from Evtuguin et al., 2003). The holocellulose was dried at room temperature (24ºC) and stored in airtight containers.

2.2.2.2 Sodium chlorite delignification

10 g of extractives-free sawdust was treated with 388 mL of water, 15 mL of acetic acid 100%, 72 mL of sodium acetate 30% (w/v), and 55 mL of sodium chlorite 30% (w/v), at 75ºC for 30 minutes, with constant stirring. After the treatment, the holocellulose was collected on a polystyrene membrane (porosity 60 μm), washed with 5 L of distilled water and, soon thereafter, with 100 mL of acetone (adapted from Magaton, 2008). The holocellulose was dried at room temperature (24ºC) and stored in airtight containers.

2.2.3 Isolation of xylans

A sample of 6 g of holocellulose (PAA-holocellulose or $NaClO_2$-holocellulose) was treated with 130 mL of DMSO, at 24ºC for 24 hours, under nitrogen atmosphere and with constant stirring (adapted from Hägglund et al., 1956). After the treatment, the suspension was filtered through a polystyrene membrane (porosity 60 μm) and washed with ~20 mL of distilled water. The supernatant liquid was added to 600 mL of ethanol at pH 3.5 (adjusted with formic acid) (adapted from Magaton et al., 2008) and left for 12 hours at 4ºC. The precipitated hemicelluloses were isolated by centrifugation (10 min at 45000 rpm) and washed 5 times with methanol (adapted from Evtuguin et al., 2003). The xylans were dried in a vacuum oven for (5 h at 50ºC).

2.3 Analyses

2.3.1 Delignification and isolation yields

The total yield was estimated gravimetrically based on the amount of starting material (extractives-free biomasses). The xylan yields after DMSO isolation was calculated taking into consideration the amount of xylose in the extractives-free and xylan samples, combined with the total yield.

2.3.2 Monosaccharide composition analyses

The monosaccharides composition, including glucose from crystalline cellulose, was determined by sulfuric hydrolysis. Samples (4 mg) were kept in a glass tube with 0.25 mL of 72% sulfuric acid for 3 hours at room temperature. Then, deionized water was added to dilute the solution to approx. 1.2-1.3 mol L^{-1} sulfuric acid and the tubes were incubated at 100ºC for 3 hours.

The content of the more labile uronic acids (glucuronic and galacturonic acid) was determined by acidic methanolysis (Appeldoorn et al., 2010; Bertaud et al., 2002). Freeze-dried samples (1 mg) were incubated with 1 mL of 2 mol L^{-1} HCl in dry methanol for 5 hours at 100°C. Subsequently, the samples were neutralized with pyridine, dried under a stream of air, and further hydrolyzed with 2 mol L^{-1} TFA at 121°C for 1 hour. The samples were again dried under a stream of air and dissolved in H_2O.

The monosaccharides were analyzed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an ICS-3000 system (Dionex) equipped with a CarboPac PA1 column (4x250 mm, Dionex). Inositol was added to all samples as an internal standard prior to hydrolysis. All experiments were performed in triplicate.

2.3.3 Klason lignin

The Klason lignin in the holocellulose samples was determined gravimetrically as insoluble residue after acid hydrolysis with 72% sulfuric acid, according to the TAPPI 222 om-02 standard method.

2.3.4 Acetyl content and degree of acetylation

The acetyl content of the xylan samples was determined after alkaline hydrolysis with NaOH at 70ºC overnight using high performance liquid chromatography (HPLC) with UV detection (adapted from Voragen et al., 1986). The HPLC instrument used was purchased from Dionex–Thermofisher, CA, USA, equipped with a UV detector (210 nm), Rezex and a ROA-Organic acid column (300 × 7.8 mm; phenomenex, Torrance, CA, USA). Runs were performed at in 2.5 mmol L^{-1} H_2SO_4, at 50ºC, and the flow rate was 0.5 mL min^{-1}. The degree of acetylation (DA) was determined from the acetyl content in the xylan samples according to Eq. 1 (Xu et al., 2010).

$$DA = \frac{132 \times \%\ acetyl}{\left(M_{acetyl} \times 100\right) - \left(M_{acetyl} - 1\right) \times \%\ acetyl} \quad (1)$$

where: DA is the degree of acetylation, % acetyl is the acetyl content determined by analysis, M_{acetyl} is the acetyl molecular weight (43 g mol^{-1}) and 132 g mol^{-1} is the xylose molecular weight.

2.3.5 Glycosidic linkage analysis

Freeze-dried xylan fractions (5 mg) were carboxyl reduced with sodium borodeuteride ($NaBD_4$) after activation with carbodiimide, in order to label the uronic acids present in the fractions, following the protocol adopted by Kim and Carpita (1992). The samples (1 mg, three technical replicates) were further swelled in anhydrous DMSO for 16h at 60°C and methylated in the presence of $NaOH/CH_3I$ (Ciucianu and Kerek, 1984) five times in order to ensure complete methylation. The samples were then hydrolyzed with 2 mol L^{-1} TFA at 121°C for 2 hours, reduced with sodium borohydride ($NaBH_4$) and acetylated with acetic anhydride in pyridine (Albersheim et al., 1967). The obtained permethylated alditol acetates (PMAAs) were separated and analyzed using gas chromatography (HP-6890, Agilent Technologies) coupled to an electron-impact mass spectrometer (HP-5973, Agilent Technologies) on a SP-2380 capillary column (30 m × 0.25 mm i.d.; Sigma–Aldrich) with a temperature range increasing from 160°C to 210°C at a rate of 1°C min^{-1}. The mass spectra of the fragments obtained from the permethylated alditol acetates were compared with those of reference polysaccharide derivatives and to available data (Carpita and Shea, 1989). Eucalyptus samples were reduced before activation. The quantification was based on

the carbohydrate composition and effective carbon response of each compound, as detected by GC-MS.

2.4.6 Size-exclusion chromatography

The apparent molar mass distributions of the xylan extracts from eucalyptus, sugarcane bagasse and straw were analyzed by size exclusion chromatography (SECcurity 1260, Polymer Standard Services, Mainz, Germany) using a refractive index detector. SEC analyses were performed at a flow rate of 0.5 mL min^{-1} using dimethyl sulfoxide (DMSO, HPLC grade, Sigma-Aldrich, Sweden) with 0.5% w/w LiBr (ReagentPlus) as mobile phase, using a column set consisting of a GRAM PreColumn, 100 and 10000 analytical columns (Polymer Standards Services, Mainz, Germany) kept at 60 °C. Prior to analyses, the xylans were dissolved directly in the SEC eluent for 16h at 60 °C. Standard calibration was performed by the injection of pullulan standards of known molar masses provided by Polymer Standards Services (PSS, Mainz, Germany). The apparent molar mass distributions relative to pullulan linear standards were obtained using WinGPC software (Polymer Standards Services, Mainz, Germany).

2.4.7 ^{1}H nuclear magnetic resonance spectroscopy

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra of xylan samples dissolved in D_2O were recorded at room temperature on a Bruker Advance 400 Hz instrument by using the Bruker pulse program. Xylanase treatment prior to ^{1}H analysis was used to improve the dissolution of xylan samples.

2.4.8 Fourier transform infrared spectrometry

Fourier transform infrared (FTIR) spectra (wavelength 4000-600 cm^{-1}) were recorded by a Perkin-Elmer Spectrum 2000 FTIR spectrometer (Waltham, MA, USA) equipped with an attenuated total reflectance (ATR) system (Spectac MKII Golden Gate Creecstone Ridge, GA, USA). The spectra were obtained from dry samples, as 16 scans at a resolution of 4 cm^{-1} and at intervals of 1 cm^{-1} at room temperature. Origin 9.1 software was used for the spectra evaluation.

3. Results and discussion

3.1 Yield and chemical composition of isolated xylan

In our previous work, eucalyptus, sugarcane bagasse and straw were chemically characterized and the results thereof, based on the complete mass balance, are set out in Figure 2 (Carvalho et al., 2015). Bagasse and straw showed to contain larger quantities of hemicelluloses and pectins than eucalyptus, expressed as other sugars (sum of xylose, galactose, mannose, arabinose, uronic acids and acetyl groups).

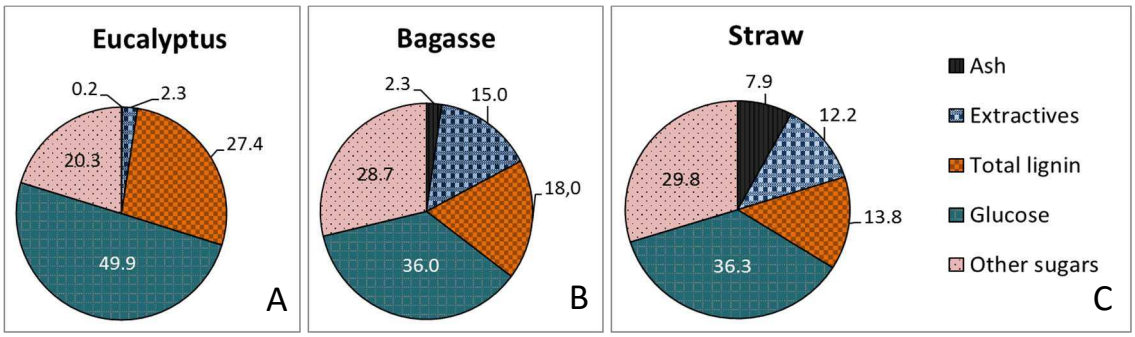

Figure 2 - Chemical composition of eucalyptus wood (**A**), sugarcane bagasse (**B**) and straw (**C**) based on the complete mass balance. Other sugars mean the sum of xylose, galactose, mannose, arabinose, uronic acids and acetyl groups (Carvalho et al., 2015).

In the present work, eucalyptus, bagasse and straw were used for xylan preparation. Two delignification methods were used prior to the extraction of xylan with DMSO: PAA and $NaClO_2$. Using PAA delignification, the loss of dry matter (including lignin, ash and, to a lesser extent, polysaccharides) from the initial amount of extractives-free biomasses was 29.0%, 24.8% and 25.7% for eucalyptus, bagasse and straw, respectively. The loss of dry matter using the $NaClO_2$ delignification process was 15.0%, 15.4% and 27.7% for eucalyptus, bagasse and straw, respectively. Results indicated that the condition for PAA delignification used in this work were more effective for lignin removal than the that used for $NaClO_2$ delignification (Fig. 3).

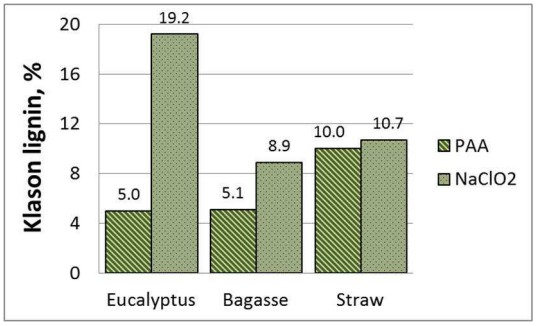

Figure 3 - Klason lignin content in holocellulose from eucalyptus wood, bagasse and straw obtained through PAA and $NaClO_2$ delignification. Klason lignin was not corrected due to silica co-precipitation.

The total yield (i.e., the combination of the delignification and xylan isolation steps) and the yield based on the content of xylose determined by sugar analysis in the extractives-free biomasses and isolated xylans are set out in Table 1. The PAA/DMSO process resulted in a higher total yield of xylan for all biomasses than the $NaClO_2$/DMSO process. Evtuguin et al. (2003) also observed higher xylan yields in eucalyptus using the PAA/DMSO process.

Table 1 - Total yield and xylose yield after xylan isolation step.

Material	Total yield,%		Xylose yield,%	
	PAA/DMSO	$NaClO_2$/DMSO	PAA/DMSO	$NaClO_2$/DMSO
Eucalyptus	4.9	0.2	29.1	0.22
Bagasse	4.3	0.7	10.2	1.1
Straw	5.7	1.1	12.6	1.7

The FTIR spectra obtained for xylan samples from eucalyptus, bagasse and straw presented typical signals for xylan, as shown in Figure 4: a sharp band at 1039 cm^{-1}, which is due to the C-O, C-C stretching or C-OH bending in sugar units (Chaikumpollert et al., 2004) and the expected bands between 1175 and 1000 cm^{-1} (Sun et al., 2004). The β-glycosidic linkages between the xylose units were evidenced by the presence of a sharp band at 897 cm^{-1} (Gupta et al., 1987). The band at 3350-3330 cm^{-1} corresponds to the hydroxyl stretching vibrations of xylans, as well as the water involved in the hydrogen bonding and the band at 2920 cm^{-1} represented C-H stretching vibrations (Sun et al., 2004). The band at 1160 cm^{-1} observed in the spectra indicated the presence of arabinose residues (Egüés et al., 2014). The presence of acetyl groups in the xylan was confirmed by the absorption at 1734 cm^{-1}, which is due to the C=O stretching (Bian et al., 2010). The band at 1241 cm^{-1}, which is due to the C-O stretching, and at 1370 cm^{-1}, which is due to the C-CH_3 stretching, were also detected

(Xu et al., 2010). The absorption at 1628 cm^{-1} assigned principally to the water absorbed by xylans (Kačuráková et al., 1998). The weak band at 1510 cm^{-1}, which is due to the aromatic skeletal vibration, indicated the presence of a small amount of lignin in the xylan samples (Sun et al., 2004). During the isolation methods, the glycosidic linkages can be disrupted and the hydroxyl groups can be oxidized, resulting in bands around 1720 cm^{-1}, which are due to ketone carbonyl stretching (Magaton et al., 2008; Sun and Tomkinson, 2002). The absence of such signals in the spectra of xylans extracted from eucalyptus, bagasse and straw in the present work ruled out oxidation reactions during the delignification and isolation procedures.

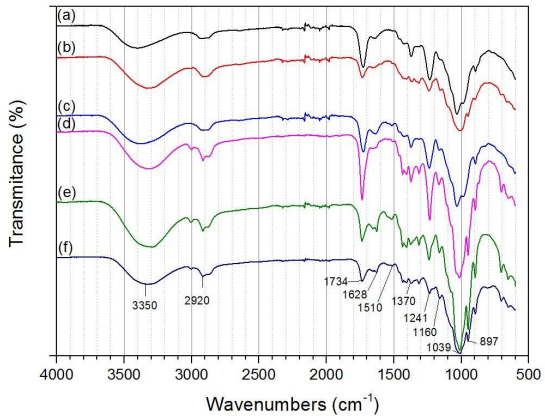

Figure 4 - FTIR spectra of xylan samples of eucalyptus PAA/DMSO (**spectrum a**), eucalyptus $NaClO_2$/DMSO (**spectrum b**), bagasse PAA/DMSO (**spectrum c**), bagasse $NaClO_2$/DMSO (**spectrum d**), straw PAA/DMSO (**spectrum e**) and straw $NaClO_2$/DMSO (**spectrum f**).

The monosaccharide composition of eucalyptus, bagasse and straw holocelluloses and xylans isolated with two different procedures are shown in Figure 5. Xylose was the main hemicellulose component for all three investigated materials, with sugarcane straw having the highest content, followed by bagasse and eucalyptus. In bagasse and straw, the xylose content was 1.5 and 2.2 times higher, respectively, than that in eucalyptus. The ratio between glucose and xylan was quite similar for the extractive-free biomasses and both types of holocelluloses, those extracted by PAA or $NaClO_2$. The composition of the xylan samples isolated from various types of holocelluloses, however, was quite different.

The sugar analysis of the PAA/DMSO samples confirmed the presence of a significant amount of arabinose in the bagasse and straw, which was also seen in the FTIR spectra (Fig. 4). Glucuronic acids (GlcA) were observed in all eucalyptus samples, confirming that the xylan in eucalyptus is the glucuronoxylan (GX). GlcA is

usually methylated in O-4 (4-O-MeGlcA) in eucalyptus xylan (Evtuguin et al., 2003). Heteroxylans in the sugarcane bagasse and straw were an arabinoxylan (AX) type, which is typical for grasses. Glucose, galactose and galacturonic acid were also present in the PAA/DMSO samples, although in much smaller amounts.

A similar sugar composition for xylan from eucalyptus isolated by the PAA/DMSO process was observed by Magaton et al. (2008).

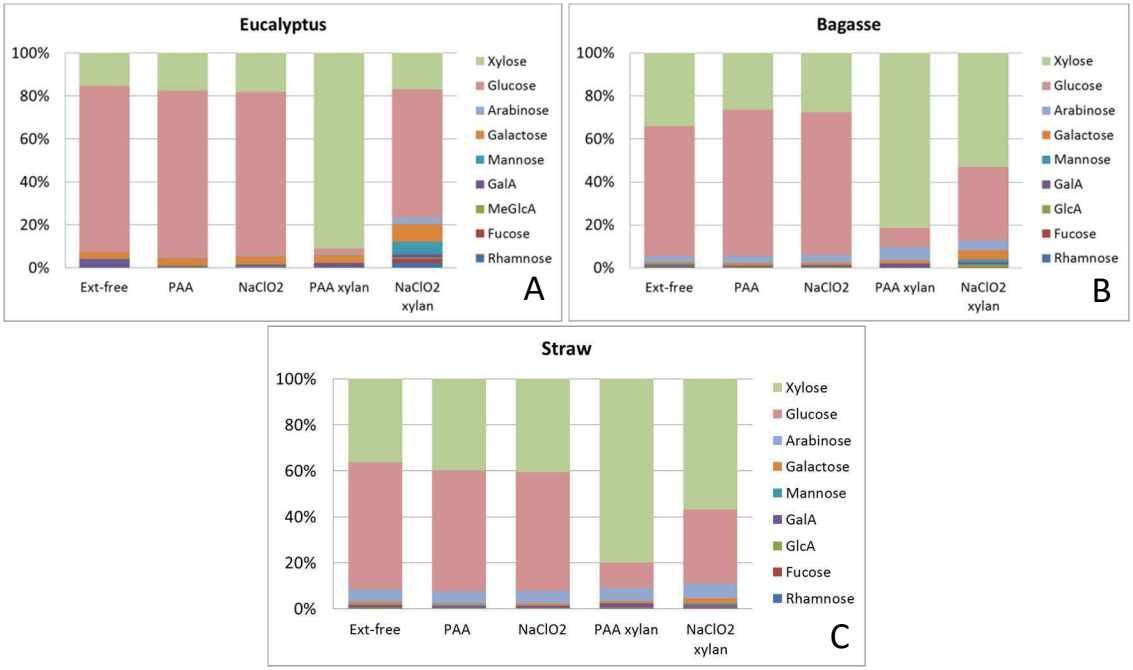

Figure 5 - Sugar composition of eucalyptus wood (**A**), bagasse (**B**) and straw (**C**) for extractive-free biomasses, PAA-holocellulose, $NaClO_2$-holocellulose and xylan isolated by PAA/DMSO and $NaClO_2$/DMSO.

The composition of the $NaClO_2$/DMSO-extracted samples, especially for eucalyptus, together with the very low yields obtained, suggested that the extraction and isolation process was unsatisfactory. High amounts of glucose, and a considerable increase in the quantity of mannose and galactose were found in $NaClO_2$/DMSO-extracted samples, especially for eucalyptus. This further indicated that isolation of non-xylan hemicelluloses, such as mannans, cellulose and other pectic material took place during the $NaClO_2$/DMSO process. Most likely, the accessibility of DMSO in the cell walls was very low due to insufficient delignification, and then, a mixture of polysaccharides was removed only from the fiber surface.

3.2 Acetyl groups content in heteroxylans

Table 2 shows the content of acetyl groups in the xylan samples and the degree of acetylation, calculated according to Xu et al. (2010). In our previous work, approximately 9.1%, 8.9% and 4.7% of acetyl groups were observed in hemicelluloses (including xylose, galactose, mannose, arabinose, uronic acids and acetyl groups) from eucalyptus, bagasse and straw, respectively (Carvalho et al., 2015). Similar quantities of acetyl groups were observed for the PAA/DMSO-extracted xylans. For PAA/DMSO-extracted xylan from eucalyptus, the acetyl group content was lower than that observed by Evtuguin et al. (2003) for *Eucalyptus globulus* (19.6%), which might be due to the use of different eucalyptus species. The degree of acetylation for the eucalyptus xylan isolated by PAA/DMSO was, however, similar to the hardwood xylans isolated from beech and birch (Teleman et al., 2002), but substantially less than the value observed by Magaton et al. (2008) for *Eucalyptus urophylla* × *Eucalyptus grandis* (0.55).

Table 2 - Acetyl content and degree of acetylation (DA) in xylans.

Sample	Acetyl content (%)		DA	
	PAA/DMSO	$NaClO_2$/DMSO	PAA/DMSO	$NaClO_2$/DMSO
Eucalyptus	11.30 (0.17)	3.31 (0.07)	0.39 (0.01)	0.10 (0.00)
Bagasse	8.72 (0.37)	5.39 (0.22)	0.29 (0.01)	0.17 (0.01)
Straw	2.42 (0.45)	1.46 (0.00)	0.08 (0.01)	0.05 (0.00)

(…) Standard deviation.

3.3 Linkage between sugars in xylan

In order to investigate the substitution patterns of the isolated xylan fractions, linkage (methylation) analysis was performed on the PAA/DMSO and $NaClO_2$/DMSO extracts (Table 3). As expected, all of the xylans were essentially composed of a linear backbone of (1 →4)-linked β-D-xylopyranosyl units, partially O-2 substituted with 4-O-methyl-β-D-glucuronosyl (OMeGlcA) units in eucalyptus, and O-3 and/or O-2 substituted with arabinosyl units in sugarcane bagasse and straw.

When the two isolation treatments were compared, it was evident that the chlorite-extracted xylans contained a significant amount of other contaminating polysaccharides, consistent with the sugar composition results. In the bagasse and straw samples, the presence of 5-Ara*f,* 2-Ara*f* , 3-Ara*f,* 2,5-Ara*f,* as well as 3-Gal*p* and other branched Gal*p* units, pointed to presence of arabinan and arabinogalactan, whereas 3-Glc*p* and 4-Glc*p* denoted presence of glucans and cellulose. These differences were more evident in the straw xylans than in the bagasse samples. Analogously, the

eucalyptus samples also showed an abundance of these non-xylan polysaccharides, mainly cellulose and other additional pectins, such as rhamnogalacturanan.

Table 3 - Linkage between sugars in xylan samples isolated from eucalyptus, sugarcane bagasse and sugarcane straw.

Linkage	Structural units deduced	Relative abundance (% mol)					
		Eucalyptus		Bagasse		Straw	
		PAA	$NaClO_2$	PAA	$NaClO_2$	PAA	$NaClO_2$
t-Ara*f*	Ara*f*-(1 →	<0.1	0.8 (0.0)	4.6 (0.1)	3.6 (0.3)	5.3 (0.4)	6.0 (0.7)
2-Ara*f*	→ 2) Ara*f*-(1 →	<0.1	n.d	n.d	0.2 (0.2)	0.5 (0.1)	0.8 (0.0)
3-Ara*p*	→ 3) Ara*p*-(1 →	n.d	n.d	n.d	0.1 (0.0)	<0.1	0.3 (0.1)
5-Ara*f*	→ 5) Ara*f*-(1 →	<0.1	0.4 (0.1)	0.2 (0.0)	0.3 (0.1)	0.5 (0.3)	0.7 (0.3)
2,5-Ara*f*	→ 2,5) Ara*f*-(1 →	<0.1	1.1 (0.6)	n.d	<0.1	<0.1	<0.1
Total Ara[a]		0,2 (0,0)	2.4 (0.8)	4.8 (0.1)	4.1 (0.5)	6.4 (0.9)	7.6 (1.0)
t-Xyl*p*	Xyl*p*-(1 →	1.6 (0.2)	2.0 (0.1)	2.5 (0.2)	2.8 (0.2)	2.5 (0.4)	2.5 (0.5)
2-Xyl*p*	→ 2) Xyl*p*-(1 →	n.d	n.d	n.d	0.2 (0.1)	0.1 (0.1)	0.4 (0.1)
4-Xyl*p*	→ 4)-Xyl*p*-(1 →	74.4 (2.3)	30.9 (3.5)	81.8 (0.7)	66.3 (2.1)	75.3 (2.4)	56.6 (0.3)
2,4-Xyl*p*	→ 2,4)-Xyl*p*-(1 →	9.5 (1.0)	3.2 (0.2)	1.2 (0.1)	1.1 (0.1)	1.0 (0.2)	1.3 (0.0)
3,4-Xyl*p*	→ 3,4)-Xyl*p*-(1 →	0.6 (0.1)	0.8 (0.0)	3.6 (0.1)	4.3 (0.3)	4.2 (0.8)	7.2 (0.7)
2,3,4-Xyl*p*	→ 2,3,4) Xyl*p*-(1 →	0.3 (0.1)	1.2 (0.5)	<0.1	<0.1	<0.1	0.2 (0.0)
Total Xyl[a]		86,4 (3,6)	38.1 (4.3)	89.1 (1.2)	74.9 (2.8)	83.4 (3.9)	68.5 (1.7)
t-Glc*p*	Glc*p*(1 →	n.d	n.d	0.2 (0.0)	0.8 (0.3)	0.2 (0.0)	0.3 (0.0)
3-Glc*p*	→ 3)- Glc*p* A-(1 →	0.3 (0.2)	2.0 (0.5)	2.6 (0.0)	6.7 (0.7)	3.4 (1.2)	5.0 (0.8)
4-Glc*p*	→ 4) Glc*p*-(1 →	0.4 (0.1)	29.4 (7.4)	2.2 (0.0)	9.9 (1.9)	4.2 (0.0)	15.3 (0.6)
4,6-Glc*p*	→ 4,6)-Glc*p*-(1 →	<0.1	2.6 (0.2)	n.d	n.d	n.d	n.d
3,4-Glc*p*	→ 3,4)-Glc*p*-(1 →	n.d	1.6 (0.3)	n.d	n.d	n.d	n.d
Total Glc[a]		0,7 (0,3)	34.6 (9.4)	5.0 (0.1)	17.3 (2.8)	7.9 (1.2)	20.6 (1.5)
4-Man*p*	→ 4) Man*p*-(1 →	0.1 (0.0)	8.4 (0.1)	n.d.	n.d.	n.d.	n.d.
4,6-Man*p*	→ 4,6) Man*p*-(1 →	n.d.	0.4 (0.1)	n.d.	n.d.	n.d.	n.d.
Total Man[a]		0,1 (0,0)	8.8 (0.2)	<0.1	0.3 (0.0)	<0.1	<0.1
t-Gal*p*	Gal*p*-(1 →	1.8 (0.2)	1.3 (0.9)	0.5 (0.2)	1.3 (0.3)	0.8 (0.0)	1.6 (0.1)
6-Gal*p*	→ 6) Gal*p*-(1 →	n.d	n.d	<0.1	0.1 (0.1)	0.2 (0.1)	0.1 (0.0)
3-Gal*p*	→ 3) Gal*p*-(1 →	n.d	n.d	<0.1	0.5 (0.1)	0.3 (0.2)	0.1 (0.0)
3,4-Gal*p*	→ 3,4) Gal*p*-(1 →	n.d	0.6 (0.1)	n.d	n.d	n.d	n.d
2,3-Gal*p*	→ 2,3) Gal*p*-(1 →	n.d	0.8 (0.3)	n.d	n.d	n.d	n.d
2,4-Gal*p*	→ 2,4) Gal*p*-(1 →	n.d	1.3 (0.2)	n.d	n.d	n.d	n.d
3,6-Gal*p*	→ 3,6) Gal*p*-(1 →	0.2 (0.0)	1.6 (0.1)	<0.1	0.2 (0.1)	<0.1	0.1 (0.1)
4,6-Gal*p*	→ 4,6) Gal*p*-(1 →	0.1 (0.0)	2.7 (0.4)	0.1 (0.0)	0.7 (0.3)	0.5 (0.2)	0.6 (0.0)
Total Gal[a]		2.2 (0.3)	8.2 (2.0)	0.9 (0.1)	2.8 (1.0)	1.8 (0.6)	2.6 (0.3)
3-Rha*f*	→ 3) Rha*f*-(1 →	0.6 (0.1)	n.d.	n.d.	n.d.	n.d.	n.d.
2,4-Rha*f*	→ 2.4) Rha*f*-(1 →	n.d.	1.6 (1.1)	n.d.	n.d.	n.d.	n.d.
Total Rha[a]		0.6 (0.1)	1.6 (1.1)	n.d.	<0.1	n.d	<0.1
2-Gal*p*A	→ 2) Gal*p*A-(1 →	1.4 (0.5)	1.1 (0.2)	n.e.	n.e.	n.e.	n.e.
4-Gal*p*A	→ 4) Gal*p*A-(1 →	1.2 (0.2)	2.4 (1.9)	n.e.	n.e.	n.e.	n.e.
Total GalA[a]		2.6 (0.6)	2.9 (2.0)	0.2 (0.0)	0.2 (0.0)	0.5 (0.0)	0.2 (0.0)
t-Glc*p*A	Glc*p*A(1 →	6.3 (1.1)	3.0(0.5)	n.e.	n.e.	n.e.	n.e.
2-Glc*p*A	→ 2) Glc*p*A-(1 →	0.8 (0.1)	0.4 (0.1)	n.e.	n.e.	n.e.	n.e.
Total GlcA[a]		7.2 (0.2)	3.4 (0.6)	<0.1	0.5 (0.0)	<0.1	0.2 (0.0)

(…) Standard deviation. [a]Monosaccharide composition as calculated by acid methanolysis. n.d: not detected. n.e: not evaluated.

When examining the eucalyptus xylans, the most relevant structural features were the degree of glucuronosyl substitution of the xylan backbone, as well as the presence of O-2 substituted 4-O-methyl-β-glucuronic acid (→2)-Glc*p*A-(1→) units, in addition to the terminal MeGlcA (Glc*p*A-(1→). Both PAA/DMSO and $NaClO_2$/DMSO xylans had the same level of glucuronosyl substitution (8-9 OMeGlcA units/100 units of the xylose backbone). Moreover, around 10-12% of the uronic acid moieties in both isolated xylans were O-2 substituted. This indicated that the use of the two different treatments does not result in one structural xylan pattern being favoured over another, regardless of the contaminating non-xylan polysaccharides and pectins that were observed in the chlorite-extracted samples. The presence of substituted glucuronic acid moieties has been reported before in PAA-extracted eucalyptus xylans (Pinto et al.,

2005; Evtuguin et al., 2003). The structures suggested in these studies involve the substitution of MeGlcA with mainly galactosyl units, as well as with glucans, representing crosslinking points with other polysaccharides. This model would fit with the results for the PAA extracts in the present work (Table 3). Linkage analysis revealed more branches in the xylose backbone (2,4-Xyl*p*) than the number of MeGlcA units. This could be due to the difficulties of quantifying MeGlcA covalently linked to lignin moieties, as it was shown in the FTIR results. The presence of small quantities of 3,4-Xyl*p* and 2,3,4- Xyl*p* units might arise from incomplete saponification of acetyl groups during the methylation process (Pinto et al., 2005).

Magaton et al. (2008) suggested a chemical linkage between the glucose and galactose to the xylan chain *via* uronic acids was responsible for the presence of glucose and galactose in the extracted xylan samples from eucalyptus. This was also supported by Evtuguin et al. (2003), who, after observing remarkable amounts of glucose, arabinose and rhamnose in xylan isolated from eucalyptus using the PAA/DMSO process, suggested that these sugars more likely came from small polysaccharides fragments of different origin, but which were chemically linked to the backbone of the xylans. These authors pointed out that the most probable origin for these xylan sugars is the chemical linkage, rather than a simple adsorption, during the xylan isolation process. In the present work the linkage of xylan to glucose and pectins was not proved by linkage analysis results.

In the case of sugarcane straw and bagasse samples extracted with PAA, the quantity of arabinosyl terminal units is proportional to the number of branched 2,4-Xyl*p* and 3,4-Xyl*p* units. Only traces of double substituted 2,3,4-Xyl*p* residues were found in both the PAA and $NaClO_2$ treated xylans, in contrast to reports on other grass xylans, such as cereals, where these double substitutions are relatively abundant (Heikkinen et al., 2013). The linkage patterns in the straw and bagasse xylan were quite similar to each other, with the straw xylan having a slightly greater degree of arabinosyl branching. To the best of our knowledge, this is the first time that linkage analysis has been performed on PAA-extracted xylan (in native form) from sugarcane bagasse or straw. Previously reported linkage analysis on alkali-extracted xylan from bagasse also showed the absence of double substitutions in this type of xylan, but indicated a 2-3 times greater degree of arabinosyl branching at O-3 (Banerjee et al, 2014; Mellinger-Silva et al., 2011). These differences might be related to deacetylation in alkaline conditions, due, for example, to higher solubility of acetylated xylan fractions with a smaller degree of arabinosyl branching.

Using the PAA/DMSO process, the molar ratio between terminal residues (t-Ara*f*) and branch units for xylans (2,4-Xyl*p*, 3,4-Xyl*p* and 2,3,4-Xyl*p*) extracted from bagasse (0.94) and straw (1.00), together with the results of the sugar analysis, indicated that no undermethylation occurred during the analysis. On the other hand, the $NaClO_2$/DMSO process revealed less conformity in the molar ratio between terminal residues and branch units for bagasse (0.65) and straw (0.69), and resulted in a lower xylose content in xylans (Fig. 5). The $NaClO_2$/DMSO processes resulted in xylan with different chemical behaviour, such as higher resistance towards methylation, highlighting the deficiencies of these processes for xylan isolation.

3.4 Average molecular weight and molecular weight distribution

The average molecular weight of the extracted xylan samples are set out in Table 4, while the molecular weight distribution is shown in Figure 6. As a whole, the different sugarcane and eucalyptus xylan samples exhibited monomodal size distributions. This suggested that the homogeneous macromolecular populations in such extracts related mostly to xylan polymers, with different substitutions attached to the xylan backbone. Only the xylan sample extracted with $NaClO_2$/DMSO from eucalyptus shows a broader and multimodal distribution (Figure 6), which could be linked to a heterogeneous population of polysaccharides, as witnesses by monosaccharide composition and linkage analysis.

Table 4 – Average molecular number (Mn), average molecular weight (Mw) and polydispersity index (D) of xylan samples isolated from eucalyptus, sugarcane bagasse and straw.

Samples	Extraction process	M_n, kDa	M_w, kDa	D
Eucalyptus	PAA/DMSO	30.8 (0.3)	42.2 (0.3)	1.4
	$NaClO_2$/DMSO	9.7 (0.3)	55.2 (2.4)	5.7
Bagasse	PAA/DMSO	25.2 (0.0)	37.6 (0.3)	1.5
	$NaClO_2$/DMSO	23.7 (0.5)	40.8 (2.9)	1.7
Straw	PAA/DMSO	17.9 (0.5)	29.5 (0.5)	1.7
	$NaClO_2$/DMSO	18.2 (1.1)	34.2 (3.2)	1.9

(…) Standard deviation.

The molecular weight value for the xylan extracted from eucalyptus using PAA/DMSO was 42 kDa, just slightly higher than the value of 36 kDa reported for the *Eucalyptus globulus* (Evtuguin et al., 2003). The average value for the eucalyptus xylan extracted with PAA/DMSO contrasted with that extracted with $NaClO_2$/DMSO. This

reinforced our view that the $NaClO_2$/DMSO extraction process did not succeed in isolating relevant xylan fractions from the eucalyptus, but, rather, isolated a mixture of shorter polysaccharide chains with a high index. The polydispersity indexes for eucalyptus were 1.4 and 5.7, for PAA/DMSO xylan samples and $NaClO_2$/DMSO xylan samples, respectively.

The average molar mass values for sugarcane bagasse and straw xylans were in the range of 30-41 kDa, which were similar to those obtained previously for sugarcane bagasse xylans (Peng et al., 2009; Sun et al., 2004) and for straw xylans from other grasses (e.g. wheat straw) (Persson et al., 2009). The polydispersity indexes of the bagasse and straw xylan samples were in the range of 1.5-1.9, which were lower than those obtained previously for sugarcane bagasse xylans (Peng et al., 2009; Sun et al., 2004).

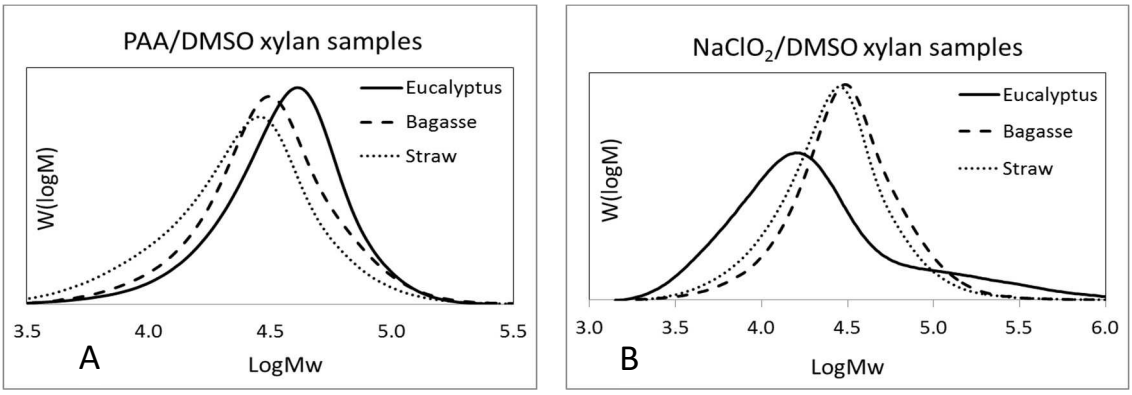

Figure 6 - Molecular weight distribution of xylan from eucalyptus wood, bagasse and straw extracted by PAA/DMSO (**A**) and $NaClO_2$/DMSO (**B**).

3.5 ^1H NMR spectra of xylan

Proton NMR spectra for the PAA/DMSO xylan from eucalyptus, bagasse and straw are set out in Figure 7. Assignments for proton resonances were evaluated by using existing data (Sun et al., 2011; Marques et al., 2010; Magaton et al., 2008; Evtuguin et al., 2003; Hoffmann et al., 1992; Izydorczyk and Biliaderis, 1992; Cavagna et al., 1984). The hydrogen from acetyl groups (CH_3-CO-) (δ_H 2.01-2.11), the D_2O (HDO) (δ_H 4.71), and solvent impurities (δ_H 1.94, 2.00, 2.16, 2.47, 2.62, 2.82, 3.07 and 3.25) were also assigned. The following different structures, common in biomasses, were identified for the xylan: non-acetylated xylose units isolated from other acetylated xylan units in the backbone; non-acetylated xylose units neighbouring the acetylated xylan units; xylose units acetylated in oxygen 2; xylose units acetylated in oxygen 3;

and xylose units acetylated in oxygen 2 and 3. This was consistent with the signals for anomeric hydrogen in the region between δ_H 4.4-5.5. In addition, for eucalyptus, the 4-O-methylglucuronic acid linked to oxygen 2 of the acetylated xylan (3-O-linked), the 4-O-methylglucuronic acid unit and the 4-O-methylglucuronic acid linked to the galactosyl unit, were assigned. For bagasse and straw, linkages between arabinose and xylose were observed in two different ways in the ^{1}H NMR spectra: arabinose linked to oxygen 3 in monosubstituted xylan (δ_H 5.39) and arabinose linked to oxygen 3 (δ_H 5.29) and 2 (δ_H 5.23) in disubstituted xylan (Izydorczyk and Biliaderis, 1992) (Table 5).

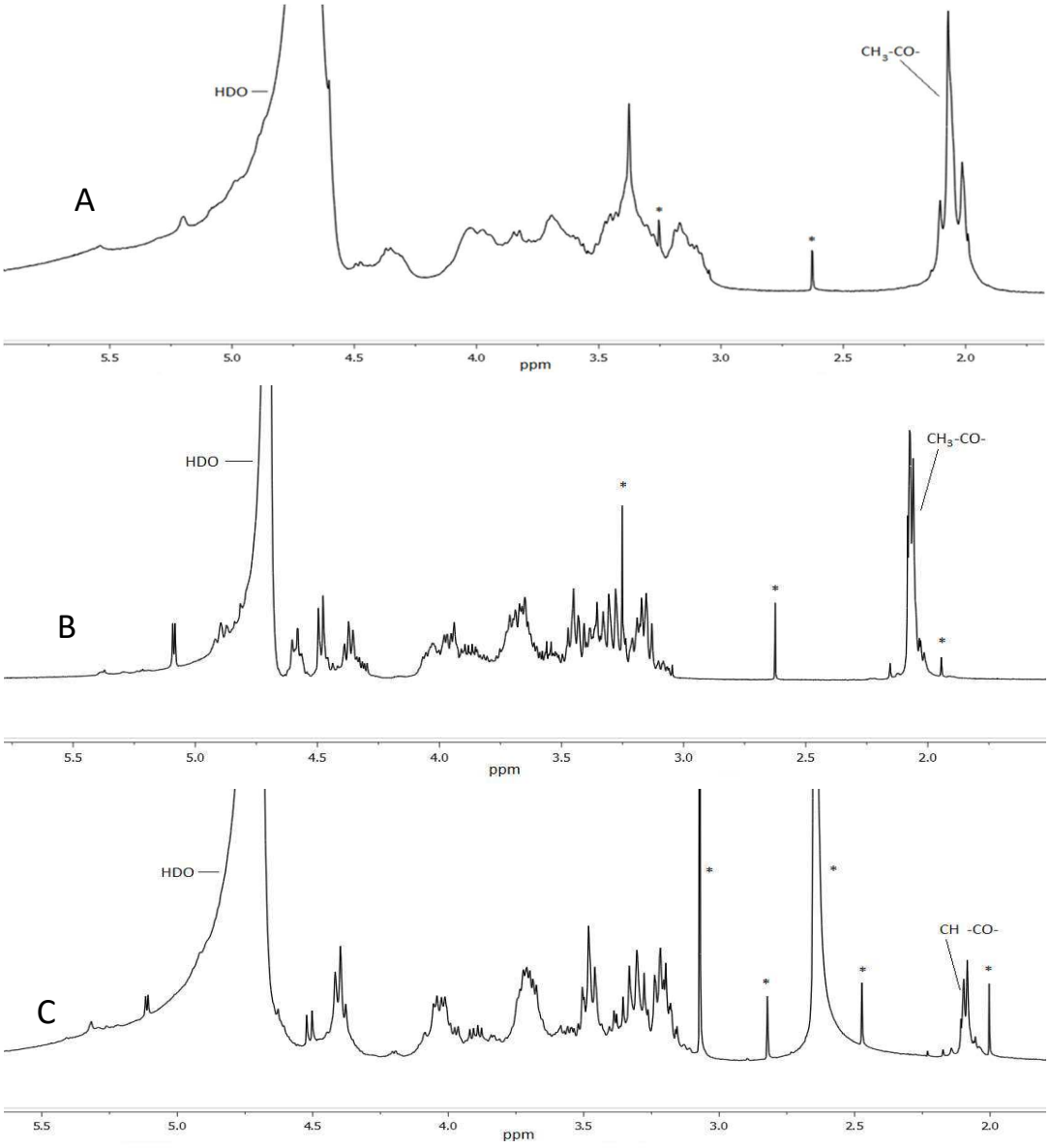

Figure 7 - ^{1}H NMR spectra of acetylated xylan from eucalyptus wood (**A**), bagasse (**B**) and straw (**C**) extracted by PAA/DMSO process. Designation for the structural fragments is presented in Table 5. *solvent impurities.

Different patterns of acetylation and branches were observed in the xylans from eucalyptus, bagasse and straw. Branches of 4-O-methylglucuronic acid in eucalyptus xylan and of arabinose in sugarcane bagasse and straw were the main difference between these xylans.

Table 5 - Proton NMR chemical shifts for structural units of acetylated xylan from eucalyptus, bagasse and straw. Xylan extracted by PAA/DMSO process.

Biomass	Structural units	H1	H2	H3	H4	H5	
						ax	eq
Eucalyptus	Xyl (isol.)	4.49	3.30	3.56	3.79	3.42	n.d.
	Xyl (Xyl-Ac)	4.37	3.19	3.54	3.69	n.d.	n.d.
	Xyl-3Ac	4.60	3.47	4.98	3.94	3.47	n.d.
	Xyl-2Ac	n.d.	n.d.	3.79	3.85	3.45	n.d.
	Xyl-2,3Ac	n.d.	n.d.	5.09	4.03	3.54	n.d.
	Xyl-3Ac-2MeGlcA	n.d.	3.69	4.99	3.97	3.47	n.d.
	MeGlcA	5.20	3.56	3.82	3.17	n.d.	n.d.
	MeGlcA-2Gal	5.45	3.76	3.82	3.19	n.d.	n.d.
Bagasse	Xyl (isol.)	4.46	3.30	3.55	3.79	3.37	n.d.
	Xyl (Xyl-Ac)	4.42	3.22	3.54	3.71	3.36	n.d.
	Xyl-3Ac	4.58	3.47	4.92	3.91	3.47	4.15
	Xyl-2Ac	4.64	n.d.	3.79	3.85	3.43	4.15
	Xyl-2,3Ac	4.84	4.82	5.19	4.05	3.51	4.20
	Xyl-3Ara	5.39	4.18	3.91	4.18	3.75	3.77
	Xyl-2,**3**Ara	5.29	4.18	3.91	4.18	3.75	3.77
	Xyl-**2,**3Ara	5.23	4.16	3.94	4.14	3.75	3.77
Straw	Xyl (isol.)	4.50	3.30	3.58	3.72	3.39	4.09
	Xyl (Xyl-Ac)	4.38	3.20	3.54	3.70	3.35	n.d.
	Xyl-3Ac	4.52	3.46	4.91	3.92	3.46	n.d.
	Xyl-2Ac	4.61	4.63	3.81	3.84	3.46	4.19
	Xyl-2,3Ac	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Xyl-3Ara	5.32	4.20	3.92	4.20	3.74	3.76
	Xyl-2,**3**Ara	5.29	4.20	3.92	4.20	3.74	3.76
	Xyl-**2,**3Ara	5.26	4.19	3.93	4.11	3.74	3.76

n.d: not determined or inexistent. The following designations used were: Xyl (isol.) → non-acetylated Xyl*p* in the backbone isolated from other acetylated Xyl*p* units; Xyl (Xyl-Ac) → non-acetylated Xyl*p* linked with neighboring acetylated Xyl*p*; Xyl-3Ac - 3-*O*-acetylated Xyl*p*; Xyl-2Ac → 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac → 2,3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2MeGlcA → MeGlcA 2-*O*-linked and 3-*O*-acetylated Xyl*p*; MeGlcA → MeGlcA unit; MeGlcA-2Gal → Gal*p* 2-*O*-linked MeGlcA; Xyl-3Ara – terminal arabinose linked to *O-3* of monosubstituted xylose; Ara Xyl-2,**3**Ara – terminal arabinose linked to *O-3* in disubstituted xylose; and Xyl-**2**,3 - terminal arabinose linked to *O-2* in disubstituted xylose.

In the eucalyptus ^{1}H NMR spectra (Fig. 7A), the anomeric proton at δ_H 5.20 and the singlet at δ_H 3.38 were assigned as 4-O-methylglucuronic acid attached *via* α-(1→2) linkage to xylose (Teleman et al., 2000; Cavagna et al., 1984). This information, together with the linkage analysis results, enabled the assignment of the 4-O-methylglucuronic acid branches in the xylose at position O-2. In addition, for eucalyptus, branches of 4-O-methylglucuronic acid have been observed elsewhere in O-2 in acetylated xylose (acetylation in O-3) (Magaton et al., 2008; Evtuguin et al., 2003) and deacetylated xylose (Shatalov et al., 1999). By integrating the proton H-3 (δ_H 4.99) of the structural element Xyl-3Ac-2MeGlcA with the proton H-1 (δ_H 5.20) of the 4-O-methylglucuronic acid, a ratio of 1:1 was found, which indicated that all 4-O-

methylglucuronic acid branches were present in xylose acetylated at position O-3, as observed by Magaton et al. (2008) for the same eucalyptus hybrid and by Evtuguin et al. (2003) for *Eucalyptus globulus*. In the ^{1}H NMR spectra, the signal of 4-O-methylglucuronic acid attached *via* α-(1→2) linkage to xylose was not completely determined due to overlapping with the solvent signal (HDO).

In the bagasse and straw ^{1}H NMR spectra (Fig. 7B and 7C), arabinosyl substitutions were assigned in O-2 and O-3, in agreement with the results from the linkage analysis. Although the presence of xylose disubstituted by arabinose was observed in the ^{1}H NMR spectra, the results of the methylation linkage analysis showed that these branches occurred in low frequency in xylan from bagasse and straw. In xylan from straw, xylose with two acetyl groups was not observed.

3.6 Empirical structure of xylan from eucalyptus, bagasse and straw

The empirical xylan structures and the designation for the structural fragments in xylans from eucalyptus, bagasse and straw (Fig. 8 and Table 6, respectively) were obtained through a combination of the results of the methylation linkage analysis, ^{1}H NMR and degree of acetylation. The acetylated heteroxylan from eucalyptus, sugarcane bagasse and sugarcane straw were shown to be both, chemically and structurally different from each other. For xylan from eucalyptus, the molar ratio between the xylose units and the branches of 4-O-methylglucuronic acid units was 10:1.1, based on the methylation linkage analysis. In addition, eucalyptus xylan contained 0.39 acetyl groups per xylose unit, with 64% of the acetyl groups being observed at position O-3 of the xylose (from which 28% in xylose substituted in position 2 by 4-O-methylglucuronic acid unit), 26% at position O-2 of the xylose and 10% at positions O-2 and O-3 of the same xylose. The 4-O-methylglucuronic acids were linked to xylose at position O-2 of acetylated xylose (Xyl-3Ac-2MeGlcA). The presence of 4-*O*-methylglucuronic acids linked to terminal galactosyl unit, in low abundance, was confirmed by ^{1}H NMR spectroscopy and methylation linkage analysis. The presence of xylose with acetyl groups at position O-3 was observed in the ^{1}H NMR spectra, while the presence of xylose acetylated at position O-2 and xylose acetylated at positions O-2,3 simultaneously were also suggested by the ^{1}H NMR spectra, although some signals were obscured by the overlapping solvent signal (HDO).

Table 6 - Empirical structure of xylan from eucalyptus, bagasse and straw.

Structural fragments and short designation	Relative abundance (per 100 Xyl*p* units)		
	Eucalyptus	Bagasse	Straw
→4)-β-D-Xyl*p*-(1 → **(Xyl)**	63	70	86
→4)[3-*O*-Ac]-β-D-Xyl*p*-(1 → **(Xyl-3Ac)**	14	17	5
→4)[2-*O*-Ac]-β-D-Xyl*p*-(1 → **(Xyl-2Ac)**	10	4	3
→4)[3-*O*-Ac] [2-*O*-Ac]-β-D-Xyl*p*-(1 → **(Xyl-2,3Ac)**	2	4	-
→4)[4-*O*-Me-α-D-Glc*p*A(1→2)] [3-*O*-Ac] -β-D-Xyl*p*-(1 → **(Xyl-3Ac-2MeGlcA)**	11	-	-
-4- *O*-Me-α-D-Glc*p*A-(1 → **(MeGlcA)**	7[a]	-	-
→ 2)-4- *O*-Me-α-D-Glc*p*A-(1 → **(MeGlcA-2Gal)**	1[a]	-	-
→4)[α-L-Arab*f*(1→3)]-β-D-Xyl*p*-(1 → **(Xyl-3Ara)**	-	4	5
→4) [α-L-Arabf(1→2)]-β-D-Xyl*p*-(1 → **(Xyl-2Ara)**	-	1	1
α-L-Ara*f*-(1 → **(Ara)**	-	5	6

[a]Value for MeGlcA units underestimated by methanolysis.

Acetylated heteroxylan from sugarcane bagasse had a molar ratio between the xylose units and the terminal arabinose units of 10:0.5, based on the methylation linkage analysis. From these results and the ^{1}H NMR, the xylan from bagasse showed to have a substantial quantity of xylose substituted by arabinose in position O-3 than in position O-2. Bagasse xylan contained 0.29 acetyl groups per xylose unit, with 60% of the acetyl groups being observed at position O-3 of the xylose, 13% at position O-2 of the xylose and 27% at positions O-2 and O-3 of the same xylose.

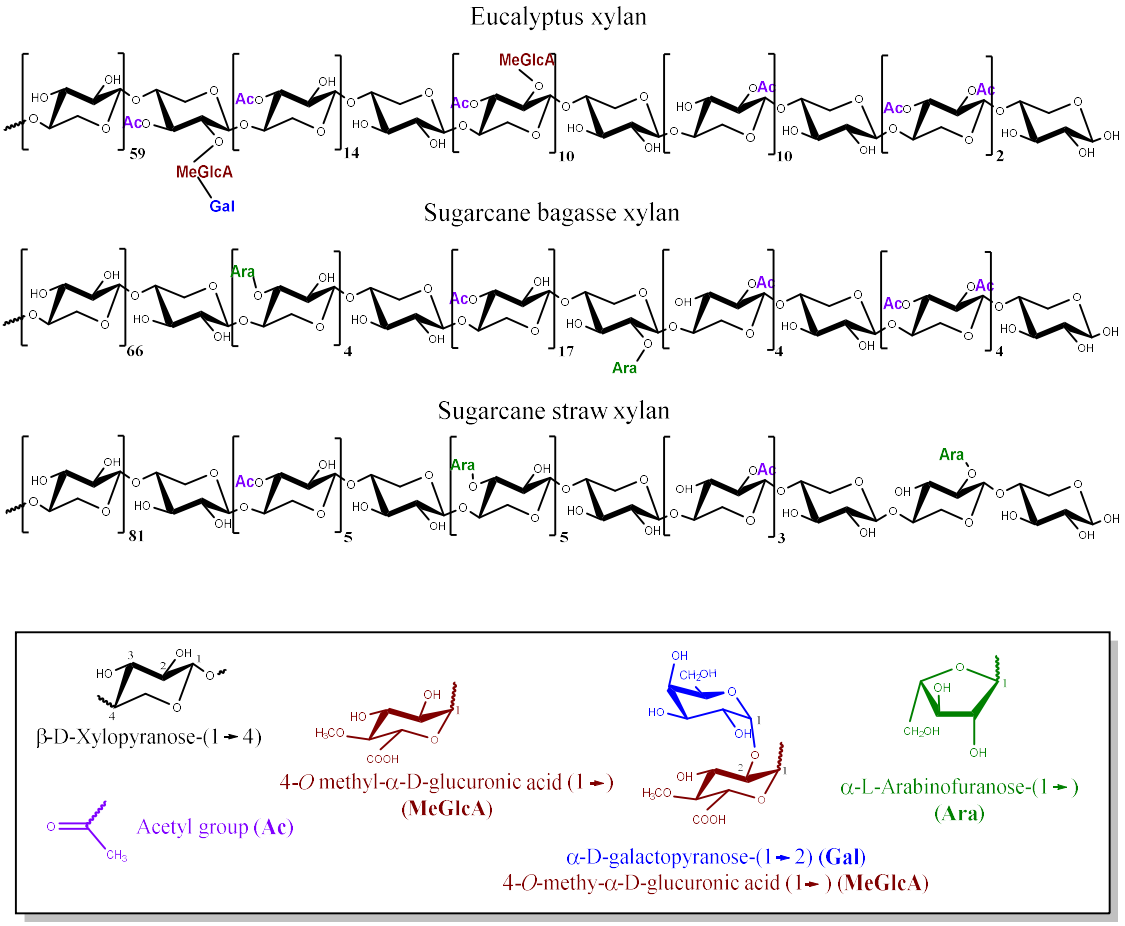

Figure 8 - Empirical structure of xylan from eucalyptus, bagasse and straw.

Acetylated heteroxylan from sugarcane straw had a ratio between the xylose units and the terminal arabinose units of 10:0.6, based on the methylation linkage analysis, which showed that the straw xylan is slightly more branched than xylan from bagasse. The xylan from straw had a larger quantity of xylose substituted by arabinose in position O-3 than in position O-2. In addition, straw xylan contained 0.08 acetyl groups per xylose unit, with 67% of the acetyl groups being observed at position O-3 of the xylose and 33% at position O-2 of the xylose.

4. Conclusions

- Xylan from sugarcane bagasse and straw were shown to be different from each other and to be less acetylated and substituted than that from eucalyptus;
- The molar ratio of xylose units to branches of 4-O-methylglucuronic acid in eucalyptus was 10:1.1, and at least a quarter of the xyloses contained at least one acetyl group (DA 0.39). The acetyl groups were attached to the xylose unit at positions O-3 (64%), O-2 (26%) and O-2,3 (10%). All xylose units having a 4-O-methylglucuronic acid at position O-2 also possessed an acetyl groups at position O-3. The molecular weight and the polydispersity index of xylan from eucalyptus (PAA/DMSO sample) were 42 kDa and 1.4, respectively;
- The xylan from bagasse was shown to contain a slightly lower molar ratio of xylose units to arabinose (10:0.5) than xylan from straw (10:0.6), but a greater degree of acetylation (0.29 and 0.08 for bagasse and straw, respectively). The acetyl groups were attached at positions O-3 (60%), O-2 (13%) and O-2,3 (27%) of xylose unit in bagasse xylan and at positions O-3 (67%) and O-2 (33%) of xylose unit in straw xylan. In xylan from bagasse and straw, xylose substituted at position O-3 (Xyl-3Ara) occurred with greater frequency than that substituted at position O-2 (Xyl-2Ara). In addition, Xyl-3Ac occurred with greater frequency than Xyl-2Ac or Xyl-2,3Ac, for all biomasses. Xylan from bagasse and straw (PAA/DMSO samples) contained a molecular weight of 38 kDa and 30 kDa, respectively, and a polydispersity index of 1.5 and 1.7, respectively; and
- The process $NaClO_2$/DMSO did not succed to isolate significative amount of xylan from biomasses.

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Article III

Assessment of hydrothermal and acid pretreatments for bioethanol production from eucalyptus, sugarcane bagasse and straw

ABSTRACT - The effect of hydrothermal and acid (1.5%, 3.0% and 4.5% H_2SO_4) pretreatments on the chemical composition of eucalyptus, sugarcane bagasse and straw was compared with a view to their subsequent bioconversion into ethanol using simultaneous saccharification and fermentation (SSF) with preliminary presaccharification step. The chemical transformations in pretreated biomasses varied as a result of the final pH of pretreatments. Decreasing the final pH reduced the pretreatment yield by improving lignin and hemicelluloses removal. For eucalyptus, however, the chemical composition, pretreatment yields and final pH were similar for acid pretreatments, irrespectively to the acid concentration. Hemicelluloses removal during the pretreatments was in the range of 63-96% for eucalyptus, 25-98% for bagasse and 23-95% for straw. Lignin removal during pretreatments was in the range of 10%-34% for eucalyptus and 10%-27% for bagasse. The analysis of lignin fragments and of polysaccharides degradation products revealed formation of pseudo-lignin from bagasse and straw and pseudo-extractives from eucalyptus during the pretreatments. Differences in lignin structure and reactivity (e.g. S, G and H lignin) may be determining factors to the preferential formation of pseudo-extractives or pseudo-lignin. Acid pretreatments resulted in biomasses with lower lignin and hemicelluloses amount compared to those pretreated using hydrothermal process. These biomasses had higher cellulose accessibility to enzymatic hydrolyses which was evidenced by the higher presaccharification yield. The acid dose that maximized glucose release and presaccharification yield for eucalyptus was 1.5% H_2SO_4, while for bagasse and straw this value was 4.5% H_2SO_4. Narrow correlations between glucose yield and ethanol yield, as well as between glucose yield and volumetric productivity of ethanol were observed for hydrothermal and acid pretreatments. The straw pretreated by acid process (4.5% H_2SO_4) presented the highest ethanol production among the biomasses, assessed based on ethanol yield (0.056 $g_{ethanol}/g_{biomass}$), volumetric productivity of ethanol (0.51 g L^{-1} h^{-1}) and ethanol concentration (5.1 g L^{-1}). The final pH proved to be an important control variable for pretreatments in acid conditions with direct effects on chemical transformations of biomasses and indirect effects on ethanol production.

Keywords: Acid pretreatment, hydrothermal pretreatment, pH, presaccharification, pseudo-extractives, simultaneous saccharification and fermentation.

RESUMO – O efeito dos pré-tratamentos hidrotérmico e ácido (H_2SO_4 1,5%, 3,0% e 4,5%) na composição química de eucalipto, bagaço de cana-de-açúcar e palha de cana-de-açúcar foi comparado com foco em sua subsequente bioconversão em etanol usando sacarificação e fermentação simultâneas (SFS) com pré-sacarificação. As transformações químicas nas biomassas pré-tratadas variaram como resultado do pH

dos pré-tratamentos. Para o eucalipto, entretanto, a composição química, rendimento dos pré-tratamentos e pH final foram similares para os pré-tratamentos ácidos, independente da concentração de ácido. A remoção de hemiceluloses durante os pré-tratamentos foi de 63%-96% para o eucalipto, 25%-98% para o bagaço e 23%-95% para a palha. A remoção de lignina durante os pré-tratamentos foi de 10%-34% para o eucalipto e 10%-27% para o bagaço. Análises de fragmentos de lignina e de produto de degradação de polissacarídeos revelaram a formação de pseudo-lignina a partir do bagaço e da palha e pseudo-extrativos a partir do eucalipto durante os pré-tratamentos. Diferenças na estrutura e reatividade da lignina (ex. ligninas S, G e H) podem ser fatores determinantes na formação preferencial de pseudo-extrativos ou pseudo-lignina. Pré-tratamentos ácidos resultaram em biomassas com menores teores de lignina e hemiceluloses comparados com biomassas pré-tratadas pelo processo hidrotérmico. Essas biomassas apresentaram maior acessibilidade da celulose à hidrólise enzimática o que foi evidenciado pelo maior rendimento da pré-sacarificação. A concentração ácida que potencializou a liberação de glicose e o rendimento da pré-sacarificação para o eucalipto foi de H_2SO_4 1,5% e de H_2SO_4 4,5% para bagaço e palha. Estreitas correlações entre rendimento em glicose e rendimento em etanol, bem como entre rendimento em glicose e produtividade volumétrica de etanol foram observadas para os pré-tratamentos hidrotérmico e ácido. A palha pré-tratada por processo ácido (H_2SO_4 4,5%) apresentou a maior produtividade de etanol dentre as biomassas, avaliada com base no rendimento em etanol (0,056 $g_{etanol}/g_{biomassa}$), produtividade volumétrica de etanol (0,51 g L^{-1} h^{-1}) e concentração de etanol (5,1 g L^{-1}). O pH final provou ser uma importante variável de controle para pré-tratamentos em condições ácidas, com efeito direto na transformação química das biomassas e indireto na produção de etanol.

Palavras-chave: Pré-tratamento ácido, pré-tratamento hidrotérmico, pH, pré-sacarificação, pseudo-extrativos, sacarificação e fermentação simultâneas.

1. Introduction

Recently bioethanol has been recognized as the most promising biofuel to replace fossil-based fuels. Bioethanol is a versatile fuel, suitable to be used in neat form or blended with gasoline for gasoline engine, due its high octane number (108). In addition, the low cetane number (8) and the high heat of vaporization (0.91 MJ/kg) of bioethanol avoids its self-ignition in the diesel engine (Balat and Balat, 2009; Dermibas, 2009; Balat et al., 2008; Demirbaş et al., 2005). Bioethanol is a primary alcohol (C_2H_5OH) and the oxygen present in its chemical structure improves the combustion process, reducing hydrocarbon, carbon monoxide and particulate emission during the burning, however, with the possibility to increase the nitrogen oxide emission (Balat et al., 2008). The feedstocks for bioethanol production are lignocellulosic biomasses, which indicates its sustainability during the use as well as in the production. Several lignocellulosic feedstock have been investigated for bioethanol production, such as

sugarcane bagasse (Souza et al., 2012; Laser et al., 2002), sugarcane straw (Santos et al., 2014; Oliveira et al., 2013), wheat straw (Petersen et al., 2009; Saha et al., 2005), corn stover (Lloyd and Wyman et al., 2005; Esteghlalian et al., 1997), switchgrass (Esteghlalian et al., 1997), eucalyptus wood (Romaní et al., 2010; Ballesteros et al., 2004), poplar wood (Negro et al., 2003; Esteghlalian et al., 1997), beech wood (Nitsos et al., 2013), among others.

In Brazil, eucalyptus and sugarcane residues stand out as potential feedstocks for bioethanol production due their high production volume. Eucalyptus is a fast-growing gender which is largely cultivated in Brazil for many forest-based industries. Sugarcane is one of the main Brazilian agricultural crops and the production of sugarcane bagasse (stalks) and sugarcane straw (leaves and tips) for 2015/16 harvest can overcome 92 million tons for each residue (Conab, 2015; Oliveira et al., 2013).

Bioethanol production through biological conversion includes pretreatments, enzymatic hydrolysis and fermentation (Rubin, 2008). Pretreatments and enzymatic hydrolysis are used to disrupt the complex network of cellulose, hemicellulose and lignin from which lignocellulosic biomasses are formed, aiming at increasing the cellulose accessibility for a more efficient enzymatic hydrolysis (Singh et al., 2015). Pretreatment conditions have been recently reviewed by various authors (Singh et al., 2015; Alvira et al., 2010; Cardona et al., 2010) and acidic conditions such as hydrothermal and dilute acid have been considered great alternatives for increasing cellulose accessibility, by hemicelluloses removal, as well as inducing chemical changes in lignin and cellulose (Lee et al., 2010; Sun and Cheng, 2005). Similar reactions occur in hydrothermal and acid pretreatment but in a lesser extent during hydrothermal pretreatments (Carvalho et al., 2015).

Hydrothermal pretreatment is a process in which no additional chemical is required besides water (Vegas et al., 2008; Garrote et al., 2007). This pretreatment is usually performed at higher temperature (150-250ºC), under pressure, during residence time from a minute to an hour (Santos et al., 2014; Lee et al., 2010; Romaní et al., 2010; Laser et al., 2002). During hydrothermal pretreatment, hydronium ions are released from hemicelluloses (Parajó et al., 2004) and comparatively, the hemicellulose from eucalyptus is better source of acid groups than those from sugarcane bagasse or straw (Carvalho, 2015). The hydronium ions promote the reaction that results in the chemical transformation in biomasses (Santos et al., 2014; Lee et al., 2010). Romaní et al. (2010) and Laser et al. (2002), highlighted the hydrothermal pretreatment as a promising method for eucalyptus and sugarcane bagasse, respectively. Romaní et al. (2010)

observed that higher severities in hydrothermal pretreatment led to decreasing in bioethanol yields, due to partial cellulose degradation during the pretreatment. Santos et al. (2014) performed hydrothermal pretreatment for sugarcane straw and the results indicated efficient hemicellulose removal providing a substrate with better cellulose accessibility for the subsequent enzymatic hydrolysis.

From a chemical point of view, hydrothermal and acid pretreatment are similar, with the difference that in acid pretreatment, an external acid source is required. Usually sulfuric acid is used. Acid pretreatments are performed at higher temperature (100-200ºC), under pressure during residence time from 2 to 300 minutes and sulfuric acid concentration from 0.6% to 6.0% (w/w) (Lloyd and Wyman, 2005; Saha et al., 2005; Sun and Cheng, 2005; Aguilar et al., 2002; Esteghlalian et al., 1997).

Aguilar et al. (2002) observed about 90% of xylan removal from sugarcane bagasse, low cellulose degradation and by-products formation by using sulfuric acid concentration and reaction time of 2% and 24 minutes, respectively, in acid pretreatment at 122ºC. One disadvantage of acid pretreatment is the need for pH neutralization before enzymatic hydrolysis (Taharzadeh and Karimi, 2007). During acid pretreatment as well as in hydrothermal pretreatment, the increasing the severity may leads to degradation of xylan to furfural, which is an inhibitor to the formation of ethanol during fermentation. Most of these inhibitors, however, are found in the filtrate, which proves the effectiveness of pretreatments for biomasses chemical transformation (Cybulska et al., 2010).

After pretreatments the conversion of biomasses into bioethanol follows through enzymatic hydrolysis and fermentation. During enzymatic hydrolysis, or simply, saccharification, a number of enzymes (cellulases) attack both, amorphous and crystalline structure of cellulose, breaking it down into cellobiose and from it to glucose. Cellulases for saccharification are produced by fungi such as *Trichoderma reesei*. During saccharification, if in high concentration, the glucose and cellobiose released can promote inhibition in β-glucosidase and cellulase, respectively (Philippidis et al., 1993). A way around this inhibition is performing the simultaneous saccharification and fermentation (SSF) instead of separate hydrolysis and fermentation (SHF). SSF consists of saccharification and fermentation in the same reactor, with coexistence of enzymes (for saccharification) and yeast (for fermentation) (Baeyens et al., 2015). In addition to decrease inhibition, SSF decreases the enzymes need and increases the volumetric productivity of ethanol compared to SHF (Santos et al., 2010). As the temperature for saccharification is higher than that for fermentation,

thermotolerant yeast strains have been continuously studied (Souza et al., 2012; Ballesteros et al., 2004; Hari Krishna, et al., 2001), which can surely increase the efficiency of SSF. Presaccharification step prior to SSF is used to supply glucose in the beginning of SSF, allowing improvements in ethanol yield (Santos et al., 2010).

Suitable pretreatments are key for efficient conversion of biomasses into bioethanol by decreasing their recalcitrance. A number of previous studies determined conditions for hydrothermal and acid pretreatment to improve chemical hydrolysis by optimization of temperature and time (Santos et al., 2014; Nitson et al., 2013; Petersen et al., 2009; Lloyd and Wyman, 2005; Aguilar et al., 2002; Esteghlalian et al., 1997). The novelty of this study is determining the effect of acid charge during the pretreatment on the chemical transformation of three different biomasses and their impact on subsequent enzymatic hydrolysis, with a focus on the bioethanol production. The objectives of this study were: (i) to evaluate hydrothermal and acid pretreatments (in three different concentrations) on the chemical composition of eucalyptus, sugarcane bagasse and straw and (ii) to evaluate the efficiency of bioethanol production from pretreated biomasses through SSF with preliminary presaccharification step. It is anticipated that this information will contribute to a better understanding of the effect of pretreatment acidity level on bioconversion of eucalyptus, bagasse and straw into bioethanol. In addition, it will provide useful guidance for researchers when deciding on the most suitable acid pretreatment technology for bioethanol production from these biomasses.

2. Experimental

2.1 Working plan

Figure 1 describes the working plan. Each biomass was chemically characterized and used in hydrothermal and acid (1.5%, 3.0% and 4.5% w/w H_2SO_4) pretreatments. The pretreated biomasses were also chemically characterized and used for bioethanol production through SSF after a presaccharification step. The time for presaccharification step was optimized based on the glucose release and presaccharification yield for the biomasses pretreated by hydrothermal processes. The same optimal time was used for acid pretreated biomasses. For the biomasses pretreated by 1.5%, 3.0% and 4.5% H_2SO_4, only the pretreated biomass produced using the amount of acid which provide the best presaccharification performance followed through the subsequent bioethanol production *via* SSF.

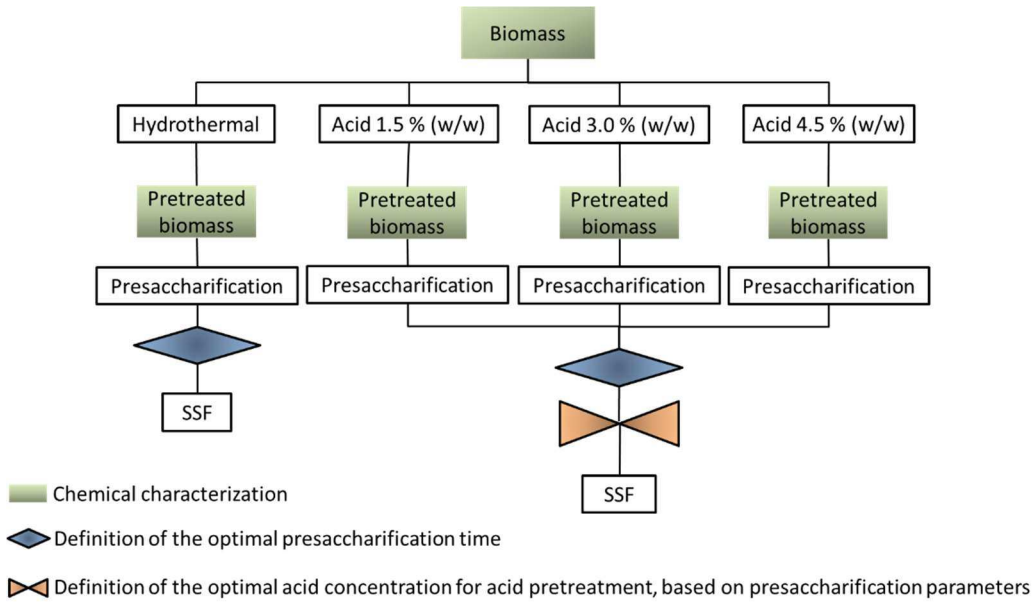

Figure 1 - Working plan for eucalyptus, bagasse and straw chemical characterization, hydrothermal and acid pretreatments, presaccharification and SSF for bioethanol production.

2.2 Materials

The lignocellulosic biomasses used were eucalyptus, sugarcane bagasse and sugarcane straw. Wood chips of a 7 years old clonal hybrid of eucalyptus (*Eucalyptus urophylla* x *Eucalyptus grandis*) were supplied by a Brazilian pulp company. Chips were screened and those with dimensions smaller than 0.5 cm x 3 cm x 3 cm were collected for chemical analyses and pretreatments. Five-months old bagasse and straw (cultivar RB867515) were supplied by Center Sugarcane Experimentation (Oratórios, Minas Gerais State, Brazil) at the Federal University of Viçosa after chipping (bagasse and straw) and juice removal (bagasse) generating particles of 10 mm diameter. Biomasses were dried to about 85% dryness and stored in polyethylene bags at room temperature prior the use. Moisture content was determined according to TAPPI T 264 cm-07. Chemicals used were sulfuric acid 95-97% (Merck Milipore, Germany), commercial cellulase Celluclast 1.5 L (from *Trichoderma reesei* ATCC 26921) (Sigma-Aldrich, Brazil) and *Saccharomyces cerevisiae* LBM-1 isolated from fermentation vast in Brazil.

2.3 Methods

2.3.1 Hydrothermal and acid treatments

One-hundred grams o.d. (oven dried equivalent) of eucalyptus wood, sugarcane bagasse and sugarcane straw were subjected to hydrothermal and to acid pretreatments in three concentrations: (i) 1.5% H_2SO_4 (w/w); (ii) 3.0% H_2SO_4 (w/w); and (iii) 4.5% H_2SO_4 (w/w). Pretreatments were performed in duplicate in a Regmed reactor (2 L capacity), under constant agitation, using the following parameters: liquor:biomass ratio = 2:1 L kg^{-1} (eucalyptus) and 7:1 L kg^{-1} (bagasse and straw); maximum temperature = 175ºC; time to maximum temperature = 90 min; and time at maximum temperature = 15 min. After the pretreatment, the reactor was cooled. The pretreated biomasses were washed with an excess of water and centrifuged at 800 rpm for 4 minutes. Before washing, a sample of liquor was collected for pH measurement. The pretreated biomasses were conditioned for 24 hours at 23 ± 1 °C and 50 ± 2% relative humidity for constant weight and then stored at room temperature in polyethylene bags.

2.3.2 Optimization of presaccharification step

Prior presaccharification, pretreated eucalyptus was converted to sawdust at a 20/80 mesh by using a Wiley mill bench model. Bagasse and straw were not grinded. The commercial cellulase preparation Celluclast 1.5 L was used for enzymatic hydrolysis. In an 125 mL erlenmeyer flask, 4 g of pretreated biomass were suspended in 50.0 mL of citrate buffer (50 mM, pH 4.8) and supplemented with 15 Filter Paper Units (FPU) of enzyme per gram of substrate (1.4 mL) (Souza et al., 2012). The erlenmeyer flask was capped and incubated in a shaker at 50ºC and 180 rpm agitation. Samples were collected every 12 hours until 72 hours of enzymatic hydrolysis, centrifuged for 10 min at 10,000 x g, and the supernatants were used for evaluation of glucose and cellobiose concentration. The optimal presaccharification time was determined as the time for maximum glucose release and the maximum presaccharification yield in the hydrothermal pretreatment. After defining the optimal presaccharification time in hydrothermal pretreatment, the same time was used for the acid pretreatment. The acid concentration for acid pretreatments in each biomass was defined as the condition with the maximum glucose release at the optimal presaccharification time.

2.3.3 Simultaneous saccharification and fermentation

In an 125 mL erlenmeyer flask, 4 g of pretreated biomass were suspended in 50.0 mL of fermentation medium (2.5 g L^{-1} yeast extract; 2.5 g L^{-1} peptone; 2 g L^{-1} NH_4Cl; 1 g L^{-1} KH_2PO_4; and 0.3 g L^{-1} $MgSO_4.7H_2O$) in citrate buffer (50 mM, pH 4.8) and supplemented with 15 Filter Paper Units (FPU) of enzyme per gram of substrate (1.4 mL) (Souza et al., 2012). The erlenmeyer flask was capped and incubated in a shaker at 50ºC and 180 rpm. The yeast was inoculated after the optimized reaction time in biomasses pretreated by hydrothermal and acid (optimal acid concentration) process (See Experimental 2.3.2). Yeast cultures (*Saccharomyces cerevisiae* LBM-1) were inoculated, in sterile conditions in the erlenmeyer flask (from presaccharification), which was capped and incubated in a shaker at 37 °C and 180 rpm for 10 hours. After SSF, samples were collected and centrifuged for 10 min at 10,000 x g, the supernatants were analyzed for glucose and ethanol concentrations. Untreated biomasses were used as control experiment for presaccharification and SSF. SSF was performed in duplicate for untreated and treated biomasses.

2.4 Analyses

2.4.1 Determination of chemical composition and pretreatment parameters

Chemical characterization was performed at 40/60 mesh sawdust, produced by using a Wiley mill bench model. Sawdust was dried at room temperature (23 ± 1ºC and 50 ± 2% relative humidity) to constant weight and saved in airtight containers. The moisture content was determined according to TAPPI T 264 cm-07. Chemical analyses for raw material were conducted in triplicate and for pretreated biomasses in duplicate.

The following chemical analyses were performed for determination of chemical composition: ash content (TAPPI 211 om-02), silica content (insoluble part of ash remained after acid hydrolysis with HCl) (TAPPI 244 cm-11), total extractives content (1:2 ethanol-toluene for 5 hours → 95% ethanol for 4 hours → hot water for 1 hour) (TAPPI T 264 cm-07), Klason lignin was determined according to Gomide and Demuner (1986) and corrected by the silica content according to Carvalho et al. (2015), soluble lignin (Goldschimid, 1971), anhydrosugars content (glucose, xylose, galactose, mannose and arabinose) (Wallis et al., 1996), uronic acids (Scott, 1979) and acetyl groups (Solar et al., 1987).

Determination of complete mass balance for raw materials and pretreated biomasses were calculated according to Carvalho et al. (2015). The pH was determined in the liquor after pretreatments and the yield was determined gravimetrically on the solid fraction.

2.4.2 Determination of enzymatic hydrolysis and fermentation parameters

Glucose and cellobiose concentrations were determined in samples collected after presaccharification by using an HPLC instrument with refractive index detector and HPX-87 H / BIORAD column (300 mm x 8.7 mm). Mobile phase was water with 0.05 mM sulfuric acid; flow rate was 0.5 mL/min; column pressure: 1200 psi; and injected volume was 20 µL.

The optimal presaccharification time was defined in function of the glucose concentration and presaccharification yield. Presaccharification yield ($\boldsymbol{Y_{G/B}}$) ($g_{glucose}/g_{biomass}$) was calculated by dividing the difference between the final ($\boldsymbol{Glu_f}$) and initial ($\boldsymbol{Glu_i}$) glucose mass (g) released from biomass during presaccharification (measured in samples collected from presaccharification medium) by the total mass of biomass (g) used for presaccharification test (***Biomass***), according to Eq. 1 (Souza et al., 2012).

$$Y_{G/B} = \frac{Glu_f - Glu_i}{Biomass} \quad (1)$$

Glucose yield ($\boldsymbol{G_Y}$) (%) was calculated by dividing the difference between the final ($\boldsymbol{Glu_f}$) and initial ($\boldsymbol{Glu_i}$) glucose mass (g) released from biomass during presaccharification (measured in samples collected from presaccharification medium) by the glucose content present in the pretreated biomass used for presaccharification test obtained by the complete mass balance (g) ($\boldsymbol{Glu_B}$), according to Eq. 2.

$$G_Y = \frac{Glu_f - Glu_i}{Glu_B} \times 100 \quad (2)$$

Glucose and ethanol concentrations were determined in samples collected after the fermentation by using a refractive index HPLC detector according the following conditions: column: HPX-87 H / BIORAD; measurement: 300 mm x 8.7 mm diameter;

mobile phase: water with 0.05 mM sulfuric acid; rate flow: 0.7 mL/min; column pressure: 1920 psi; and injected volume: 10 µL.

Biothanol production was evaluated based on ethanol yield and volumetric productivity of ethanol, according to Souza et al. (2012). Ethanol yield ($\boldsymbol{Y_{E/B}}$) ($g_{ethanol}/g_{biomass}$) was calculated at the end of the fermentation (10 hours) by dividing the difference between the final ($\boldsymbol{EtOH_f}$) and initial ($\boldsymbol{EtOH_i}$) ethanol mass (g) (measured in samples collected from fermentation medium) by the total mass of biomass (g) used for SSF (***Biomass***), according to the Eq. 3.

$$Y_{E/B} = \frac{EtOH_f - EtOH_i}{Biomass} \quad (3)$$

Volumetric productivity of ethanol ($\boldsymbol{Q_P}$) (g L^{-1} h^{-1}) was calculated by dividing the maximum concentration of ethanol ($\boldsymbol{EtOH_f}$ g L^{-1}) achieved (measured in samples collected from fermentation medium), by the time of fermentation (***t***) in hours, according to the Eq. 4.

$$Q_P = \frac{EtOH_f}{t} \quad (4)$$

3. Results and discussion

3.1 Effects of pretreatments on the processing yield and chemical composition of biomasses

Eucalyptus wood contains naturally more acid groups in the xylan structure than bagasse and straw (Carvalho, 2015), which explains the lower end pH of hydrolizate after hydrothermal pretreatment (Table 1). Decreasing in final pH reduced the pretreatment yields, irrespectively to the biomass. Eucalyptus wood presented higher pretreatment yields than bagasse and straw, regardless of the pretreatment condition. Increasing acid concentration over 1.5% had insignificant impact on yield for the eucalyptus wood but a large effect on the yields of bagasse and straw. Using the maximum acid concentration (4.5% H_2SO_4), the final pH of pretreatments was similar for all biomasses.

Table 1 - Correlation coefficient between final pH and yield of pretreatments.

Biomass	Parameters	Hydrothermal	Acid 1.5%	Acid 3.0%	Acid 4.5%	CC[a]
Eucalyptus	pH	3.4 (0.1)	1.3 (0.1)	1.5 (0.1)	1.5 (0.3)	0.97
	Yield, %[a]	91.5 (0.6)	80.8 (1.0)	80.1 (2.6)	78.5 (3.3)	
Bagasse	pH	4.5 (0.0)	2.3 (0.0)	1.4 (0.0)	1.2 (0.0)	0.99
	Yield, %[a]	84.5 (0.5)	66.7 (2.9)	57.5 (2.3)	50.8 (0.1)	
Straw	pH	5.3 (0.0)	4.0 (0.0)	3.2 (0.1)	1.5 (0.0)	0.93
	Yield, %[a]	80.2 (0.8)	70.5 (0.3)	58.2 (0.8)	55.8 (0.3)	

(…) Standard deviation.

[a] Correlation coefficient.

The complete mass balance for untreated and pretreated biomasses (Table 2) was combined with pretreatments yield to obtain the chemical composition of biomasses after pretreatments (Fig. 2).

Table 2 - Chemical composition (lignin, anhydrosugar, ashes and extractives/pseudo-extractives) of the untreated and pretreated biomasses reported based on the complete mass balance[a].

Biomass	Pretreatment	Lignin, %[b]	Glucose, %	Other sugars, %[c]	Ash, %	Extractives,%[d]
Eucalyptus	Raw material	27.4	49.9	20.3	0.2	2.3
	Hydrothermal	25.2	54.0	8.2	0.2	12.4
	Acid 1.5%	23.5	57.0	1.3	0.3	17.9
	Acid 3.0%	23.6	57.5	1.0	0.8	17.2
	Acid 4.5%	22.9	55.8	1.3	1.1	18.9
Bagasse	Raw material	18.0	36.0	28.7	2.3	15.0
	Hydrothermal	25.5	42.6	25.4	1.0	5.6
	Acid 1.5%	24.1	53.5	6.1	1.4	14.8
	Acid 3.0%	24.5	58.4	1.9	1.4	13.8
	Acid 4.5%	25.7	58.7	1.0	1.5	13.2
Straw	Raw material	13.8	36.3	29.8	7.9	12.2
	Hydrothermal	22.1	40.4	28.4	5.2	3.8
	Acid 1.5%	22.5	46.0	18.6	5.1	7.8
	Acid 3.0%	25.2	52.3	6.0	5.5	11.0
	Acid 4.5%	27.8	52.4	2.9	5.4	11.5

[a] Calculated from average of chemical components.

[b] Pseudo-lignin formed for bagasse and straw during hydrothermal and acid pretreatments.

[c] Sum of xylose, galactose, mannose, arabinose, uronic acids and acetyl groups. Uronic acids and acetyl groups measured only in raw material.

[d] Pseudo-extractives formed for eucalyptus during hydrothermal and acid pretreatments.

The hemicelluloses were the main chemical component removed by hydrothermal and acid pretreatments. Hemicelluloses removal from biomasses is very important to bioethanol production because increases the enzymes accessibility to cellulose which favors the subsequent enzymatic hydrolysis (Alvira et al., 2010). An inverse correlation between final pH and hemicelluloses removal in pretreatments was observed, irrespectively of biomass type (Table 3). For eucalyptus wood, values of 63%, 95%, 96% and 95% of hemicelluloses were removed from the raw material by hydrothermal, acid 1.5%, acid 3.0% and acid 4.5% pretreatments, respectively. In

bagasse the percentages were 25%, 86%, 96% and 98%, respectively, and in straw percentages were 23%, 56%, 88% and 95%, respectively. Similar to the trend observed for yield, there was no significant impact of acid concentration over 1.5% on hemicelluloses removal for eucalyptus wood because final pH was similar for all amount of acid in acid pretreatments.

About 90% hemicelluloses removal from bagasse was observed by Aguilar et al. (2002) by using 2% sulfuric acid at 122ºC for 24 minutes. Esteghlalian et al. (1997) also observed about 90% hemicelluloses removal from corn stover, switchgrass and poplar wood after within the first minute of reaction by using 0.9% sulfuric acid at 180ºC. In present study, results indicated that the hemicelluloses removal from biomasses was dependent on final pH (Table 3).

Table 3 - Correlation coefficient between final pH of pretreatments and the hemicelluloses amount remaining in the pretreated biomasses.

Biomass	Parameters	Raw material	Hydrothermal	Acid 1.5%	Acid 3.0%	Acid 4.5%	CC[b]
Eucalyptus	pH	-	3.4	1.3	1.5	1.5	0.99
	Hemic., g[a]	20.3	7.5	1.0	0.8	1.0	
Bagasse	pH	-	4.5	2.3	1.4	1.2	0.99
	Hemic., g[a]	28.7	21.5	4.1	1.1	0.5	
Straw	pH	-	5.3	4.0	3.2	1.5	0.93
	Hemic., g[a]	29.8	22.8	13.1	3.5	1.6	

[a] Hemicelluloses represented by the sum of xylose, galactose, mannose and arabinose present in biomasses after pretreatments expressed in grams (obtained by the combination of complete mass balance and actual yield of each biomass). Hemicelluloses amount in raw materials includes also uronic acid and acetyl groups.
[b] Correlation coefficient.

Lignin structure was transformed during hydrothermal and acid pretreatments. It has been claimed that lignin can be removed and/or converted into condensed structures during hydrothermal and acid pretreatments (Alvira et al., 2010; Lora and Wayman, 1987). The formation of pseudo-lignin from lignin and polysaccharides degradation products may be possible during acid pretreatment. The presence of pseudo-lignin in biomass may impair the enzymes activity during saccharification (Sannigrahi et al., 2011).

In order to avoid the formation of pseudo-lignin, milder acidic conditions have been suggested (Hu et al., 2012; Sannigrahi et al., 2011). However, in present study, the formation of pseudo-lignin was observed in bagasse and straw even in milder acidic conditions (hydrothermal pretreatment). Higher amount of pseudo-lignin was observed at higher acid pH for bagasse and straw. Native lignin from bagasse and straw presents significant amount of guaiacyl (G), syringyl (S) and the *p*-hydroxyphenyl (H) units of

lignin. In eucalyptus wood only guaiacyl (G) and syringyl (S) units are present (Brandt et al., 2013). Because of methoxy groups in position C3 (for S and G lignin) and C5 (for S lignin), S lignin generates less condensed structures and structures more easily removed from lignocellulosic biomasses than G lignin. Similarly, S and G lignins are more reactive and easily removed from lignocellulosic biomasses than H lignin (absence of methoxy groups in the lignin structure) (Santos et al., 2011). The lignin removal from eucalyptus wood was 16% (hydrothermal), 31% (acid 1.5% and 3.0%) and 34% (acid 4.5%). In bagasse the lignin removal was 10% (acid 1.5%), 22% (acid 3.0%) and 27% (acid 4.5%). In bagasse pretreated by hydrothermal pretreatment and straw pretreated by hydrothermal and acid pretreatments pseudo-lignin was generated (Table 4).

Table 4 - Correlation coefficient between end pH of pretreatments and the lignin amount remained in the pretreated biomasses.

Biomass	Parameters	Raw material	Hydrothermal	Acid 1.5%	Acid 3.0%	Acid 4.5%	CC[b]
Eucalyptus	pH	-	3.4	1.3	1.5	1.5	0.96
	Lignin, g [a]	27.4	23.1	19.0	18.9	18.0	
Bagasse	pH	-	4.5	2.3	1.4	1.2	1.00
	Lignin, g [a]	18.0	21.5	16.1	14.1	13.1	
Straw	pH	-	5.3	4.0	3.2	1.5	0.72
	Lignin, g [a]	13.8	17.7	15.9	14.7	15.5	

[a] Lignin amount present in biomasses after pretreatments expressed in grams (obtained by the combination of complete mass balance and actual yield of each biomass).
[b] Correlation coefficient.

Glucose amount in biomasses decreased significantly less than the hemicelluloses or lignin during pretreatments. Glucose removal increased with decreasing final pH and the maxima glucose removal for eucalyptus, bagasse and straw were 12.2%, 17.2% and 19.5%, respectively.

Table 5 - Correlation coefficient between final pH of pretreatments and the glucose amount remained in the pretreated biomasses.

Biomass	Parameters	Raw material	Hydrothermal	Acid 1.5%	Acid 3.0%	Acid 4.5%	CC[b]
Eucalyptus	pH	-	3.4	1.3	1.5	1.5	0.86
	Glucose, g [a]	49.9	49.4	46.1	46.1	43.8	
Bagasse	pH	-	4.5	2.3	1.4	1.2	0.73
	Glucose, g [a]	36.0	36.0	35.7	33.6	29.8	
Straw	pH	-	5.3	4.0	3.2	1.5	0.93
	Glucose, g [a]	36.3	32.4	32.4	30.4	29.2	

[a] Glucose amount present in biomasses after pretreatments expressed in grams (obtained by the combination of complete mass balance and actual yield of each biomass).
[b] Correlation coefficient.

For eucalyptus, the formation of pseudo-extractives was observed in both, hydrothermal and acid pretreatments (Fig. 2). Pseudo-extractives are structures formed during pretreatments from fragments of lignin and polysaccharides that re-precipitated on fibers. Pseudo-extractives present similar solubility to extractives from original raw material (Carvalho et al., 2015).

Although in pretreatments of bagasse and straw the formation of pseudo-extractives was not observed, the amounts of extractives determined in these biomasses after acid pretreatments were higher than that in hydrothermal pretreated material. This result was consistent to that observed for eucalyptus, in which the amounts of pseudo-extractives formed during hydrothermal pretreatment were lower than those formed in the acid pretreatments (1.5%, 3.0% and 4.5% H_2SO_4). The acid concentration had little effect on the amount of eucalyptus pseudo-extractives above 1.5% w/w concentration and the same happened for yield loss and hemicelluloses removal. This trend is explained by the insignificant change in final pH when acid concentration was increased from 1.5% up to 4.5%.

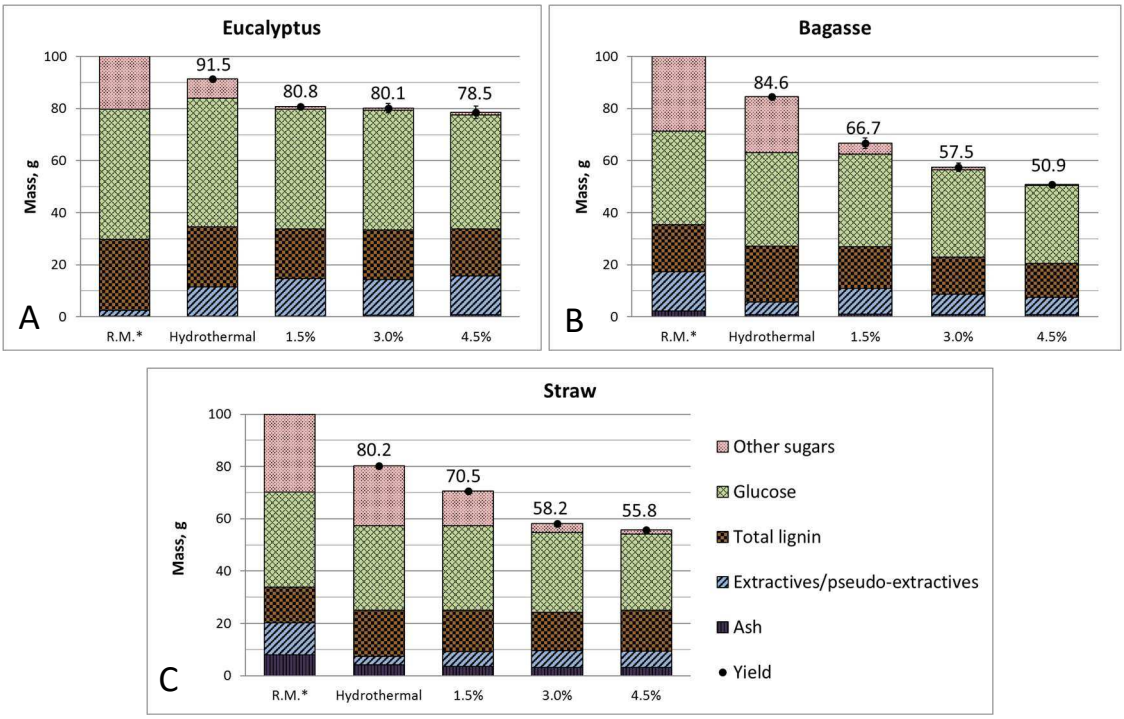

Figure 2 - Chemical composition of eucalyptus (**A**), bagasse (**B**) and straw (**C**) before (raw material - R.M.*) and after the pretreatments (obtained by the combination of complete mass balance and actual yield of each biomass).

Fragments of polysaccharides and lignin were responsible for both, pseudo-lignin and pseudo-extractives formation, but the results suggested that in bagasse and

straw the formation of pseudo-lignin seems to be the preferred path for these fragments. Differences in lignin structure and reactivity (e.g. S, G and H lignin) may be determining factors to the preferential formation of pseudo-extractives or pseudo-lignin.

3.2 Simultaneous saccharification and fermentation

3.2.1 Presaccharification tests

It is not surprising that a positive effect of the pretreatment on the enzymatic hydrolysis was observed for all tested biomasses. Glucose release by untreated biomasses during presaccharification was extremely low and only slightly affected by the time (Fig. 3), most likely due to the natural low porosity and, as consequence, low accessibility towards enzymes of untreated biomasses. In addition, in untreated biomasses the cellulose presents a limited accessibility to enzymes by virtue of a natural barrier building by lignin and hemicelluloses. This barrier justifies the need of pretreatments in order to improve sugar release during process for bioethanol production from lignocellulosic materials (Öhgren et al., 2007; Taharzadeh and Karimi; 2007). The time of 24 hours for presaccharification was fixed for all pretreated biomasses because this time reached the maximum glucose release for biomasses hydrothermically pretreated and also achieved satisfactory glucose release for biomasses pretreated using acid pretreatments, in spite of not being actually the maximum glucose concentration (Fig. 3).

Glucose concentration for eucalyptus, bagasse and straw hydrothermally pretreated were 12.9, 13.2 and 11.5 g L^{-1}, respectively. At the presaccharification time of 24 hours, the maximum glucose release in acid pretreated materials occurred at the concentration of 1.5% for eucalyptus and 4.5% for bagasse and straw. At this condition, the glucose concentration for eucalyptus pretreated with 1.5% acid was 15.7 g L^{-1}. For bagasse and straw pretreated with 4.5% acid the glucose concentrations were 20.5 g L^{-1} and 22.0 g L^{-1}, respectively. Unlike the eucalyptus pretreated with 1.5% acid, for which the glucose release stabilized after 24 hours presaccharification, for bagasse and straw pretreated with 4.5% acid, the glucose release kept increasing from 24 to 72 hours presaccharification, but in a lower rate than that from 0 to 24 hours.

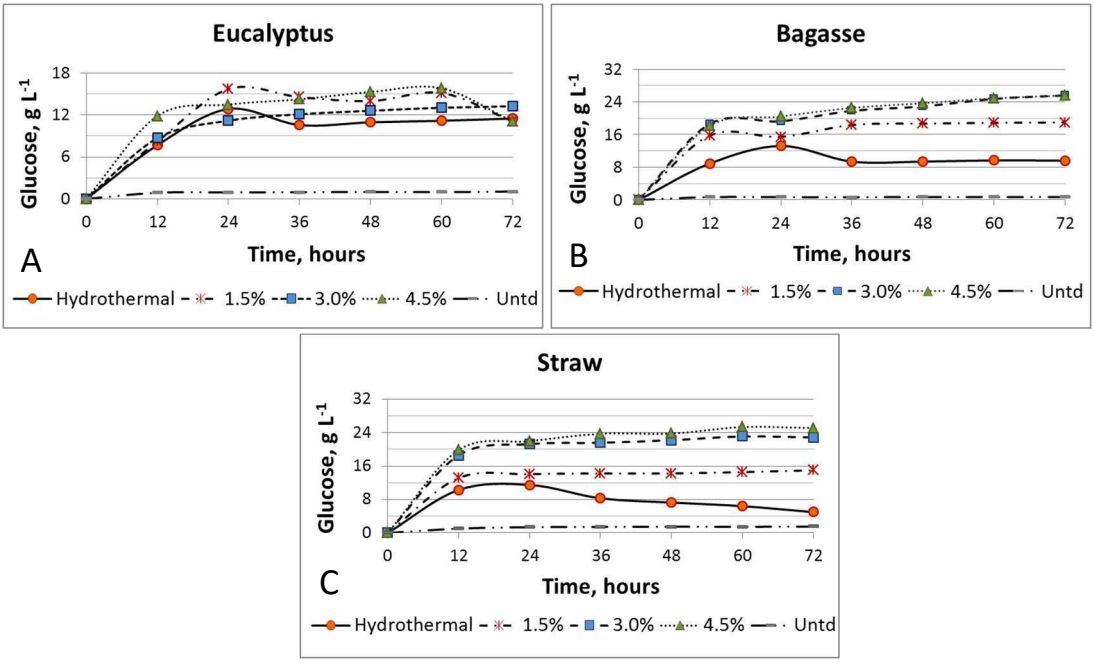

Figure 3 - Glucose content released from eucalyptus (**A**), bagasse (**B**) and straw (**C**) for untreated biomasses (Untd) and biomasses pretreated by hydrothermal and acid (1.5%, 3.0% and 4.5% H_2SO_4) pretreatments after 0, 12, 24, 36, 48, 60 and 72 hours of presaccharification.

Souza et al. (2012) obtained similar results when studying delignified bagasse. These authors obtained about 20.0 g L^{-1} glucose at 24 hours presaccharification. They noted a trend of decreasing glucose release rate with increasing time of enzymatic hydrolysis over 24 hours.

During enzymatic hydrolysis the cellulose chain can be cut off in dimers of glucose named as cellobiose before the complete glucose individualization (Baeyens et al., 2015). Cellobiose is not fermentable to bioethanol and can also inhibit cellulases during SSF (Philippidis et al., 1993). At 24 hours of presaccharification, cellobiose concentration lower than 0.13 g L^{-1} was recorded for the pretreated biomasses (Table 6). No cellobiose was detected for the untreated biomasses during presaccharification, irrespectively of enzymatic hydrolysis time.

Table 6 - Cellobiose concentration at 24 hours of presaccharification for pretreated biomasses.

Cellobiose, g L^{-1}	Eucalyptus	Bagasse	Straw
Hydrothermal	0.10	0.09	0.07
Acid 1.5%	0.09	0.08	0.08
Acid 3.0%	0.12	0.10	0.08
Acid 4.5%	0.11	0.10	0.13

Presaccharification yield was calculated from glucose concentration after 24 hours of presaccharification. The acid concentrations in acid pretreatment that maximized the presaccharification yield were 1.5% acid for eucalyptus wood and 4.5% acid for bagasse and straw. Very low presaccharification yield was observed for untreated biomasses (Fig. 4). An increase in presaccharification yields were observed from hydrothermal to the various acid pretreatment, irrespective of biomass type, which can be explained by the lower amount of lignin and hemicelluloses remained in pretreated biomasses, that caused improvements in cellulose accessibility. Eucalyptus and bagasse presented similar presaccharification yield after hydrothermal pretreatment.

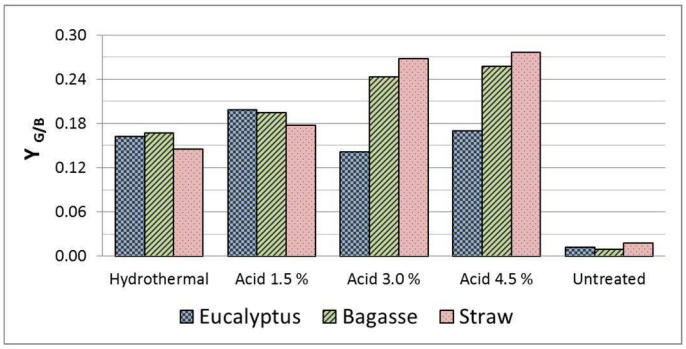

Figure 4 - Presaccharification yield ($Y_{G/B}$) measured at 24 hours for pretreated and untreated biomasses.

Eucalyptus, bagasse and straw have xylan with different structures, including different amount of acid groups on structure (Carvalho, 2015) and these differences impact the pretreatments. The eucalyptus, which contains higher amount of acid groups, achieved the highest removal of hemicelluloses (63%) and lignin (16%) amoung the biomasses during hydrothermal pretreatment, which most likely improved the cellulose accessibility to enzymes during presaccharification. In bagasse and straw, besides the lowest hemicellulose removal (25% and 23%, respectively) pseudo-lignin was also generated (Fig. 2). Besides that, the type of lignin (S, G and H lignin), and not only the amount, affects differently in the enzymatic hydrolysis. Berlin et al. (2005) investigated the effect of hardwood and softwood lignin on inhibition of cellulose hydrolysis during enzymatic process by using cellulase from *T. reesei* and observed that the different lignins drive to different levels of enzyme inhibition. The aforementioned authors demonstrated also the existence of unproductive binding of enzymes to lignin, which decreased the enzymes efficiency during the hydrolysis of cellulose.

A narrow correlation between the final pH in pretreatments and presaccharification yield was observed for bagasse and straw (Table 7), which

suggested that for these biomasses, the pH control is an important tool to improve presaccharification process.

Table 7 - Correlation coefficient between the final pH in pretreatments and presaccharification yield. All presaccharification tests were performed using buffer at pH of 4.8.

Biomass	Parameters	Hydrothermal	Acid 1.5%	Acid 3.0%	Acid 4.5%	CC [c]
Eucalyptus	pH[a]	3.4	1.3	1.5	1.5	-0.24
	$Y_{G/B}$[b], ($g_{glucose}/g_{biomass}$)	0.162	0.198	0.141	0.170	
Bagasse	pH[a]	4.5	2.3	1.4	1.2	-0.93
	$Y_{G/B}$[b], ($g_{glucose}/g_{biomass}$)	0.166	0.195	0.243	0.258	
Straw	pH[a]	5.3	4.0	3.2	1.5	-0.91
	$Y_{G/B}$[b], ($g_{glucose}/g_{biomass}$)	0.145	0.177	0.268	0.277	

[a] Final pH in pretreatments.

[b] Presaccharification yield.

[c] Correlation coefficient. Negative values indicate negative correlation, i.e., by decreasing pH in pretreatments the presaccharification yield of biomasses increased.

Results of chemical composition of pretreated biomasses and presaccharification yield suggested that bagasse and straw, which contained less lignin and hemicelluloses, were more adequate to enzymatic hydrolysis process. No clear trends were observed in the case of eucalyptus wood, which showed very little variations in chemical composition and yield in the acid pretreatment regardless of acid concentration.

After 24 hours presaccharification, the highest glucose yields were observed for biomasses pretreated by acid processes compared to hydrothermal processes (Fig. 5), indicating better cellulose accessibility than biomasses pretreated by hydrothermal process. The cellulose accessibility in straw (untreated) was naturally higher than that for bagasse or eucalyptus (Fig. 5). Straw pretreated by 4.5% acid achieved 52.8% glucose release during presaccharification. Glucose release from biomasses can be even higher if the SSF process is considered, because glucose keeps being released continuously by enzymatic hydrolysis as long as it is consumed to produce ethanol, but in the case of SSF, with reduced enzymes inhibition.

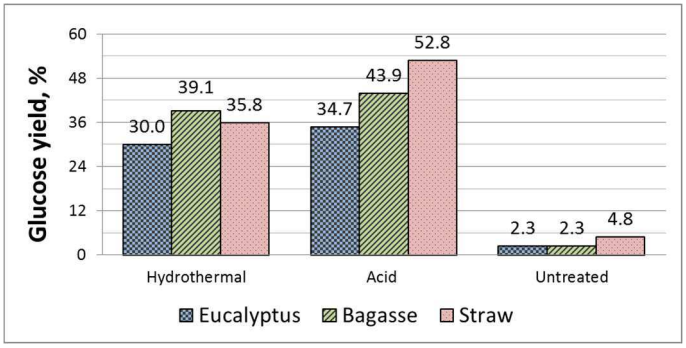

Figure 5 - Glucose yield (G_Y) measured at 24 hours for untreated biomasses and biomasses pretreated by hydrothermal and acid pretreatments at optimal acid condition (1.5% acid for eucalyptus and 4.5% acid for bagasse and straw).

3.2.2 Assessment of bioethanol production

As expected, besides improving enzymatic hydrolysis of biomasses, the pretreatments enhance bioethanol production (Fig. 6). The ethanol yields from bagasse and straw after acid pretreatment (acid 4.5%) were respectively 7.7 and 8.1 times higher than the ethanol yield for these biomasses pretreated by hydrothermal process. However, the eucalyptus pretreated by hydrothermal process generated ethanol yield only 1.2 times higher than the eucalyptus pretreated by 1.5% acid.

Untreated eucalyptus and eucalyptus pretreated *via* hydrothermal process presented ethanol yield and volumetric productivity of ethanol significant higher than those from bagasse and straw. These results differed from those from presaccharification in which similar presaccharification yields were observed for untreated biomasses and also between biomasses pretreated *via* hydrothermal process. This results suggested that chemical composition of biomasses affects the performance of SSF and the process of inhibition of yeast. Eucalyptus, bagasse and straw pretreated *via* hydrothermal process produced ethanol yields 5.1, 3.6 and 4.3 times higher than untreated counterparts.

The acid process was more efficient as pretreatment for bioethanol production from bagasse and straw. Ethanol yield increased by 28.3 and 35.1 times for bagasse and straw, respectively, compared to untreated biomasses. For eucalyptus pretreated by acid process, ethanol yield increased only 4.2 times compared to untreated biomass and also decreased compared to eucalyptus pretreated by hydrothermal process, which confirmed the effect of pretreatment on the chemical composition of pretreated eucalyptus and its inhibition of yeast. Straw presented the highest ethanol yield and volumetric productivity of ethanol among the biomasses. Straw pretreated with 4.5% acid produced

ethanol concentration of 5.1 g L^{-1}. Sugarcane bagasse and eucalyptus produced only 3.6 g L^{-1} and 1.3 g L^{-1} ethanol, respectively.

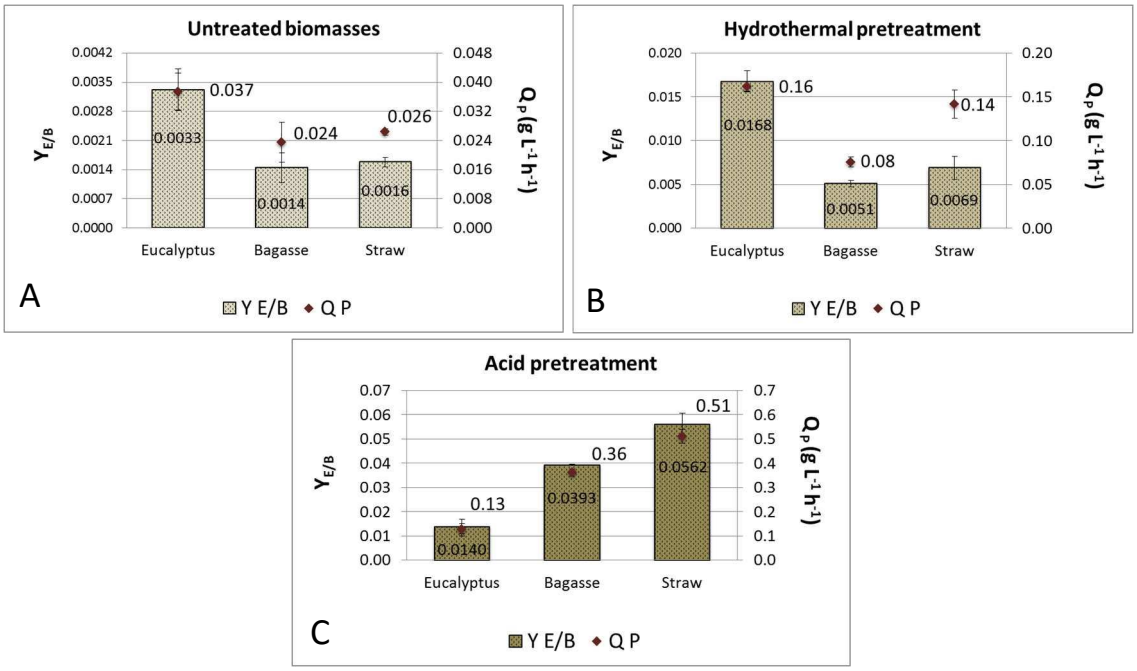

Figure 6 - Column graphic shows the ethanol yield ($Y_{E/B}$) and scatter graphic shows the volumetric productivity of ethanol (Q_P) after 24 hours of presaccharification and 10 hours of simultaneous saccharification and fermentation for untreated biomasses (**A**) and biomasses pretreated by hydrothermal (**B**) and acid (**C**) pretreatment. Acid pretreatment was performed at 1.5% acid for eucalyptus and 4.5% acid for bagasse and straw.

Santos et al. (2010) achieved ethanol concentration for sugarcane bagasse almost threefold higher than that obtained in the present study by performing 24 hours SSF after 16 hours presaccharification; however, these authors used alkaline pretreatment for bagasse delignification, which most likely favored the enzymatic hydrolysis by improving the cellulose accessibility. The volumetric productivity of ethanol achieved by these aforementioned authors for bagasse (0.30 g L^{-1} h^{-1}) was higher than the observed in the present study after hydrothermal pretreatment (0.08 g L^{-1} h^{-1}) but lower than the observed after acid pretreatment (0.36 g L^{-1} h^{-1}). However, these authors considered the time for volumetric productivity of ethanol as the sum of presaccharification time (16 hours) and SSF (24 hours). Higher volumetric productivity of ethanol for delignified bagasse was also observed by Souza et al. (2012) (~ 1.1 g L^{-1} h^{-1}) for 24 hours presaccharification and 8 hours SSF. This information combined to the results in present study confirms the observation of Cardoso et al. (2013), that residual lignin affects enzymatic hydrolysis more significantly than residual hemicelluloses. The

ethanol yield achieved by Souza et al. (2012) was higher than that obtained in the present study, but these authors used alkaline pretreatment.

The glucose yield (measured after presaccharification) effect on SSF parameters differed between hydrothermal and acid pretreatments. For acid pretreatment, a positive correlation was observed between glucose yield and ethanol yield as well as between glucose yield after presaccharification and volumetric productivity of ethanol (Table 8). These results indicated similar behavior of enzymatic hydrolysis during presaccharification and SSF. Surprisingly, for hydrothermal pretreatment a negative correlation was observed between these aforementioned parameters. These results indicated not only differences in enzymatic hydrolysis in presaccharification and SSF as well suggested different inhibition processes on yeast between biomasses, most likely due their different chemical composition, including residual hemicelluloses and residual lignin, in addition to the presence of the potential inhibitors generated during pretreatments: pseudo-lignin (as described by Sannigrahi et al., 2011) and pseudo-extractives. This study did not prove the inhibition mechanism of pseudo-extractives on SSF, but the increasing in pseudo-extractives content in eucalyptus pretreated by acid process compared to that pretreated by hydrothermal processes suggested a negative effect of the pseudo-extractives amount on the ethanol production.

Table 8 - Correlation coefficient between glucose yield (G_Y) after presaccharification and ethanol yield ($Y_{E/B}$) after SSF and between glucose yield (G_Y) after presaccharification and volumetric productivity of ethanol (Q_P) after SSF.

Parameters	Eucalyptus	Bagasse	Straw	CC[a]
Hydrothermal pretreatment				
G_Y, %	30.0	39.1	35.8	-
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.0168	0.0051	0.0069	-0.98
Q_P, g L^{-1} h^{-1}	0.16	0.08	0.14	-0.91
Acid pretreatment				
G_Y, %	34.7	43.9	52.8	-
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.0140	0.0393	0.0562	0.99
Q_P, g L^{-1} h^{-1}	0.13	0.36	0.51	0.99

[a] Correlation coefficient between G_Y and $Y_{E/B}$ and between G_Y and Q_P. Negative values indicate negative correlation.

Residual glucose was observed for all the samples and they were somewhat similar (Table 9), irrespective of pretreatment type. The residual glucose concentrations of this study were lower than those observed by Santos et al. (2010) in a similar study considering 16 hours presaccharification and 24 hours SSF for delignified bagasse. Souza et al. (2012) found no residual glucose after 10 hours SSF.

Table 9 – Residual glucose concentration in fermentation medium after SSF.

Glucose, g L^{-1}	Eucalyptus	Bagasse	Straw
Untreated biomass	$2.07^{(0.32)}$	$2.65^{(0.14)}$	$1.97^{(0.17)}$
Hydrothermal pretreatment	$2.25^{(0.18)}$	$2.88^{(0.01)}$	$2.73^{(0.01)}$
Acid pretreatment[a]	$2.21^{(0.11)}$	$1.88^{(0.25)}$	$2.56^{(0.59)}$

$^{(\ldots)}$ Standard deviation.
[a]1.5% acid for eucalyptus and 4.5% acid for bagasse and straw.

4. Conclusions

- The fragments of lignin and polysaccharides degradation products generated during pretreatments (acid and hydrothermal) lead to formation of pseudo-lignin in bagasse and straw or pseudo-extractives in eucalyptus;
- The removal of lignin and hemicelluloses from bagasse and straw increased with increasing acid concentration in the range of 1.5% - 4.5% w/w H_2SO_4, whereas for eucalyptus wood no improvement was seen over 1.5% H_2SO_4;
- Maximum ethanol yield was achieved with hydrothermal pretreatment for eucalyptus wood and with acid pretreatment at 4.5% w/w H_2SO_4concentration for sugarcane bagasse and straw;
- The glucose released during presaccharification was negatively influenced by the remaining amount of lignin and hemicelluloses in biomasses after pretreatments;
- Eucalyptus pretreated in hydrothermal condition was more effective for producing ethanol (0.017 $g_{ethanol}/g_{biomass}$) than eucalyptus pretreated in acid condition (0.014 $g_{ethanol}/g_{biomass}$) most likely due to the highest formation of pseudo-extractives, a potential SSF inhibitor;
- Acid condition was more effective than hydrothermal condition to pretreat bagasse and straw, generating ethanol yields of 0.039 $g_{ethanol}/g_{biomass}$ and 0.056 $g_{ethanol}/g_{biomass}$, respectively, most likely due to the lower amount of remaining hemicellulose and lignin and lower pseudo-lignin formation; and
- Among the three biomasses evaluated, the straw pretreated with 4.5% acid provided the highest ethanol yield (0.056 $g_{ethanol}/g_{biomass}$), ethanol concentration (5.1 g L^{-1}) and volumetric productivity of ethanol (0.51 g L^{-1} h^{-1}).

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Article IV

Assessment of alkaline pretreatment for bioethanol production from eucalyptus, sugarcane bagasse and straw

ABSTRACT - The impact of alkaline pretreatment at different alkaline charges (5%, 10% and 15% w/w NaOH, on dry basis) on the chemical composition of eucalyptus, sugarcane bagasse and straw was compared with a view to their subsequent bioconversion into ethanol using simultaneous saccharification and fermentation (SSF) with preliminary presaccharification step. The effectiveness of removal of lignin, hemicelluloses and cellulose from biomass increased with increasing in alkaline charge. Pretreatments promoted delignification in the range of 11%-51% for eucalyptus, 22%-90% for bagasse and 60%-99% for straw for the alkaline charges in the range of 5%-15% (w/w NaOH). Lignin removal from bagasse and straw was higher than that from eucalyptus due to the combined effect of higher frequency of both, free phenolic groups and ester bonds in grass lignins, which increases the lignin solubility in alkaline conditions. It was also observed that bagasse had a removal by 37%-45% hemicelluloses and 0.8%-11% cellulose. For straw, higher amount of carbohydrates was removed, in the range of 55%-66% hemicelluloses and 19%-36% cellulose. For eucalyptus, fragments of lignin and carbohydrates were converted into pseudo-extractives, thus increasing the total extractives contents by 3.3 (5% NaOH), 3.5 (10% NaOH) and 2.9 (15% NaOH) times, in relation to the original raw material. Maximum ethanol yield and maximum volumetric productivity of ethanol were achieved for eucalyptus pretreated using 10% NaOH, bagasse pretreated using 15% NaOH and straw pretreated using 5% NaOH. At the optimal pretreatment condition, approximately 51% glucose was released from pretreated bagasse. Similar behavior (reactions/inhibitions) in enzymatic hydrolysis during presaccharification and SSF was observed for biomasses with positive correlation between glucose yield of presaccharification and ethanol yields, and also between glucose yield of presaccharification and volumetric productivity of ethanol. Sugarcane bagasse presented the highest values for the respective parameters, namely: 8.8 g L^{-1} ethanol concentration, 0.101 $g_{ethanol}/g_{biomass}$ ethanol yield and 0.88 g L^{-1} h^{-1} volumetric productivity of ethanol. The results suggest that the ethanol production from bagasse can be improved by increasing the SSF time, since after 10 hours SSF approximately 5.3 g L^{-1} glucose had still remained in fermentation medium. Alkaline charge proved to be an important control variable for alkaline pretreatments, with determinant effect on chemical transformations of biomasses and result on ethanol production.

Keywords: Alkaline charge, eucalyptus wood, pseudo-extractives, sugarcane bagasse, sugarcane straw.

RESUMO - O impacto do pré-tratamento alcalino em diferentes cargas alcalinas (5%, 10% e 15% NaOH m/m, em base seca) na composição química de eucalipto, bagaço e palha de cana-de-açúcar foi comparado com foco em sua subsequente bioconversão em

etanol usando sacarificação e fermentação simultânea (SFS) com pré-sacarificação. A eficiência de remoção de lignina, hemiceluloses e celulose das biomassas aumentou com o aumento da carga alcalina. Os pré-tratamentos promoveram deslignificação de 11%-51% para o eucalipto, 22%-90% para o bagaço e 60%-99% para a palha para cargas alcalinas entre 5%-15% (NaOH m/m). A remoção de lignina do bagaço e da palha foi maior que do eucalipto devido ao efeito combinado de maior frequência dos grupos fenólicos livres e de ligações éster na lignina das gramíneas, o que aumenta a solubilidade da lignina em condições alcalinas. No bagaço, remoções de 37%-45% de hemiceluloses e 0.8%-11% de celulose foram observadas. Na palha maior teor de carboidratos foi removido, entre 55%-66% de hemiceluloses e 19%-36% de celulose. No eucalipto, fragmentos de lignina e carboidratos produziram pseudo-extrativos aumentando a quantidade de extrativos em 3,3 (5% NaOH), 3,5 (10% NaOH) and 2,9 (15% NaOH) vezes em relação à matéria-prima. O rendimentos em etanol máximo e a máxima produtividades médias de etanol foram obtidas para eucalipto pretratado usando 10% NaOH, para bagaço pretratado usando 15% NaOH e para palha pretratada usando 5% NaOH. Na condição ótima de pré-tratamento, aproximadamente 51% de glicose foi liberada do bagaço pré-tratado. Comportamento similar (reação/inibição) na hidrólise enzimática durante a pré-sacarificação e durante a SFS foi observada para as biomassas com correlação positiva entre rendimento em glicose na pré-sacarificação e rendimento em etanol, bem como entre rendimento em glicose na pré-sacarificação e produtividade volumétrica de etanol. O bagaço de cana apresentou os maiores valores para os respectivos parâmetros, a saber: concentração de etanol de 8,8 g L^{-1}, rendimento em etanol de 0,101 $g_{etanol}/g_{biomassa}$ e produtividade volumétrica de etanol de 0,88 g L^{-1} h^{-1}. Os resultados indicaram que a produção de etanol a partir de bagaço pode ser melhorada com o aumento do tempo de SFS uma vez que, após 10 horas de SFS, aproximadamente 5,3 g L^{-1} de glicose ainda tinha permanecido no meio de fermentação. A carga alcalina provou ser uma importante variável de controle para pré-tratamentos alcalinos, com efeito determinante nas transformações químicas das biomassas e, como resultado, na produção de etanol.

Palavras-chave: Carga alcalina, madeira de eucalipto, pseudo-extrativos, bagaço de cana, palha de cana.

1. Introduction

Bioethanol production from lignocellulosic biomasses, consist of sugar release from cell wall by hydrolysis (pretreatments and saccharification) and ethanol generation by fermentation of the sugars released (Rubin et al., 2008). The pretreatments are considered key steps for bioethanol production in which the increasing on cellulose accessibility is one of the major goals. The most appropriate condition for pretreatment, however, varies depending on the raw material.

Recently, a number of lignocellulosic biomasses have been studied for bioethanol production, including agricultural residues (Santos et al., 2014; Oliveira et

al., 2013; Souza et al., 2012; Petersen et al., 2009; Lloyd and Wyman et al., 2005; Saha et al., 2005), energy crops (Esteghlalian et al., 1997) and woods (Nitsos et al., 2013; Romaní et al., 2010; Ballesteros et al., 2004; Negro et al., 2003; Esteghlalian et al., 1997). In Brazilian scenario, the residues of sugarcane bagasse and sugarcane straw from sugarcane industries appear as potential feedstocks for bioethanol production, with annual generation of 92 million tons (approximately) for each residue (Conab, 2015; Oliveira et al., 2013). In addition, the eucalyptus stands out as a potential woody feedstock due its high productivity and adaptability to Brazilian climate compared to other wood species.

Lignocellulosic biomasses are basically comprised of lignin, cellulose, hemicelluloses, extractives and ash. The first three components are arranged in cell wall forming a complex network in which lignin and hemicelluloses build a physical protection around the cellulose. The objective of several pretreatments is to improve the cellulose accessibility by removing lignin and hemicelluloses (Esteghlalian et al., 1997). However, the effects of residual lignin and residual hemicelluloses on enzymatic hydrolysis are not the same. Cardoso et al. (2013) observed that the effect of residual hemicelluloses on enzymatic hydrolysis was irrelevant compared to the residual lignin effect for sorghum straw pretreated by acid (H_2SO_4), alkaline (NaOH) and acid (H_2SO_4) followed by alkaline (NaOH) conditions, due to the physical barrier built by lignin. This chemical component also participates in nonproductive binding with enzymes, which contributes to the decrease in performance of enzymatic hydrolysis (Berlin et al., 2005). In addition, pseudo-lignin, can be generated during pretreatments, especially under acid conditions (Alvira et al., 2010; Lora and Wayman, 1987). Pseudo-lignin has been reported a powerful inhibitor for enzymes activity (Sannigrahi et al., 2011).

Alkaline pretreatments are common for the biomass delignification, with some effects on the increasing of surface area, accessibility, cellulose swelling and hemicelluloses removal. The alkaline source most often used for alkaline pretreatments is the sodium hydroxide (NaOH). This pretreatment can be performed at room temperature or at higher temperature (20-121ºC) during time ranging from minutes to hours (15 minutes - 12 hours) and alkaline concentration from 0.5% to 20% (Menezes et al., 2014; Alvarez et al., 2013; Sheikh et al., 2013; Souza et al., 2012; Alvira et al., 2010; Cardona et al., 2010; Santos et al., 2010; Xu et al., 2010). Menezes et al. (2014) observed removal around of 75%, 56% and 31% for lignin, hemicelluloses and cellulose, respectively, from coffee pulp after alkaline pretreatment at 121ºC for 25 minutes by using 4% NaOH (w/v) with a pretreatment yield of approximately 18%.

This pretreated biomass was used for ethanol production by saccharification and fermentation generating 12 g L^{-1} ethanol. Xu et al. (2010) performed alkaline pretreatments under different conditions (0.25 - 1 hour at 121ºC, 1 - 48 hours at 50ºC and 1 - 96 hours at 21ºC) and alkaline charge (0.5% - 2% w/v NaOH) and observed higher lignin removal for alkaline pretreatment at 121ºC for 30 minutes and 1% NaOH with a positive effect of pretreatment temperature on lignin removal.

For bioconversion of sugars to ethanol the combination of presaccharification and simultaneous saccharification and fermentation (SSF) is usually used. Presaccharification is an enzymatic hydrolysis used for releasing sugars from the biomasses by providing sugars in the beginning of SSF. Presaccharification is performed before SSF and allows improvements in ethanol yield (Santos et al., 2010). SSF is a combined process in which enzymes for saccharification and yeasts for fermentation are provided and act together in the same reactor. During SSF process, the enzymes inhibition due to saccharification products is reduced and improvements in ethanol production is also achieved (Baeyens et al., 2015; Santos et al., 2010).

However, the temperature is one limitation of saccharification and fermentation in SSF process. The optimal temperature for enzymes (for saccharification) is usually higher than that for yeasts (for fermentation). To overcome this limitation, thermotolerant yeast strains have been investigated and have demonstrated improvements in ethanol production by SSF process (Ballesteros et al., 2004).

The production of bioethanol by SSF with initial presaccharification from biomasses pretreated by alkaline processes was purposed in the present study. The novelty of this study was to identify the effect of alkaline charge in alkaline pretreatments on the chemical transformation of biomasses and their subsequent behavior in enzymatic hydrolysis, with focus on the bioethanol production. The objectives of this study were: (i) to evaluate alkaline pretreatments (5, 10 and 15% w/w NaOH) with respect to their impact on the chemical composition of eucalyptus, sugarcane bagasse and straw and (ii) to evaluate the behaviour of the pretreated biomasses during bioethanol production by using presaccharification and SSF in sequence. It is anticipated that the results of this study will serve as a guidance for researchers and industrialists when deciding on the most suitable pretreatment technology for bioethanol production from eucalyptus wood, sugarcane bagasse and straw.

2. Experimental

2.1 Working plan

Figure 1 depicts the working plan. Eucalyptus, sugarcane bagasse and straw were chemically characterized and used in alkaline pretreatments (5%, 10% and 15% NaOH on dry basis). The pretreated biomasses were also chemically characterized and used for bioethanol production through presaccharification step followed by SSF. The optimum time for presaccharification step was defined based on the following parameters: maximum glucose release and maximum presaccharification yield. For each biomass, only the pretreated biomass produced using the amount of alkali which provide the best performance in presaccharification step followed through the subsequent bioethanol production *via* SSF.

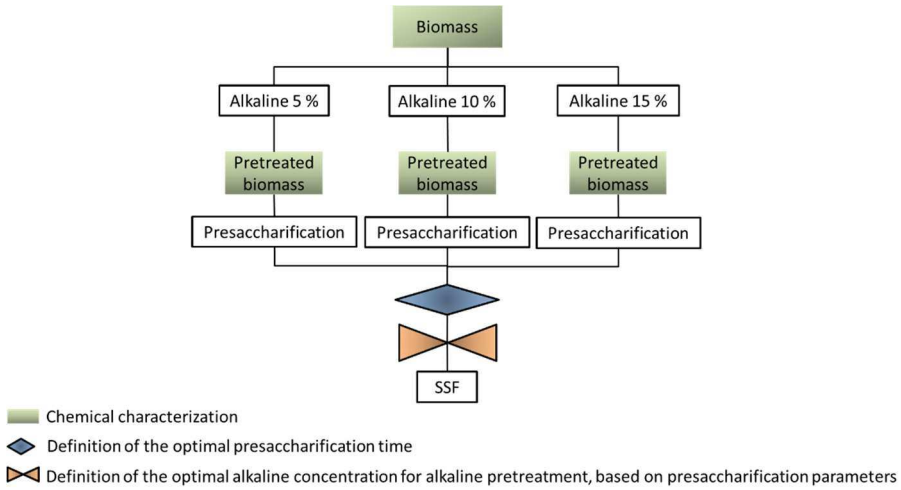

Figure 1 - Working plan for eucalyptus, bagasse and straw chemical characterization, alkaline pretreatment, presaccharification and SSF for bioethanol production.

2.2 Materials

The 7 years old clonal hybrid of eucalyptus (*Eucalyptus urophylla* x *Eucalyptus grandis*) was supplied by a Brazilian pulp company as wood chips. Chips were screened and those with dimensions smaller than 0.5 cm x 3 cm x 3 cm were collected for chemical analyses and pretreatments. Sugarcane bagasse and straw aged five-months old (cultivar RB867515) were supplied by Center Sugarcane Experimentation (Oratórios, Minas Gerais State, Brazil) at the Federal University of Viçosa after chipping (bagasse and straw) and juice removal (bagasse) (10 mm diameter). The biomasses were dried to about 85% dryness and stored in polyethylene bags at room temperature prior the use. Moisture content was determined according to TAPPI T 264

cm-07. Chemicals used were sodium hydroxide of lentils analytical grade (Merck Milipore, Germany), commercial cellulase Celluclast 1.5 L (Sigma-Aldrich, Brazil) (from *Trichoderma reesei* ATCC 26921) and *Saccharomyces cerevisiae* LBM-1 isolated from fermentation vast in Brazil.

2.3 Methods

2.3.1 Alkaline Pretreatments

One-hundred grams o.d. (oven dried equivalent) of eucalyptus wood, bagasse and straw were used for alkaline pretreatment in three alkaline charges: 5%, 10% and 15% NaOH (on a dry biomass basis). Pretreatments were performed in duplicate in a Regmed reactor (2 L capacity), under constant agitation, using the following parameters: liquor:biomass ratio = 2:1 L kg^{-1} (eucalyptus) and 7:1 L kg^{-1} (bagasse and straw); maximum temperature = 175ºC; time to maximum temperature = 90 min; and time at maximum temperature = 15 min. After the pretreatment, the reactor was cooled, and the pretreated biomasses were washed with an excess of water and centrifuged at 800 rpm for 4 minutes. Before washing, a sample of liquor was collected for pH measurement. The pretreated biomasses were conditioned for 24 hours at 23 ± 1ºC and 50 ± 2% relative humidity to constant weight and then stored at room temperature in polyethylene bags.

2.3.2 Optimization of presaccharification step

Pretreated eucalyptus was converted to sawdust at 20/80 mesh by using a Wiley mill bench model for presaccharification, on the other hand, bagasse and straw were used without grinding. In an 125 mL erlenmeyer flask, 4 g of pretreated biomass were suspended in 50.0 mL of citrate buffer (50 mM, pH 4.8) and supplemented with the commercial cellulase preparation Celluclast 1.5 L in the ratio of 15 Filter Paper Units (FPU) per gram of substrate (1.4 mL) (Souza et al., 2012). The erlenmeyer flask was capped and incubated in a shaker at 50ºC and 180 rpm agitation. Samples were collected at 0, 12, 24, 36, 48, 60 and 72 hours of enzymatic hydrolysis, centrifuged for 10 min at 10,000 x g, and the supernatants were used for glucose and cellobiose determination. Glucose concentration in hydrolysates and presaccharification yield were used for fixing of the optimal presaccharification time and alkaline charge for pretreatments. Untreated biomasses were used as control experiment.

2.3.3 Simultaneous saccharification and fermentation

In an 125 mL erlenmeyer flask, 4 g of pretreated biomass were suspended in 50.0 mL of fermentation medium (2.5 g L^{-1} yeast extract; 2.5 g L^{-1} peptone; 2 g L^{-1} NH_4Cl; 1 g L^{-1} KH_2PO_4; and 0.3 g L^{-1} $MgSO_4.7H_2O$) in citrate buffer (50 mM, pH 4.8) and supplemented with 15 Filter Paper Units (FPU) of enzyme per gram of substrate (1.4 mL) (Souza et al., 2012). The erlenmeyer flask was capped and incubated in a shaker at 50ºC and 180 rpm for presaccharification step during the time predefined previously (See Experimental 2.3.2). Yeast cultures (*Saccharomyces cerevisiae* LBM-1) were inoculated, under sterile conditions, into the presaccharification products, the erlenmeyer flask was capped and incubated in a shaker at 37 °C and 180 rpm for 10 hours for SSF. Samples were collected after SSF, centrifuged for 10 min at 10,000 x g, and the supernatants were analyzed for glucose and ethanol determinations. SSF was performed in duplicate. Untreated biomasses were used as control experiment.

2.4 Analyses

2.4.1 Determination of chemical composition and pretreatment parameters

Biomasses sawdust (40/60 mesh) was produced by using a Wiley mill bench model and used for chemical characterization. Sawdust was dried (23 ± 1ºC and 50 ± 2% relative humidity) to constant weight and saved in airtight containers. The moisture content was determined according to TAPPI T 264 cm-07. Chemical analyses for raw material were conducted in triplicate and for pretreated biomasses in duplicate.

The chemical analyses performed were: ash content (TAPPI 211 om-02), silica content (insoluble part of ash remained after acid hydrolysis with HCl) (TAPPI 244 cm-11), total extractives content (1:2 ethanol-toluene for 5 hours → 95% ethanol for 4 hours → hot water for 1 hour) (TAPPI T 264 cm-07), Klason lignin was determined according to Gomide and Demuner (1986) and corrected by the silica content according to Carvalho et al. (2015), soluble lignin (Goldschimid, 1971), anhydrosugars content (glucose, xylose, galactose, mannose and arabinose) (Wallis et al., 1996), uronic acids (Scott, 1979) and acetyl groups (Solar et al., 1987). Complete mass balance of biomasses was calculated from the results of chemical composition according to Carvalho et al. (2015).

The pretreatments parameters evaluated were: pH – measured after pretreatment from liquor; and pretreatment yield – determined gravimetrically on the solid fraction of pretreated biomasses.

2.4.2 Determination of enzymatic hydrolysis and fermentation parameters

Glucose and cellobiose concentrations after presaccharification were determined by using an HPLC instrument with refractive index detector and HPX-87 H / BIORAD column (300 mm x 8.7 mm). The HPLC conditions were: water with 0.05 mM sulfuric acid as mobile phase; flow rate of 0.5 mL/min; column pressure: 1200 psi; and injected volume of 20 µL.

In presaccharification the parameters evaluated were presaccharification yield and glucose yield. Presaccharification yield ($\boldsymbol{Y_{G/B}}$) ($g_{glucose}/g_{biomass}$) was calculated by dividing the difference between the final ($\boldsymbol{Glu_f}$) and initial ($\boldsymbol{Glu_i}$) glucose mass (g) released from biomass during presaccharification (measured in samples collected from presaccharification medium) by the total mass of biomass (g) used for presaccharification test (***Biomass***), according to Eq. 1 (Souza et al., 2012).

$$Y_{G/B} = \frac{Glu_f - Glu_i}{Biomass} \quad (1)$$

Glucose yield ($\boldsymbol{G_Y}$) (%) was calculated by dividing the difference between the final ($\boldsymbol{Glu_f}$) and initial ($\boldsymbol{Glu_i}$) glucose mass (g) released from biomass during presaccharification (measured in samples collected from presaccharification medium) by the glucose content present in the pretreated biomass used for presaccharification test obtained by the complete mass balance (g) ($\boldsymbol{Glu_B}$), according to Eq. 2.

$$G_Y = \frac{Glu_f - Glu_i}{Glu_B} \times 100 \quad (2)$$

Glucose and ethanol concentrations after SSF were determined by using a refractive index HPLC detector and HPX-87 H / BIORAD column. The HPLC conditions were: column measure - 300 mm x 8.7 mm diameter; mobile phase - water with 0.05 mM sulfuric acid; rate flow - 0.7 mL/min; column pressure - 1920 psi; and injected volume - 10 µL.

In SSF, the parameters evaluated were ethanol yield and volumetric productivity of ethanol, according to Souza et al. (2012). Ethanol yield ($\boldsymbol{Y_{E/B}}$) ($g_{ethanol}/g_{biomass}$) was calculated at the end of the fermentation (10 hours) by dividing the difference between the final ($\boldsymbol{EtOH_f}$) and initial ($\boldsymbol{EtOH_i}$) ethanol mass (g) (measured in samples collected from fermentation medium) by the total mass of biomass (g) used for SSF (***Biomass***), according to the Eq. 3.

$$Y_{E/B} = \frac{EtOH_f - EtOH_i}{Biomass} \quad (3)$$

Volumetric productivity of ethanol ($\boldsymbol{Q_P}$) (g L^{-1} h^{-1}) was calculated by dividing the maximum concentration of ethanol ($\boldsymbol{EtOH_f}$ g L^{-1}) achieved (measured in samples collected from fermentation medium), by the time of fermentation (***t***) in hours, according to the Eq. 4.

$$Q_P = \frac{EtOH_f}{t} \quad (4)$$

3. Results and discussion

3.1 Effects of alkaline pretreatments on the processing yield and chemical composition of biomasses

Alkaline pretreatments are recognized to promote removal and/or changes in lignin in biomasses (Alvira et al., 2010). The delignification performed before enzymatic hydrolysis is relevant not only for increasing cellulose accessibility, since lignin is a natural barrier to enzymes (Öhgren et al., 2007), but also for decreasing desactivation of enzymes due to their binding to lignin (Berlin et al., 2005).

The increase in alkaline charge in the pretreatments resulted in decreasing in pretreatments yield and a narrow correlation between these parameters was observed (Table 1). After pretreatments, the final pH were 9.1 (5% NaOH), 12.7 (10% NaOH) and 14.0 (15% NaOH) for eucalyptus, 7.6 (5% NaOH), 10.7 (10% NaOH) and 12.7 (15% NaOH) for bagasse and 9.4 (5% NaOH), 11.2 (10% NaOH) and 12.4 (15% NaOH) for straw. Straw had lower yield in alkaline pretreatments than eucalyptus or bagasse, irrespectively to the alkaline charge.

Table 1 - Correlation coefficient between alkaline charge (5%, 10% and 15% NaOH) in pretreatments and yield of pretreatments.

Biomass	Parameter	5% NaOH	10% NaOH	15% NaOH	CC[a]
Eucalyptus	Yield, %	95.0 (0.1)	88.2 (0.4)	77.1 (0.3)	-0.99
Bagasse		72.2 (0.5)	56.1 (4.2)	50.9 (0.5)	-0.96
Straw		55.9 (0.5)	46.4 (0.4)	37.3 (1.7)	-1.00

(...) Standard deviation.

[a] Correlation coefficient. Negative values indicate negative correlation, i.e., by increasing alkaline charge in pretreatments the yield of pretreatments decreased.

The complete mass balance (Table 2) was used to determine the chemical composition of biomasses after alkaline pretreatments (Fig. 2) by combination with the pretreatments yield. During alkaline pretreatments, besides lignin removal, chemical transformation on hemicelluloses and extractives were also observed. The hemicelluloses removal and, especially, the lignin removal were the main causes of mass loss during alkaline pretreatments.

Table 2 - Chemical composition (lignin, anhydrosugar, ash and extractives/pseudo-extractives) of the pretreated biomasses reported based on the complete mass balance[a].

Biomass	Pretreatment	Lignin, %	Glucose, %	Other sugars, %[b]	Ash, %	Extractives [c], %
Eucalyptus	Raw material	27.4	49.9	20.3	0.2	2.3
	Alkaline 5%	25.8	52.2	11.3	2.8	7.9
	Alkaline 10%	21.9	54.0	10.3	4.7	9.1
	Alkaline 15%	17.4	58.6	10.2	5.1	8.7
Bagasse	Raw material	18.0	36.0	28.7	2.3	15.0
	Alkaline 5%	19.4	49.5	25.0	1.9	4.2
	Alkaline 10%	8.3	57.6	29.1	1.4	3.6
	Alkaline 15%	3.6	62.9	30.8	1.2	1.5
Straw	Raw material	13.8	36.3	29.8	7.9	12.2
	Alkaline 5%	9.9	52.6	23.9	7.2	6.4
	Alkaline 10%	1.7	57.0	25.2	6.4	9.7
	Alkaline 15%	0.3	62.5	26.8	4.5	5.8

[a] Calculated from average of chemical components.

[b] Sum of xylose, mannose, galactose, arabinose, uronic acids and acetyl groups. Uronic acids and acetyl groups measured only in raw material.

[c] Generation of pseudo-extractives for eucalyptus during alkaline pretreatments.

Lignin removal from biomasses before enzymatic hydrolysis has been considered as a way to improve the enzymatic hydrolysis of cellulose (Mosier et al., 2005). By increasing the pretreatments severity (alkaline charge) the lignin removal increased (Table 3). For eucalyptus wood, the lignin removal was of 11%, 30% and 51% for alkaline pretreatment with 5%, 10% and 15% NaOH, respectively. For bagasse the lignin removal was 22%, 74% and 90% and for straw it reached values of 60%, 94%

and 99% for the alkaline pretreatment with 5%, 10% and 15% NaOH, respectively. Bagasse and straw achieved higher lignin removal through the alkaline pretreatments than the wood. This result is likely explained by the higher alkaline solubility of grass lignins in relation to wood lignins due to some structural characteristcs of these lignins. Bagasse and straw, contain guaiacyl (G), syringil (S) lignin and p-hydroxyphenyl (H) lignins (Brandt et al., 2013). The amount of H-lignin present in hardwood, such as eucalypt wood, is insignificant, being the lignin mostly of the G- and S-types (Boerjan et al., 2003). The latter lignin is considered more easily removed from biomasses by chemical treatment, such as kraft pulping, than the G-lignin (Santos et al., 2011). In addition, the hydroxycinnamic acid residues (namely *p*-coumaric acid and ferulic acid), present in substantial amount in grass lignin, stablish cross-link *via* ester bond with hemicelluloses. In alkaline conditions, the ferulate ester is hydrolyzed. As a result, the solubility of grass lignin increases due to the decreasing of cross-link between lignin and arabinoxylans (Grabber et al., 2004; Hammel, 1997). The higher amount of free phenolic hydroxyl groups present in grass lignins compared to wood lignin also contributes to improve the lignin solubility (Grabber et al., 2004; Granata and Argyropoulos, 1995; Nimz et al., 1981).

Table 3 - Correlation coefficient between alkaline charge (5%, 10% and 15% NaOH) in pretreatments and the lignin amount in pretreated biomasses.

Biomass	Parameter	Raw material	5% NaOH	10% NaOH	15% NaOH	CC[b]
Eucalyptus		27.4	24.5	19.3	13.4	-1.00
Bagasse	Lignin, g [a]	18.0	14.0	4.7	1.8	-0.96
Straw		13.8	5.5	0.8	0.1	-0.92

[a] Lignin amount present in biomasses after pretreatments expressed in grams (obtained by the combination of complete mass balance and actual yield of each biomass).

[b] Correlation coefficient. Negative values indicate negative correlation, i.e., by increasing alkaline charge in pretreatments the lignin amount decreased.

Part of hemicelluloses was removed from biomasses by alkaline pretreatment. By hemicelluloses removal from biomasses, the cellulose accessibility increases with consequent improvements in enzymatic hydrolysis (Alvira et al., 2010). The hemicelluloses removal increased by increasing the alkaline charge in pretreatments (Table 4). The hemicelluloses removal from eucalyptus was 47%, 55% and 61% in the alkaline pretreatment with 5%, 10% and 15% NaOH, respectively. For bagasse, these values were 37%, 43% and 45%, and for straw they were 55%, 61% and 66% respectively.

Table 4 - Correlation coefficient between alkaline charge (5%, 10% and 15% NaOH) in pretreatments and the hemicellulose amount in pretreated biomasses.

Biomass	Parameter	Raw material	5% NaOH	10% NaOH	15% NaOH	CC[b]
Eucalyptus		20.3	10.7	9.1	7.9	-1.00
Bagasse	Hemic., g [a]	28.7	18.1	16.3	15.7	-0.96
Straw		29.8	13.4	11.7	10.0	-1.00

[a] Hemicelluloses represented by the sum of xylose, galactose, mannose and arabinose present in biomasses after pretreatments expressed in grams (obtained by the combination of complete mass balance and actual yield of each biomass). Hemicelluloses amount in raw materials includes also uronic acid and acetyl groups.

[b] Correlation coefficient. Negative values indicate negative correlation, i.e., by increasing alkaline charge in pretreatments the hemicelluloses amount decreased.

Glucose removal also increased with increasing pretreatment alkaline charge, but to lesser extend in relation to lignin and hemicelluloses (Table 5). For eucalyptus the glucose removal values were 0.6%, 4.5% and 9.5% for the alkaline charges of 5%, 10% and 15%, respectively. Values of 0.8%, 10% and 11% were obtained for bagasse. The effect more significant was observed for straw, for which 19%, 27% and 36% glucose removal was achieved with the alkaline charges of 5%, 10% and 15%, respectively. In bagasse, and especially in straw, the highest glucose removal was most likely due to glucans removal from biomasses.

Table 5 - Correlation coefficient between alkaline charge (5%, 10% and 15% NaOH) in pretreatments and the glucose amount in pretreated biomasses.

Biomass	Parameter	Raw material	5% NaOH	10% NaOH	15% NaOH	CC[b]
Eucalyptus		49.9	49.6	47.6	45.2	-1.00
Bagasse	Glucose, g [a]	36.0	35.7	32.3	32.0	-0.90
Straw		36.3	29.4	26.4	23.3	-1.00

[a] Glucose amount present in biomasses after pretreatments expressed in grams (obtained by the combination of complete mass balance and actual yield of each biomass).

[b] Correlation coefficient. Negative values indicate negative correlation, i.e., by increasing alkaline charge in pretreatments the cellulose amount decreased.

For eucalyptus, the extractives amount in raw material was lower than in alkaline pretreated biomasses which indicate the formation of pseudo-extractives during the pretreatment. Pseudo-extractives are organic structures formed from fragments of lignin and polysaccharides during pretreatments, which present similar solubility to the native extractives from biomasses (Carvalho et al., 2015). Pseudo-extractives increased by factors of 3.3 (5% NaOH), 3.5 (10% NaOH) and 2.9 (15% NaOH) times in relation to the extractives contents of the original raw materials. The ash amount increased in eucalyptus compared to the raw material most likely due to sodium (from NaOH), which remained in biomasses after pretreatment and washing.

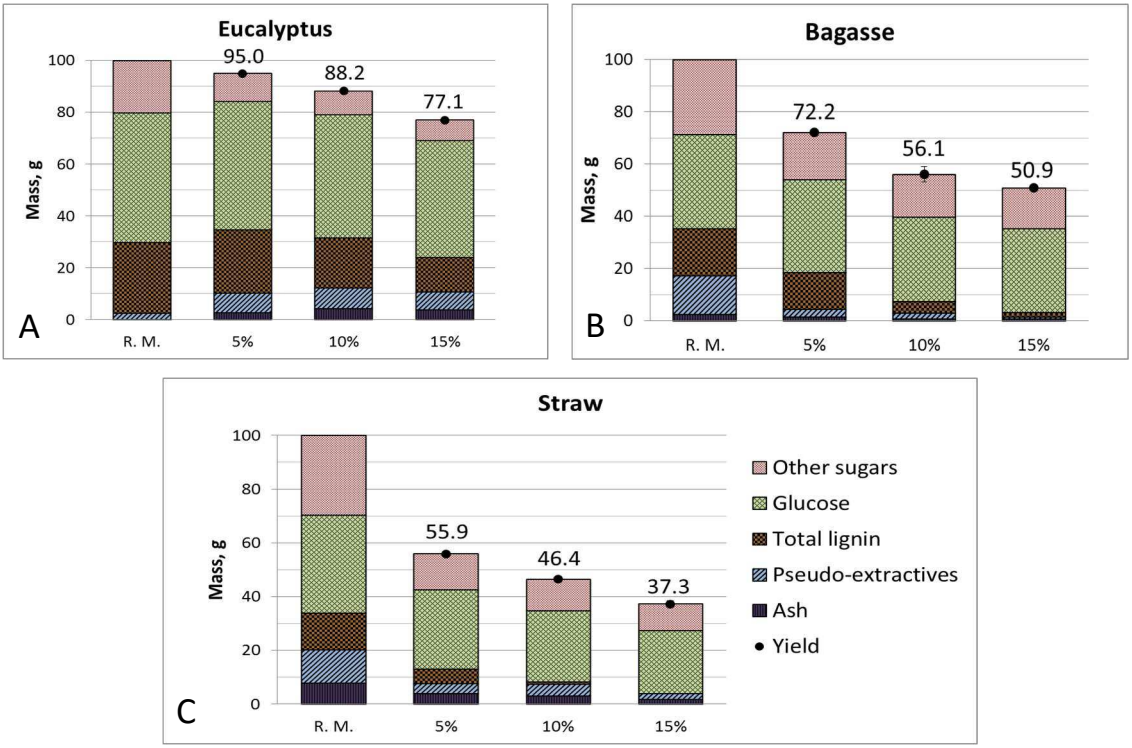

Figure 2 - Complete mass balance for eucalyptus (**A**), bagasse (**B**) and straw (**C**) before (R. M.) and after the pretreatments (5, 10 and 15% NaOH).

3.2 Simultaneous saccharification and fermentation

3.2.1 Presaccharification tests

Pretreated biomasses were more suitable for enzymatic hydrolysis than untreated biomasses, increasing at least 7.4 times the glucose release after 24 hours presaccharification (Fig. 3). Besides removing hemicelluloses (including acetyl groups and uronic acids), lignin and silica, alkaline pretreatment also promote the swelling of cellulose and fiber surface area increase (Alvira et al., 2010; Cardona et al., 2010). By decreasing the lignin amount in biomasses, improvements are expected in enzymatic hydrolysis by the decreasing in nonproductive binding between lignin and enzymes, an important factor for enzymes inactivation (Berlin et al., 2005). For eucalyptus and straw, the maximum glucose release occurred at 24 hours presaccharification. For bagasse the glucose release kept increasing in the period of 24 to 72 hours, but in a considerable lower range than that from 0 to 24 hours. Similar profile of glucose release during presaccharification of delignified bagasse was observed by Souza et al. (2012) with higher glucose releasing in the first 24 hours than in the last 24 hours of presaccharification. At 24 hours presaccharification these authors achieved glucose concentration of 20.0 g L^{-1}. The alkaline pretreatments which provided the maximum

glucose release during presaccharification at 24 hours for eucalyptus, bagasse and straw were alkaline 10%, 15% and 5%, respectively. At the optimal alkaline charge and 24 hours presaccharification, the glucose concentrations for eucalyptus, bagasse and straw were 14.3 g L^{-1}, 22.5 g L^{-1} and 16.4 g L^{-1}, respectively. Pretreated biomasses with the lowes content of lignin and hemicelluloses obtained by the higher alkaline charges in pretreatments (10-15%) (Tables 3-4), which in principle should favors the enzymatic hydrolysis, had the opposite effect in the case of straw. The highest glucose releasing was observed for straw pretreated using 5% NaOH (Fig. 3C). This result is probably related to the higher content of glucose remaining in straw pretreated using 5% NaOH.

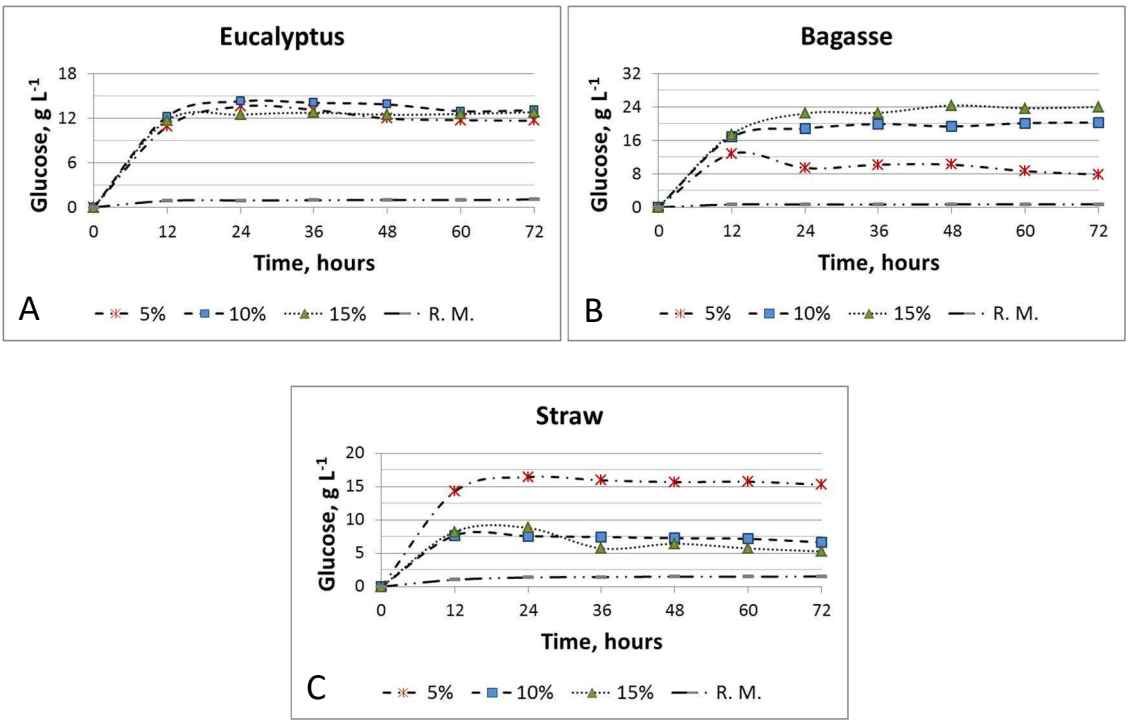

Figure 3 - Results of glucose content generated for eucalyptus (**A**), bagasse (**B**) and straw (**B**) raw material (R.M.) and biomasses pretreated by alcaline (5%, 10% and 15% NaOH) pretreatments after 0, 12, 24, 36, 48, 60 and 72 hours of presaccharification.

The low cellobiose concentration in hydrolysates indicated the efficient action of β-glucosidase during presaccharification (Table 6). In the present study the cellobiose concentrations for all biomasses and pretreatments were significantly lower than that measured by Santos et al., (2010) after 16 hours presaccharification for delignified bagasse. Cellobiose concentration was lower than the limit of detection in hydrolysates from untreated biomasses, irrespectively to the presaccharification time.

Enzymatic hydrolysis is performed by different enzymes with cellulolytic activity, usually formed by endo-glucanases, celobiohydrolases and β-glucosidases,

each one is responsible for hydrolyzing the cellulose chain in different fragments. Cellobiose is a dimer of glucose formed during enzymatic hydrolysis and hydrolyzed by β-glucosidase to glucose. The β-glucosidase action is improved by decreasing the cellobiose concentration in hydrolysates (Cardona et al., 2010; Santos et al., 2010; Palmqvist and Hahn-Hägendal, 2000). The cellobiose is unfermentable by yeasts during SSF and can also inhibit the cellulase action (Philippidis et al., 1993).

Table 6 - Cellobiose concentration at 24 hours of presaccharification for pretreated eucalyptus, bagasse and straw.

Cellobiose, g L^{-1}	Eucalyptus	Bagasse	Straw
Alkaline 5%	0.10	0.12	0.10
Alkaline 10%	0.11	0.12	0.18
Alkaline 15%	0.09	0.11	0.21

The values of glucose concentration in presaccharification medium (after 24h) (Fig. 3) were correlated to the presaccharification yields (Fig. 4), but with different presaccharification effects for each biomass. For eucalyptus, only a slight variation in presaccharification yield was observed, with the lowest value obtained after the 15% NaOH pretreatment. Higher alkaline charge increased the presaccharification yield for bagasse, with the maximum value (0.28 $g_{glucose}/g_{biomass}$) achieved in biomasses pretreated by 15% NaOH. For straw pretreatment with higher alkaline charge was not favorable, most likely due the significant glucose loss. The maximum presaccharification yield (0.21 $g_{glucose}/g_{biomass}$) was observed when the straw was pretreated with 5% NaOH. At the optimal NaOH charge, 10% for eucalyptus, 15% for bagasse and 5% for straw, the alkaline pretreatments increased presaccharification yield by 15, 35 and 11 times compared to untreated biomasses.

In general, the chemical composition of eucalyptus was less changed by alkaline pretreatment than the other raw materials, with alkaline charge over than 10% being insufficient to increase accessibility. Bagasse, on the other hand, was clearly favored by lower lignin and hemicelluloses content. Bagasse pretreated using 15% NaOH presented the best results in presaccharification. Low glucose losses during pretreatment was observed for bagasse even using the higher alkaline charge. For straw, higher NaOH charges (10-15%) resulted significant glucose losses and that negatively affected the process efficiency; hence, the 5% alkaline charge was the recommended value.

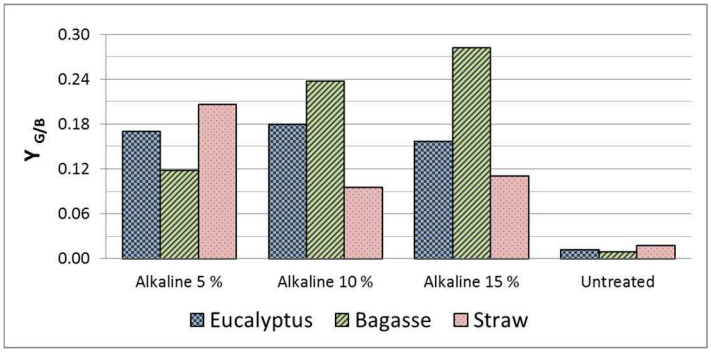

Figure 4 - Presaccharification yield ($Y_{G/B}$) measured at 24 hours for pretreated and untreated biomasses.

The glucose concentration was calculated based on the potential hydrolysis of cellulose to glucose during presaccharification (ratio between glucose release and glucose in pretreated biomass). The results showed that untreated straw presented higher cellulose accessibility than untreated bagasse or eucalyptus (Fig. 5). However, after pretreatments the chemical transformations performed in straw limited its enzymatic hydrolysis in comparison with other pretreated biomasses, even in the lowest alkaline charge. Based on the results, a milder alkaline condition is suggested for straw pretreatment. Bagasse was the more suitable biomass for glucose release by presaccharification, reaching 51.1% glucose yield after 24 hours.

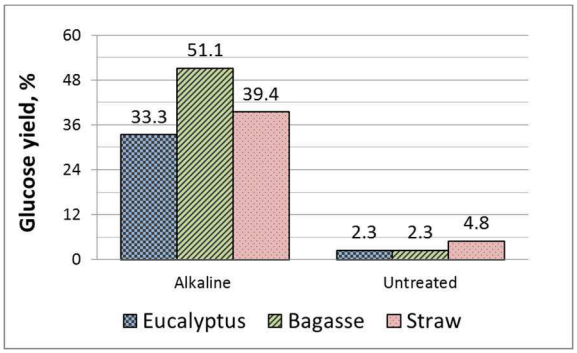

Figure 5 - Glucose yield (G_Y) measured at 24 hours for untreated biomasses and biomasses pretreated by alkaline pretreatments (10% NaOH for eucalyptus, 15% NaOH for bagasse and 5% NaOH straw).

3.2.2 Assessment of bioethanol production

Compared to untreated biomasses, the pretreatment improved the ethanol yield by 11, 72 and 48 times and the volumetric productivity of ethanol by 9, 37 and 25 times for eucalyptus, bagasse and straw, respectively, (Fig. 6). The ethanol concentration after

SSF was 3.4 g L^{-1}, 8.8 g L^{-1} and 6.4 g L^{-1}, for eucalyptus, bagasse and straw, respectively. Pretreated bagasse presented the highest ethanol yield, volumetric productivity of ethanol and ethanol concentration which indicated great possibilities for ethanol production from this biomass by using alkaline pretreatment and SSF with previous presaccharification.

By using 24 hours presaccharification followed by 8 hours SSF, Souza et al. (2012) achieved approximately 1.1 g L^{-1} h^{-1} and 0.12 for volumetric productivity of ethanol and ethanol yield respectively, for delignified bagasse. However, the aforementioned authors used a thermotolerant yeast strain (*Kluyveromyces marxianus*) and the SSF was conducted at 42ºC. So the SSF could be favored by the temperature increasing since improvements in saccharification could be gained without impairing the yeast activity. In addition, before bagasse deslignification by alkaline treatment (121ºC, 30 min, 4% w/v NaOH), these authors pretreated the bagasse with acid (121ºC, 30 min, 0.5% v/v H_2SO_4), which definetely helped to improve the accessibility of enzymes to biomass during presaccharification and SSF.

Santos et al. (2010) observed volumetric productivity of ethanol of 0.3 g L^{-1} h^{-1} for delignified bagasse by alkaline pretreatment (100ºC, 60 min, 10 g L-^{1} NaOH). Although this value is lower than the values found in the present study, these authors used the time of 40 hours to calculate the volumetric productivity of ethanol (16 hours presaccharification + 24 hours SSF) and performed the SSF at 30ºC. This information combined with the results observed in the present study indicate the possibility of improving the SSF by using thermotolerant yeast strains.

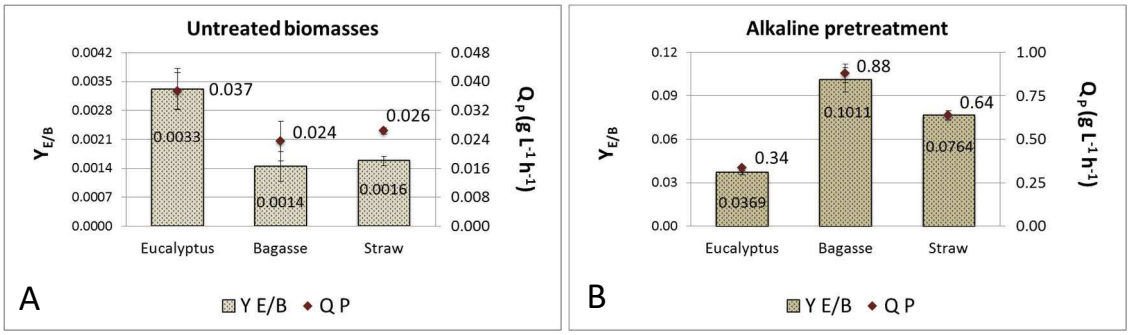

Figure 6 - Column graphic shows the ethanol yield ($Y_{E/B}$) and scatter graphic shows the volumetric productivity of ethanol (Q_P) after 24 hours of saccharification and 10 hours of simultaneous saccharification and fermentation for untreated biomasses (**A**) and biomasses treated by alkaline pretreatment (**B**). Alkaline pretreatment was performed at 10% alkaline for eucalyptus, 15% alkaline for bagasse and 5% alkaline for straw.

Positive correlations between glucose yield after presaccharification and ethanol yield as well as between glucose yield after presaccharification and volumetric productivity of ethanol were observed for all pretreated biomasses (Table 7). The results suggested that during SSF the enzymatic hydrolysis followed similar behavior than that observed during presaccharification.

Table 7 - Correlation coefficient between glucose yield (G_Y) after presaccharification and ethanol yield ($Y_{E/B}$) after SSF and between glucose yield (G_Y) after presaccharification and volumetric productivity of ethanol (Q_P) after SSF.

Parameters	Eucalyptus	Bagasse	Straw	CC[a]
G_Y,%	33.3	51.1	39.4	-
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.0369	0.1011	0.0764	0.95
Q_P, g L^{-1} h^{-1}	0.34	0.88	0.64	0.97

[a] Correlation coefficient between G_Y and $Y_{E/B}$ and between G_Y and Q_P.

Glucose concentration values after SSF (residual glucose) observed in the present study differed from those of other studies. Souza et al. (2012) observed total glucose consumption after 24 hours presaccharification at 50ºC and 10 hours SSF at 37ºC for bagasse pretreated by acid and alkaline processes in sequence (acid pretreatment performed at 121ºC for 30 min and 0.5% v/v H_2SO_4 and then alkaline treatment performed at 121ºC for 30 min and 4% w/v NaOH). In the beginning of SSF, these authors reported about 20 g L^{-1} glucose. About 7 g L^{-1} glucose were observed by Santos et al. (2010) in SSF hydrolysate after 16 hours presaccharification and 24 hours SSF for delignified bagasse (100ºC, 60 min, 10 g L^{-1} NaOH). By increasing SSF time, the aforementioned authors observed an increasing in glucose consumption until the total consumption around 25 hours SSF, same time in which the ethanol generation stabilized. In the present study about 2 g L^{-1} glucose was found after 24 hours presaccharification and 10 hours SSF for eucalyptus and straw (Table 8). For pretreated bagasse the residual glucose concentration was twofold (5.29 g L^{-1}), indicating that, in order to promote total glucose consumption and increase the ethanol production, the SSF time must be increased.

Table 8 - Glucose concentration after SSF.

Glucose, g L^{-1}	Eucalyptus	Bagasse	Straw
Untreated biomass	$2.07^{(0.32)}$	$2.65^{(0.14)}$	$1.97^{(0.17)}$
Alkaline pretreatment[a]	$2.70^{(0.35)}$	$5.29^{(0.50)}$	$2.66^{(0.14)}$

$^{(\cdots)}$ Standard deviation.

[a] 10% NaOH for eucalyptus, 15% NaOH for bagasse and 5% NaOH for straw.

4. Conclusions

- The fragments of lignin and polysaccharides degradation products generated during alkaline pretreatments lead to the formation of pseudo-extractives in eucalyptus;
- The removal of lignin, hemicelluloses and glucose from eucalyptus, bagasse and straw increased with increasing alkaline charge in the range of 5% - 15% NaOH (on dry basis);
- The alkaline pretreatment was more efficient to remove lignin from bagasse and straw than from eucalyptus, most likely due to the higher amount of free phenolic groups and ester bonds typically present in grass lignin, compared to wood lignin.
- The glucose released during presaccharification was negatively influenced by the remaining amount of lignin and hemicelluloses in pretreated eucalyptus and bagasse, whereas for straw the glucose removal during pretreatment impaired the glucose releasing during presaccharification; and
- Among the three biomasses evaluated, the bagasse pretreated with 15% alkaline charge provided the highest ethanol yield (0.101 $g_{ethanol}/g_{biomass}$), ethanol concentration (8.8 g L^{-1}) and volumetric productivity of ethanol (0.88 g L^{-1} h^{-1}).

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Article V

Cold alkaline extraction applied as pretreatment for bioethanol production from eucalyptus, sugarcane bagasse and sugarcane straw

ABSTRACT - The optimal conditions for the cold alkaline extraction (CAE) pretreatment of eucalyptus, sugarcane bagasse and sugarcane straw are proposed in view of their subsequent bioconversion into ethanol using the simultaneous sacharification fermentation (SSF) method. The optimum conditions, identified based on the factorial experimental central composite design, resulted in the removal of 46%, 52% and 61% of xylan and 15%, 37% and 45% of lignin, for eucalyptus, bagasse and straw, respectively. The formation of pseudo-extractives was observed during the CAE of eucalyptus. Despite the similar glucose concentration and yield for all biomasses after 12 hours of presascharification, the highest yield (0.065 $g_{ethanol}/g_{biomass}$), concentration (5.74 g L^{-1}) and volumetric productivity of ethanol (0.57 g L^{-1} h^{-1}) were observed for the sugarcane straw. It was most likely due to the improved accessibility of cellulose resulted from the removal of the largest amount of xylan and lignin.

Keywords: Factorial experimental central composite design, presaccharification, pseudo-extractives, *Saccharomyces cerevisiae* yeast, simultaneous saccharification and fermentation (SSF), *Trichoderma reesei* cellulase.

RESUMO – As condições ótimas para a o pré-tratamento por extração alcalina a frio (EAF) para eucalipto, bagaço de cana-de-açúcar e palha de cana-de-açúcar foram propostas com foco nas suas subsequentes bioconversões em etanol usando o método de sacarificação e fermentação simultâneas (SFS). As condições ótimas, identificadas pelo desenho experimental fatorial de composição central, resultaram em remoção de 46%, 52% e 61% de xilanas e 15%, 37% e 45% de lignina em eucalipto, bagaço e palha, respectivamente. A formação de pseudo-extrativos foi observada durante a EAF para o eucalipto. Apesar das similares concentrações de glicose e rendimentos para todas as biomassas após 12h de pré-sacarificação, os maiores rendimentos de etanol (0.065 $g_{etanol}/g_{biomassa}$), concentração de etanol (5.74 g L^{-1}) e produtividade volumétrica de etanol (0.57 g L^{-1} h^{-1}) foram observados para a palha de cana-de-açúcar. Isso provavelmente ocorreu devido à melhora na acessibilidade da celulose proporcionada pela remoção de maior quantidade de xilanas e lignina.

Palavras-chave: Desenho esperimental fatorial de composição central, pré-sacarificação, pseudo-extrativos, sacarificação e fermentação simultanêas (SFS), levedura *Saccharomyces cerevisiae*, celulase *Trichoderma reesei*.

1. Introduction

The global demand for energy continues to increase rapidly and exceeds the growth in total energy supply (Baeyens et al., 2015). Nowadays, the main focus is on the transformation of traditional energy production methods to more sustainable and environmentally friendly processes, in particular, the replacement of certain fossil-derived fuels by renewable resources (Vincent et al, 2014; Oliveira et al., 2013; Souza et al., 2012; Ragauskas et al., 2006). The production of bioethanol from non-food biomass is one sustainable alternative, as it is generated from renewable resources and reduces greenhouse gas emissions (Demirbaş et al., 2005).

Ethanol is an important fuel which is used either in neat form or as additive to gasoline (Demirbaş et al., 2005). Bioethanol production from non-food sources such as sugarcane bagasse (Souza et al., 2012; Santos et al., 2010), sugarcane straw (Santos et al., 2014; Oliveira et al., 2013; Hari Krishna et al, 2001), eucalyptus (Ballesteros et al., 2004), wheat straw (García et al., 2013), corn stover (Vincent et al., 2014) and sweet sorghum (Ballesteros et al., 2004) has been studied by several researchers. Brazil has great potential as a supplier of such non-food sources. Sugarcane is one of the country's main agricultural crops and generates a large amount of wastes: both bagasse and straw. Indeed, the forecast for the 2015/16 harvest is that about 92 million tons of bagasse and 92 million tons of straw will be generated (Conab, 2015; Oliveira et al., 2013). Brazil is also an important producer of fast-growing wood, and the most widely cultivated gender of which is *Eucalyptus*. Both eucalyptus wood and sugarcane wastes are suitable feedstock for bioethanol production (Santos et al., 2014; González-García, et al., 2012; Souza et al., 2012).

There are, however, a number of challenges that need to be overcome in order to make bioethanol production economically feasible. One is the recalcitrance of cellulose to enzymatic degradation, which is related to the chemical composition and morphology of any type of biomass (Foston and Ragauskas, 2012). Another is the need for improved methods for saccharification and fermentation of sugar molecules into ethanol, which are currently inefficient and expensive (Rubin, 2008).

Typically, some sort of chemical pretreatment is required prior to enzymatic hydrolysis in order to improve the accessibility and digestibility of cellulose present in lignocellulosic biomass (Meng and Ragauskas, 2014). Alkaline pretreatment is one of the methods that has been studied recently (Peng et al., 2009). This pretreatment can be carried out at low temperatures (below 40ºC) and promotes the fractionation of the chemical constituents of non-wood biomass into alkali-soluble lignin, hemicelluloses

and other residues with fewer chemical and physical changes (García et al., 2013; Glasser et al., 2000). The alkaline solution promotes the swelling of cellulose, which improves its accessibility for enzymes (Jackson, 1977). The main effect of alkaline pretreatment at high temperature is the delignification, but a significant amount of hemicellulose is also solubilized, which led to improvements on the enzymatic hydrolysis of cellulose (Hu and Ragauskas, 2012; Silverstein et al., 2007). In the other hand, alkaline pretreatments at low temperature are more efficient to remove hemicelluloses from lignocellulosic biomasses. For wood, however, alkaline pretreatment alone is not suitable for the extraction of sugars, and usually results in low total extraction and xylan extraction yields (García et al., 2013; Longue Júnior et al., 2010). Additional measures, such as mechanical treatment to reduce the size of the wood chips, may be necessary to improve the efficiency of alkaline pretreatment.

The next step, the saccharification, is performed through enzymatic hydrolysis, in which cellulases are used to promote hydrolysis of cellulose chain generating cellobiose and hydrolysis of the cellobiose to glucose, simultaneously. Sugars released during the saccharification steps are then converted to biofuels during the fermentation step (Rubin, 2008).

Saccharomyces cerevisiae is a facultative anaerobic yeast, which is typically used for fermentation in bioethanol production. Although it is able to convert C6 sugars to ethanol, it is not efficient for the conversion of C5 sugars derived from hemicelluloses (Baeyens et al., 2015).

One problem regarding to the saccharification process is the inhibition of enzymes caused by the saccharification products themselves (glucose and cellobiose in high concentration). In order to avoid this inhibition, it is recommended to perform the simultaneous saccharification and fermentation (SSF). In this way, the enzymes (used for saccharification) and yeast (used for fermentation) are placed in the same reactor and glucose is converted to ethanol as soon as it is released (Baeyens et al., 2015). In addition to suppressing the inhibition, SSF also results in a smaller demand for enzymes and in a higher volumetric productivity of ethanol, when compared with the traditional process (separate hydrolysis and fermentation, or SHF) (Santos et al., 2010). Typically, enzymes require higher temperatures than yeasts can withstand (Souza et al., 2012). Therefore, several recent studies have been performed with thermotolerant yeast strains, and promising results have been presented for strains such as *Kluyveromyces fragilis* NCIM 3358 (Hari Krishna, et al., 2001), *Kluyveromyces marxianus* CECT 10875 (Ballesteros et al., 2004) and *Saccharomyces cerevisiae* LBM-1 (Souza et al., 2012).

Researchers have suggested a presaccharification step prior to SSF, whereby glucose is used at the beginning of the SSF process. This can result in an increased ethanol yield and shorter processing time, as well as for higher cellulose to ethanol conversion than occurs with SSF alone (i.e., without the presaccharification step) (Santos et al., 2010).

In the present study, the cold alkaline extraction (CAE) pretreatment for eucalyptus, sugarcane bagasse and sugarcane straw was optimized by using a factorial experimental central composite design that generated second-order polynomial models based on independent variables. The objectives were: (i) to find the optimal conditions (temperature, reaction time and NaOH concentration) for CAE for eucalyptus, sugarcane bagasse and sugarcane straw in order to improve xylose removal from biomasses; and (ii) to evaluate the efficiency of bioethanol production from the pretreated biomasses through a simultaneous saccharification and fermentation (SSF) process after using a presaccharification step. To the best of our knowledge, such an approach (pretreatment optimization combined with the SSF process to assess the ethanol yield from biomasses) has not been reported before. By using mathematical models such as those employed in the present study, however, various pretreatment technologies for the bioconversion of cellulosic biomass into transportation fuels can be assessed in a much faster and more efficient way.

2. Experimental

2.1 Materials

Eucalyptus, sugarcane bagasse and sugarcane straw were used. Wood chips from a 7-year old clonal hybrid of eucalyptus (*Eucalyptus urophylla* x *Eucalyptus grandis*) were supplied by a pulp company and fragmented in a hammer mill (10 mm diameter). Bagasse and straw aged five-months old (cultivar RB867515) were supplied by Center of Sugarcane Experimentation (Oratórios, Minas Gerais State, Brazil) after chipping (bagasse and straw) and juice removal (bagasse) into particles with a 10 mm diameter. Biomasses were stored in airtight plastic bags at room temperature prior to use, and their moisture contents were determined according to TAPPI T 264 cm-07. The following chemicals were used: sodium hydroxide of lentils (analytical grade) (Merck Milipore, Germany), acetic acid (glacial) (Merck Milipore, Germany), a commercial cellulase preparation (Celluclast 1.5 L) (Sigma-Aldrich, Brazil) and the *Saccharomyces cerevisiae* LBM-1 thermotolerant yeast strain isolated from fermentation vats in Brazil.

2.2 Methods

2.2.1 Cold alkaline extraction and washing

Cold alkaline extractions were performed in a water bath with controlled temperature. A 50 g biomass was treated with 500 mL of NaOH in a 1-liter beaker. The initial liquor:biomass ratio was 10:1 (dry weight basis). The tests were carried out under the following conditions: temperature (20, 30 and 40ºC), reaction time (10, 35 and 60 min) and NaOH concentration (70, 90 and 110 g L^{-1}). Treatments were conducted with periodic agitation with a glass rod. After treatment, the sample was washed twice with 1 liter of distilled water and filtered in a Buchner funnel containing cloth as a filtering device. The pH of the treated biomass was neutralized to 6-7 with 2 N acetic acid. The treated biomass was dried to constant weight at room temperature (23 ± 1ºC and 50 ± 2% relative humidity) and saved into airtight containers. The moisture content was determined according to TAPPI T 264 cm-07. Optimal conditions for CAE of each biomass were replicated five times to test the models.

2.2.2 Experimental design

A factorial experimental central composite design was used, generating second-order polynomial models based on the independent variables. This approach allows correlating the dependent variables (solid material yield, extraction yield, xylose content in solid material, xylose retained in solid material after pretreatment and xylose extracted from solid material after pretreatment) with the independent variables (temperature, reaction time and NaOH concentration) of the cold alkaline extraction by using the minimum number of experiments. Independent variables were normalized by using the Eq. 1, where: $\boldsymbol{Xn}$ is the normalized value; $\boldsymbol{X}$ is the absolute value of the independent variable to be normalized; $\boldsymbol{X_{ave}}$ is the average value of the variables; and $\boldsymbol{X_{max}}$ and $\boldsymbol{X_{min}}$ are the maximum and minimum values, respectively.

$$Xn = \frac{X - X_{ave}}{(X_{max} - X_{min}) / 2} \quad (1)$$

The number of different cold alkaline extractions was estimated according to Eq. 2, where: $\boldsymbol{n}$ is the number of trials done; $\boldsymbol{K}$ is the number of independent variables used

(if K < 5; p = 0, if K ≥ 5, p = 1); ***2 x K*** represents the axial trials; and n_c is the number of repetitions in the central point (2 repetitions in this study).

$$n = 2^{K-p} + 2 \times K + n_c \quad (2)$$

The results were used to generate second-order polynomial models (Eq. 3) (García et al., 2013). The models presented regression terms such as: coefficients for the main effects, coefficients for quadratic main effects and coefficients for factor interaction effects.

$$Y = a_0 + \sum_{i=1}^{n} b_i * X_{ni} + \sum_{i=1}^{n} c_i * X_{ni}^2 + \sum_{\substack{i=1 \\ j=1}}^{n} d_{ij} * X_{ni} * X_{nj} \quad (i < j) \quad (3)$$

Model adjustments were performed using the software Statistica (version 8.0). Only statistically significant coefficients were used for the models (those not exceeding the significance level of 0.05 in the Student`s T-test < 2 and having a 95% confidence interval, excluding zero). The response surface construction was performed using the software SigmaPlot (version 11.0).

2.2.3 Optimization of presaccharification step

Enzymatic hydrolysis was performed using the commercial cellulose preparation Celluclast 1.5 L (Sigma-Aldrich, Brazil). In a 125 mL Erlenmeyer flask, 3 g of pretreated biomass were suspended in 37.5 mL of citrate buffer (50 mM, pH 4.8) and supplemented with 15 Filter Paper Units (FPU) of enzyme per gram of substrate (1.05 mL) (Souza et al., 2012). The Erlenmeyer flask was capped and incubated in a shaker at 50ºC and 180 rpm. Samples were collected every 12 hours, from time 0 to 72 hours, and centrifuged for 10 min at 10,000 x g, and the supernatants were used for glucose and cellobiose tracking as a function of presaccharification time.

2.2.4 Simultaneous saccharification and fermentation

In a 125 mL Erlenmeyer flask, 4 g of pretreated biomass were suspended in 50.0 mL of fermentation medium (2.5 g L^{-1} yeast extract; 2.5 g L^{-1} peptone; 2 g L^{-1} NH_4Cl; 1 g L^{-1} KH_2PO_4; and 0.3 g L^{-1} $MgSO_4.7H_2O$) and in citrate buffer (50 mM, pH 4.8) and supplemented with 15 Filter Paper Units (FPU) of enzyme per gram of substrate (1.4 mL) (Souza et al., 2012). The Erlenmeyer flask was capped and incubated in a shaker at 50ºC and 180 rpm. Presaccharification was implemented for the duration defined in the presaccharification preliminary tests (optimized by maximum glucose liberation and maximum presaccharification yield).

After the presaccharification, yeast cultures (*Saccharomyces cerevisiae* LBM-1) were inoculated, in sterile conditions, for the SSF step. The Erlenmeyer flask was capped and incubated in a shaker at 37°C and 180 rpm for 10 hours. After SSF, samples were collected and centrifuged for 10 min at 10,000 x g, and the supernatants were analyzed for ethanol determination. Untreated biomasses were used as control experiments for presaccharification and SSF.

2.3 Analyses

2.3.1 Determination of chemical composition

Raw material and pretreated biomasses were converted to sawdust (40/60 mesh) by using a Wiley mill bench model, dried at room temperature (23 ± 1ºC and 50 ± 2% relative humidity) and saved into airtight containers. The moisture content was determined according to TAPPI T 264 cm-07. Chemical analyses for raw material were conducted in triplicate and for pretreated biomasses in duplicate.

Ash content was determined in oven at 575ºC until constant weight was achieved (TAPPI 211 om-02). Silica was measured in the insoluble part of the ash that remained after acid hydrolysis with HCl (TAPPI 244 cm-11). The sequence 1:2 ethanol-toluene (5 h) and 95% ethanol (4 hours) in a Soxhlet extractor, followed by hot water extraction (1 hour) was used to determine the total extractives content (TAPPI T 264 cm-07).

Extractive-free biomasses were used for lignin and sugar analyses after acid hydrolysis. Klason lignin content was determined gravimetrically (Gomide and Demuner, 1986) and the soluble lignin was measured by UV-spectroscopy (Cary 50 Probe, Varian) at 215 and 280 nm wavelengths (Goldschimid, 1971). Klason lignin

corrected for silica was performed according to Carvalho et al. (2015). Anhydrosugar content was determined from supernatant generated after acid hydrolysis according to Wallis et al. (1996), by ion chromatography (IC) with a Dionex ICS3000, using a pulsed amperometric detector, a CarboPac PA1 column (Thermo Scientific, USA), an injection volume of 25 µL and a flow rate of 1 mL min^{-1}. External sugar standards used for calibration were glucose (Merck, Germany), xylose (Merck, Germany), galactose (Sigma-Aldrich, Germany), mannose (Merck, Germany) and arabinose (Sigma, USA). Fucose (Sigma, Slovakia) was used as an internal standard.

Uronic acids were determined from supernatant generated after acid hydrolysis with sulfuric acid by UV spectroscopy at 450 nm wavelength (Scott, 1979).

The acetyl group contents in biomasses were determined after hydrolysis with oxalic acid at 120ºC for 80 minutes by using high performance liquid chromatography (HPLC) with UV detection (Solar et al., 1987). The HPLC instrument used was from Shimadzu, with an SCL-10A system controller, LC-18.5 mm column, 25 cm (18.5 mm x 25 cm) (Shimadzu LC Shim-pack CLC-ODS, octadecyl). The temperature was 40ºC, injection volume was 20 µL, and flow rate was 0.6 mL min^{-1}. The mobile phase was H_3PO_4, 37 mmol L^{-1}, pH 2.2, adjusted with NaOH.

Determination of the mass balance of raw material and pretreated biomass was performed according to Carvalho et al. (2015).

2.3.2 Determination of cold alkaline extraction parameters

Xylose retained in solid material (***XynR***) after pretreatments was calculated as a percentage according to Eq. 4, where: ***Xi*** is the xylose content in the biomass after pretreatment (based on extractive-free biomass) (%); ***Yield*** is the solid yield (%); and ***xi*** the xylose content in the untreated biomass (based on extractive-free biomass) (%).

$$XynR = [(Xi \times Yield)/xi] \quad (4)$$

The content of xylose extracted from solid material ($\boldsymbol{X_E}$) was estimated mathematically, as a percentage, according to Eq. 5, where: and ***XynR*** is the xylose retained in the biomass after pretreatment (%).

$$X_E = 100 - XynR \quad (5)$$

In addition, pretreatment trials were evaluated with respect to the yield. Solid yield was determined gravimetrically (in duplicate). The extraction yield (***E_Y***) was estimated mathematically, as a percentage, according to Eq. 6, where: ***S_Y*** is the solid yield (%).

$$E_Y = 100 - S_Y \quad (6)$$

2.3.3 Determination of enzymatic hydrolysis and fermentation parameters

Samples collected after presaccharification were used to quantify glucose and cellobiose contents by using an HPLC instrument with a refractive index detector and HPX-87 H / BIORAD column (300 mm x 8.7 mm). The mobile phase was water with 0.05 mM sulfuric acid; the flow rate was 0.5 mL/min; the column pressure was 1200 psi; and the injected volume was 20 µL. The presaccharification yield (***Y_G/B***) ($g_{glucose}/g_{biomass}$) was calculated (Eq. 7) based on the glucose content (Souza et al., 2012), and by dividing the difference between the final (***Glu_f***) and initial (***Glu_i***) glucose mass (g) by the initial mass of biomass (g) (***Biomass***).

$$Y_{G/B} = \frac{Glu_f - Glu_i}{Biomass} \quad (7)$$

Samples collected after the fermentation were used to quantify the ethanol content using an HPLC instrument with a refractive index detector under the following conditions: column: HPX-87 H / BIORAD; measurement: 300 mm x 8.7 mm diameter; mobile phase: water with 0.05 mM sulfuric acid; flow rate: 0.7 mL/min; column pressure: 1920 psi; and injected volume: 10 µL. Bioethanol production was analyzed based on the ethanol yield and the volumetric productivity (Souza et al., 2012). The ethanol yield (***Y_E/B***) was calculated at the end of the fermentation (10 hours) by dividing the difference between the final (***EtOH_f***) and initial (***EtOH_i***) ethanol mass (g) by the ***Biomass*** mass (g), according to Eq. 8.

$$Y_{E/B} = \frac{EtOH_f - EtOH_i}{Biomass} \quad (8)$$

Volumetric productivity of ethanol ($\boldsymbol{Q_P}$) was calculated by dividing the maximum concentration of ethanol ($\boldsymbol{EtOH_f}$ g L^{-1}) achieved by the time of fermentation ($\boldsymbol{t}$), in hours, according to Eq. 9.

$$Q_P = \frac{EtOH_f}{t} \quad (9)$$

3. Results and discussion

3.1 Chemical composition of raw materials

The chemical composition of eucalyptus, sugarcane bagasse and sugarcane straw is shown in Table 1.

Table 1 - Chemical composition of eucalyptus, sugarcane bagasse and straw.

Constituents	Eucalyptus	Sugarcane bagasse	Sugarcane straw
Ash,%	0.155a (0.01)	2.31b (0.02)	7.91c (0.02)
Silica,%	n.d.[a]	1.44a (0.01)	5.77b (0.03)
Total extractive [b],%	2.30a (0.04)	15.0c (0.25)	12.2b (0.26)
Klason lignin [c],%	24.0c (0.1)	19.5b (0.1)	14.0a (0.3)
Soluble lignin [c],%	3.97c (0.02)	1.87a (0.06)	2.17b (0.08)
Total lignin [c],%	28.0c (0.1)	21.4b (0.1)	16.2a (0.3)
Glucose [c],%	49.4b (0.7)	41.8a (0.8)	41.4a (1.18)
Xylose [c],%	12.0a (0.2)	24.8b (0.2)	26.0c (0.2)
Galactose [c],%	1.20b (0.10)	0.87a (0.12)	0.93a (0.06)
Mannose [c],%	0.90b (0.05)	0.93b (0.12)	0.30a (0.10)
Arabinose [c],%	0.30a (0.10)	2.27b (0.21)	3.90c (0.05)
Uronic acids [c],%	4.00c (0.05)	1.48b (0.05)	1.30a (0.02)
Acetyl groups [c],%	1.90b (0.04)	3.04c (0.02)	1.65a (0.01)

(…) Standard deviation.

[a] non-determined: value under the limit of detection.

[b] ethanol/toluene (1:2)⇨ethanol⇨hot water.

[c] based on extractive-free biomass.

The averages in the row followed by the same letter do not differ from each other at a probability level of 5% by the Tukey test.

The nonwoody biomasses (bagasse and straw) contained significantly more ash and extractives than the wood biomass (eucalyptus). Based on the extractive-free biomass, the glucose content was quite similar in the bagasse and straw, but less than in

the eucalyptus. The large amount of lignin in eucalyptus can harm the saccharification process, since lignin acts as a physical barrier against enzymes and hinders the accessibility of cellulose (Öhgren et al., 2007). On the other hand, the bagasse and straw contained substantially more xylose and arabinose (C5 sugars) than the eucalyptus. The traditional yeasts used in the fermentation process, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, cannot convert C5 sugars derived from hemicelluloses into ethanol (Baeyens et al., 2015). As a result, a lower ethanol yield can be expected for these biomasses.

3.2 Pretreatment optimization and chemical composition of pretreated biomasses

Fifteen different trials of cold alkaline extractions were performed using the factorial experimental central composite design. This was done in order to optimize temperature (20, 30 and 40ºC), reaction time (10, 35 and 60 min) and NaOH concentration (70, 90 and 110 g L^{-1}) conditions and, therefore, to maximize xylose extraction from biomasses. Results for solid material yield, extraction yield, xylose content in solid material, xylose retained in solid material after pretreatment and xylose extracted from solid material after pretreatment for each trial are shown in Appendix A. Based on these results, second-order polynomial models were generated, with an error between the observed and calculated values lower than 15% and coefficient of determination up to 95% for all models (Table 2).

The response surfaces were plotted at two extreme levels for one of the independent variables and at the axes for the other two independent variables. To facilitate comparisons, the variables were used in the same position on the axes for each parameter used to compare various biomasses.

The conditions that achieved the highest extraction yields from biomasses were quite similar for sugarcane bagasse and straw: about 30ºC, 60 min and 110 g L^{-1} NaOH for both materials. High NaOH concentration (110 g L^{-1} NaOH) improved the extraction yield, as was evidenced in the models for the bagasse and straw by the positive signal associated with the NaOH concentration variable (Fig. 1). A similar positive correlation between reaction time and extraction yield was verified for bagasse and straw, but in this case the quadratic terms had a negative signal. The extraction yield for bagasse and straw was especially influenced by temperature, which was evidenced by the high coefficient, in quadratic terms and negative signal associated with this variable in the models. As result, similar extraction yields were verified at

extreme temperatures (20ºC and 40ºC), but these temperatures resulted only in minimum extraction yields. Matter extractions were improved by using an intermediate temperature (30ºC) for bagasse and straw. The present study confirms the results published by García et al. (2013) for wheat straw, where a positive correlation between extraction yield and reaction time was observed, but results differed according to temperature. The aforementioned authors reported that the best extraction yields were obtained at 40ºC.

Table 2 - Second-order polynomial models adjusted from normalized independent variables.

	Eucalyptus	R^2	F-Snedecor
10A	$Y_{SY} = 96.47 - 1.03\ X_C X_C - 0.49\ X_T X_t + 0.35\ X_t X_t + 0.18\ X_t$	95.1	53.7
11A	$Y_{YE} = 3.53 + 1.03\ X_C X_C + 0.49\ X_T X_t - 0.35\ X_t X_t - 0.18\ X_t$	95.1	53.7
12A	$Y_X = 9.96 - 0.63\ X_C X_C - 0.34\ X_t - 0.26\ X_T - 0.14\ X_T X_T + 0.12\ X_t X_C + 0.11\ X_T X_C$	97.6	60.3
13A	$Y_{XR} = 79.83 - 6.29\ X_C X_C - 2.54\ X_t - 2.16\ X_T + 1.02\ X_t X_C + 0.85\ X_T X_C$	96.7	58.5
14A	$Y_{XE} = 20.17 + 6.29\ X_C X_C + 2.54\ X_t + 2.16\ X_T - 1.02\ X_t X_C - 0.85\ X_T X_C$	96.7	58.5
	Sugarcane bagasse	R^2	F-Snedecor
10B	$Y_{SY} = 75.57 - 3.33\ X_t + 2.96\ X_T X_T - 2.23\ X_C + 2.14\ X_t X_t - 0.75\ X_T - 0.54\ X_T X_C - 0.35\ X_T X_t$	99.7	350.1
11B	$Y_{YE} = 24.43 + 3.33\ X_t - 2.96\ X_T X_T + 2.23\ X_C - 2.14\ X_t X_t + 0.75\ X_T + 0.54\ X_T X_C + 0.35\ X_T X_t$	99.7	370.1
12B	$Y_X = 20.24 - 1.26\ X_t X_t - 0.66\ X_C - 0.48\ X_T + 0.29\ X_T X_t - 0.26\ X_t + 0.16\ X_C X_C + 0.13\ X_T X_C$	99.6	294.0
13B	$Y_{XR} = 61.66 - 3.87\ X_C - 3.41\ X_t + 2.36\ X_T X_T - 2.29\ X_t X_t - 2.16\ X_T + 0.76\ X_T X_t + 0.64\ X_C X_C$	99.9	753.0
14B	$Y_{XE} = 38.34 + 3.87\ X_C + 3.41\ X_t - 2.36\ X_T X_T + 2.29\ X_t X_t + 2.16\ X_T - 0.76\ X_T X_t - 0.64\ X_C X_C$	99.9	753.0
	Sugarcane straw	R^2	F-Snedecor
10C	$Y_{SY} = 68.94 + 8.50\ X_T X_T - 7.38\ X_t + 3{,}82\ X_t X_t - 1{,}87\ X_t X_C - 1{,}75\ X_C$	99.0	204.9
11C	$Y_{YE} = 31.06 - 8.50\ X_T X_T + 7.38\ X_t - 3.82\ X_t X_t + 1.87\ X_t X_C + 1.75\ X_C$	99.0	204.9
12C	$Y_X = 18.51 + 2.38\ X_T X_T - 1.13\ X_C X_C - 0.95\ X_t X_t - 0.93\ X_C - 0.85\ X_t - 0.47\ X_T + 0.19\ X_T X_C$	99.2	148.6
13C	$Y_{XR} = 49.18 + 13.36\ X_T X_T - 8.33\ X_t + 5.84\ X_t X_t - 4.05\ X_C - 3.51\ X_C X_C - 1.71\ X_T - 1.50\ X_t X_C$	99.6	276.1
14C	$Y_{XE} = 50.82 - 13.36\ X_T X_T + 8.33\ X_t - 5.84\ X_t X_t + 4.05\ X_C + 3.51\ X_C X_C + 1.71\ X_T + 1.50\ X_t X_C$	99.6	276.1

Description of equations: A- eucalyptus; B- sugarcane bagasse; and C- sugarcane straw. 10- Y_{SY}: solid yield (%); 11- Y_{EY}: extraction yield (%); 12- Y_X: xylose content in solid material (%); 13- Y_{XR}: xylose retained in solid material (%); and 14- Y_{XE}: xylose extracted from solid material (%). The independet variables were: X_T: temperature; X_t: reaction time; and X_C: NaOH concentration.

For the eucalyptus, the conditions that maximized the extraction yield were 20ºC, 10 min and both 70 and 110 g L^{-1} NaOH. The extraction yield for the eucalyptus was especially influenced by the NaOH concentration, as evidenced by a high positive coefficient, in quadratic terms, associated with this variable in the models. As result, similar extraction yields were verified using extreme NaOH concentrations (70 and 110 g L^{-1}), with both concentrations resulting in maximum extraction yields. By combining

the minimum reaction time with the minimum temperature, an improvement in the extraction yield was observed. The extraction yields for bagasse and straw evidenced similar behavior during cold alkaline extraction, with the highest values being obtained by using the maximum reaction time and NaOH charge and an intermediate temperature. Under similar cold alkaline conditions, the bagasse and straw had a much higher extraction yield than the eucalyptus.

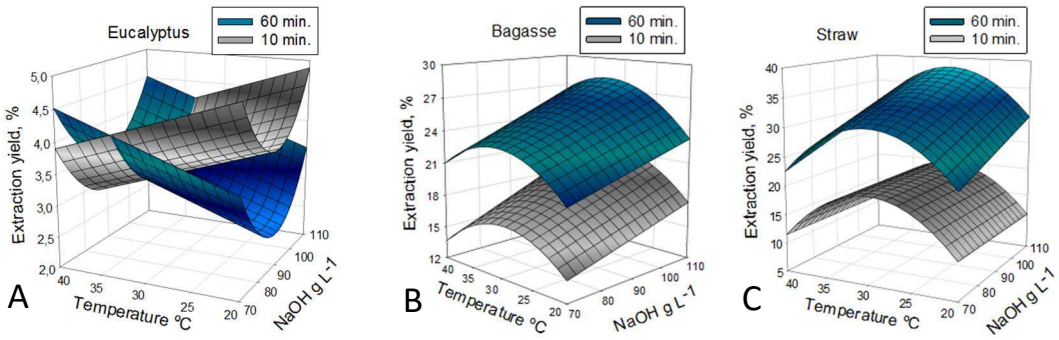

Figure 1 - Effect of temperature, reaction time and NaOH concentration on eucalyptus (**A**), bagasse (**B**) and straw (**C**) extraction yield after cold alkaline extraction.

Use of the maximum reaction time (~60 min) had a positive effect on xylose extraction for all tested biomasses. Similarly, the highest NaOH concentration (110 g L^{-1}) improved the xylose extraction for bagasse and straw (Fig. 2). For eucalyptus, a NaOH concentration of 70 g L^{-1} resulted in the highest xylose extractions. The influence of the temperature, however, differed between the materials. For bagasse and straw, a temperature of approximately 30ºC resulted in the best xylose removal. For eucalyptus, however, a higher temperature (40ºC) was required for maximum xylose extraction. Cold alkaline extraction was more efficient for xylose extraction from bagasse and straw than from eucalyptus.

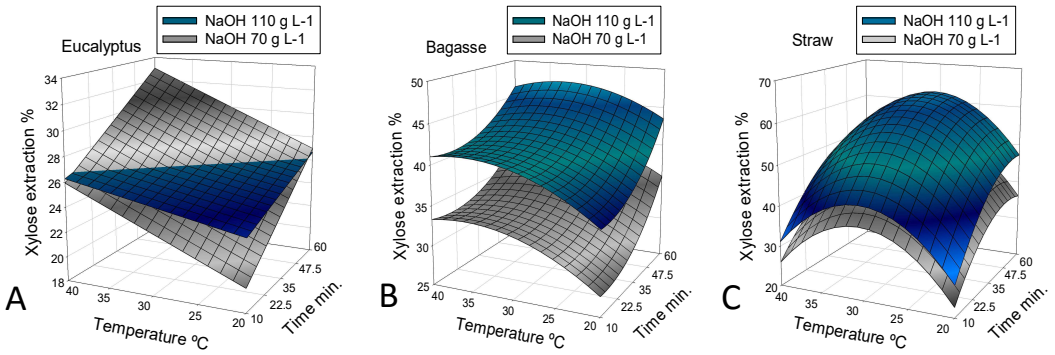

Figure 2 - Effect of temperature, time and NaOH concentration on eucalyptus (**A**), bagasse (**B**) and straw (**C**) xylose extraction after cold alkaline extractions.

Results from the present study showed that the best conditions for obtaining maximum xylose extraction were: 40ºC, 60 min and 70 g L^{-1} NaOH for eucalyptus; 33ºC, 60 min and 110 g L^{-1} NaOH for sugarcane bagasse; and 31ºC, 55 min and 110 g L^{-1} NaOH for sugarcane straw. Under these conditions, cold alkaline extraction was more efficient (based on xylose removal) and, thus, a more promising pretreatment method for sugarcane straw.

The results presented in Table 3 suggest that the theoretical approach used to evaluate the parameters for cold alkaline extraction (i.e., Eq. 10-14) were useful to predict the effect of cold alkaline extraction conducted in accordance with the experimental design. Differences between the observed values (obtained by analysis after cold alkaline extraction) and calculated values (obtained by equations) were less than 15% for all dependent variables and biomasses.

Table 3 - Results for eucalyptus, sugarcane bagasse and straw pretreated in the optimized condition for cold alkaline extraction.

Estimated by the equation					
Material	Solid yield (%)	Extraction yield (%)	Xylose content (%)	Xylose retained (%)	Xylose extracted (%)
Eucalyptus [a]	95.5	4.52	8.36	67.0	33.0
Bagasse[b]	71.9	28.1	18.2	52.5	47.5
Straw [c]	62.3	37.7	15.2	37.5	62.5
Obtained by 5 replication experiments at the optimized condition					
Material	Solid yield (%)	Extraction yield (%)	Xylose content (%)	Xylose retained (%)	Xylose extracted (%)
Eucalyptus [a]	92.2c (0.3)	7.82a (0.3)	8.38a (0.1)	64.4c (1.1)	35.6a (1.1)
Bagasse[b]	69.0b (0.4)	31.0b (0.4)	17.3c (0.3)	47.5b (0.9)	52.5b (0.9)
Straw [c]	62.6a (0.4)	37.4c (0.4)	14.5b (0.3)	34.9a (0.9)	65.1c (0.9)

(...) Standard deviation.

[a] Optimized condition: 40ºC, 60 min and 70 g L^{-1} NaOH.

[b] Optimized condition: 33ºC, 60 min and 110 g L^{-1} NaOH.

[c] Optimized condition: 31ºC, 55 min and 110 g L^{-1} NaOH.

The averages in the column followed by the same letter do not differ from each other at a probability level of 5% by the Tukey test.

Results of complete chemical characterization of biomasses that were pretreated under optimal conditions for cold alkaline extraction are presented in Appendix B. Based on these results, the quantity of each chemical component (per 100 g of biomass) was calculated using the method described by Carvalho et al. (2015), taking into account the actual yields of the pretreated samples (Fig. 3).

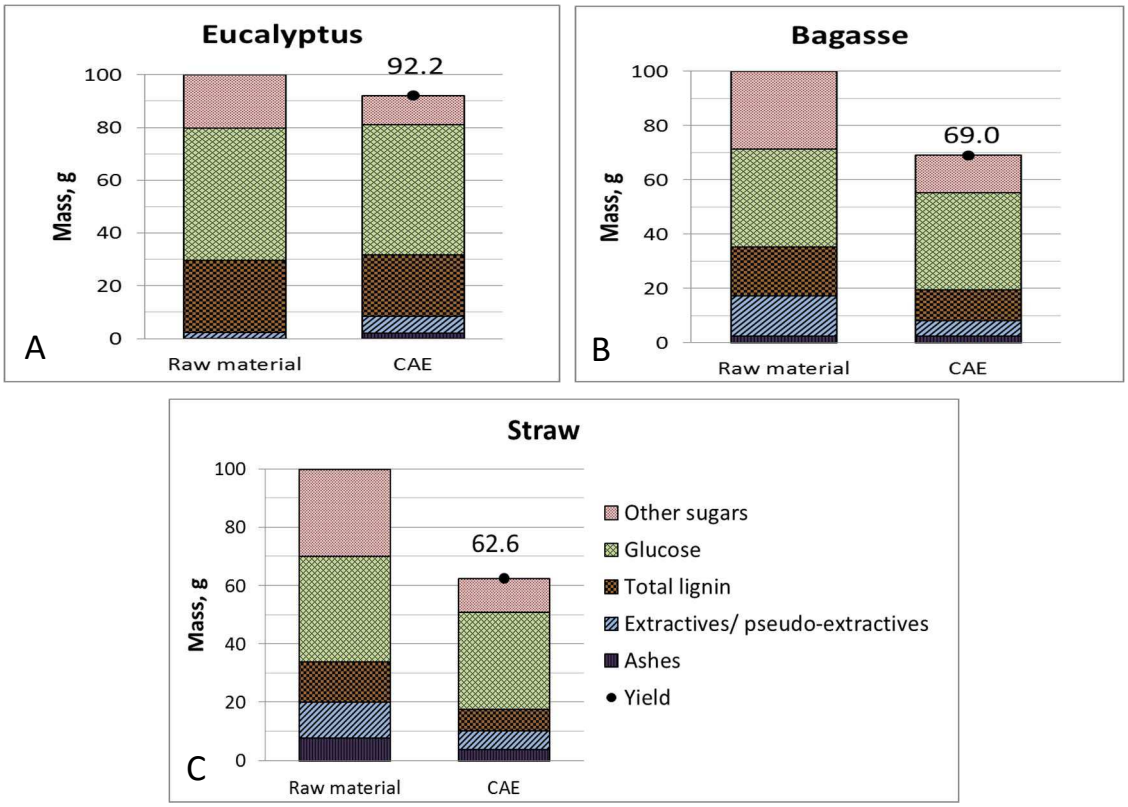

Figure 3 - Chemical composition of eucalyptus (**A**), bagasse (**B**) and straw (**C**) before and after the cold alkaline extraction (obtained by the combination of mass balance and actual yield of each biomass).

The analysis of chemical composition confirmed that the main effect of the cold alkaline extraction process was the removal of hemicelluloses and lignin. The optimal conditions for eucalyptus resulted in the removal of 46% of non-glucosidic sugars (xylose, galactose, mannose and arabinose), while those for bagasse and straw resulted in the removal of 52% and 61% of sugars, respectively. Under the optimal conditions, lignin was also extracted in the amounts of 15%, 37% and 45% for eucalyptus, bagasse and straw, respectively. The higher solubility of lignin from bagasse and straw compared to that from eucalyptus, in alkaline conditions, can be explained by the amount of free phenolic groups and ester bonds, usually higher in grass lignins (Grabber et al., 2004; Hammel, 1997; Granata and Argyropoulos, 1995; Nimz et al., 1981).

The highest amount of non-glucosidic sugars and lignin removed during the cold alkaline pretreatment was observed for straw. Significant hemicellulose (56.1%) and lignin (59.1%) removal was also observed by García et al. (2013) under optimal conditions for cold alkaline pretreatment of wheat straw. In the present study, the glucose content did not appear to change in any of the biomasses during the pretreatment.

Eucalyptus was the biomass least affected by the cold alkaline extraction process, showing the highest solid yield (92.2%). Bagasse and straw were clearly more susceptible to the cold alkaline extraction process, showing solid yields of 69.0% and 62.6%, respectively, and greater removal of non-glucosidic sugars and lignin.

A 2.8 times increasing in the amount of eucalyptus extractives was observed during cold alkaline extraction. Our previous study reported a similar phenomenon that occurred during alkaline treatment using a high temperature (175ºC), and introduced the term "pseudo-extractives" (Carvalho et al., 2015). These pseudo-extractives are structures with solubility similar to that of native extractives from lignocellulosic biomasses, which can be removed by neutral organic solvents. The results of the present study expanded our knowledge of these structures, demonstrating that pseudo-extractives are also generated from eucalyptus under cold alkaline extraction conditions. The source of the pseudo-extractives could have been modified or re-condensed carbohydrates and/or lignin that re-precipitated on the surface of fibers. Similar to the carbohydrate-derived pseudo-lignin that is formed during the various pretreatments (Hu et al., 2012), pseudo-extractives may have a negative effect on cellulose digestibility by enzymes, and the exact mechanism of their formation requires further investigation.

In the bagasse and straw, on the other hand, the quantity of extractives decreased by 62% and 48%, respectively, during pretreatments.

The increase in the ash content in the pretreated eucalyptus and bagasse was probably due to the residual amount of sodium derived from NaOH in these materials. In straw, on the other hand, the ash content decreased by approximately 50%.

3.3 Simultaneous saccharification and fermentation evaluation

3.3.1 Optimization of presaccharification step

Cold alkaline extraction improved the presaccharification performance of all biomasses, when compared with the untreated raw materials (Fig. 4A), which was caused by the increase in the cellulose accessibility derived from the partial removal of

hemicellulose and lignin. After 12 hours of presaccharification, the average glucose concentration stabilized for all pretreated biomasses. From 12 to 72 hours, the average of glucose concentrations were 7.93 g L^{-1}, 8.11 g L^{-1} and 8.35 g L^{-1} for eucalyptus, bagasse and straw, respectively, and these values were significantly different between them by Tukey test (comparison done at α=0.05).

The ceiling in the glucose concentration obtained after 12 hours was probably due to the inhibition of enzymes resulting from a particular glucose concentration in the medium, as suggested by Santos et al. (2010).

Cellobiose is a dimer that is formed from cellulose during enzymatic hydrolysis, which acts as an intermediary between the cellulose chain and the individual glucose (Baeyens et al., 2015). In addition, cellobiose acts as a fermentation inhibitor. In the present study, cellobiose concentrations were lower than 0.60 g L^{-1} for all pretreated biomasses, but did increase from time 0 to 72 hours (Fig. 4B). At 12 hours, a very low amount of cellobiose was generated for all the biomasses. For the untreated biomasses, which released a low glucose concentration, no cellobiose formation was observed in the presaccharificated samples. This suggests that, in this particular case, all the cellobiose generated by enzymatic hydrolysis was hydrolyzed to glucose. In less than 24 hours, the glucose concentration for untreated biomasses stabilized, which was probably due to limited accessibility of cellulose in these materials. Untreated straw was clearly more hydrolyzed by enzymes than untreated bagasse or eucalyptus, generating at least 1.5 times more glucose at the same presaccharification time.

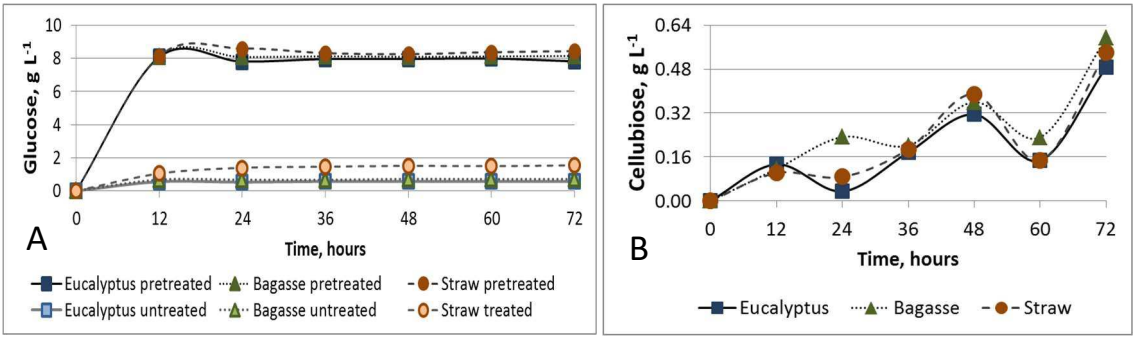

Figure 4 - Results of glucose content for untreated and pretreated biomasses (**A**) and cellobiose content for pretreated biomasses (**B**) generated during presaccharification to 0, 12, 24, 36, 48, 60 and 72 hours for eucalyptus, bagasse and straw.

The presaccharification yields (Fig. 5), as well as the glucose concentration, stabilized after 12 hours for all pretreated biomasses. The need for pretreatment was

evident for all tested biomasses based on the presaccharification yield. Untreated biomasses had approximately 14.0, 11.6 and 5.9 times lower glucose yields after presaccharification than the pretreated eucalyptus, bagasse and straw, respectively. Although untreated straw had the highest presaccharification yield among the biomasses, it did not appear to have any advantage over the other biomasses after pretreatment. From 12 to 72 hours the presaccharification yield stabilized for all pretreated biomasses and the average of presaccharification yield were quite similar among biomasses: 0.097, 0.099 and 0.102 $g_{glucose}/g_{biomass}$ for pretreated eucalyptus, bagasse and straw, respectively. As both the maximum glucose concentration and the stabilization of the presaccharification yield for all pretreated biomasses occurred after 12 hours, this amount of time was chosen for the presaccharification step before the SSF for both cold alkaline pretreated and untreated biomasses.

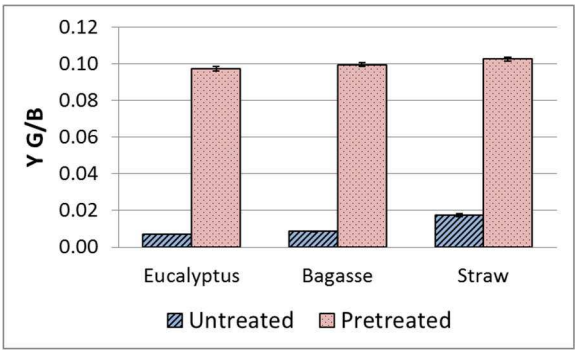

Figure 5 – Average of presaccharification yield ($Y_{G/B}$) measured from 12 to 72 hours.

3.3.2 Simultaneous saccharification and fermentation

Ethanol production from the untreated biomass was very low (Fig. 6A) and pretreatment clearly had a positive effect in this regard (Fig. 6B). Among the tested biomasses, the sugarcane straw was the most suitable for ethanol production as it had the highest ethanol yield and volumetric productivity of ethanol after cold alkaline extraction pretreatment. When considering the untreated biomasses, the straw also presented the best results.

By using 24 hours presaccharification followed by 8 hours SSF, Souza et al. (2012) achieved values substantially higher than those observed in the present study for both, ethanol yield and volumetric productivity of ethanol for sugarcane bagasse pretreated firstly by acid (121ºC, 30 min, 0.5% v/v H_2SO_4) and then by alkaline (121ºC, 30 min, 4% w/v NaOH) conditions which, definetly, helped to improve the accessibility of enzymes towards biomass during presaccharification and SSF. The aforementioned

authors obtained results of approximately 1.1 g L^{-1} h^{-1} and 0.12 $g_{ethanol}/g_{biomass}$ for volumetric productivity of ethanol and ethanol yield, respectively, for pretreated bagasse using a thermotolerant yeast strain (*Kluyveromyces marxianus*) during SSF conducted at 42ºC. Lower volumetric productivity of ethanol than that observed in the present study was found by Santos et al. (2010), who observed volumetric productivity of ethanol of only 0.3 g L^{-1} h^{-1} for bagasse pretreated by alkaline condition (100ºC, 60 min, 10 g L^{-1} NaOH), despite taking 40 hours for volumetric productivity of ethanol (16 h presaccharification + 24 hours SSF) and performing the SSF at 30ºC.

Considering the treated biomasses, the results of the present study suggest that, during the SSF, the sugarcane straw released more glucose than the other biomasses as a result of enzymatic hydrolysis. The glucose concentration after presaccharification was similar between all three biomasses, but a substantial difference in the ethanol yield was verified, most likely due to differences in the chemical composition of the pretreated biomasses. This suggests that, due to their chemical composition after pretreatment, sugarcane bagasse and, especially, sugarcane straw, are more suitable for bioethanol production then eucalyptus. Sugarcane straw showed the highest ethanol concentration (5.74 g L^{-1}), followed by sugarcane bagasse (2.90 g L^{-1}). Eucalyptus, however, only had an ethanol concentration of 0.39 g L^{-1}.

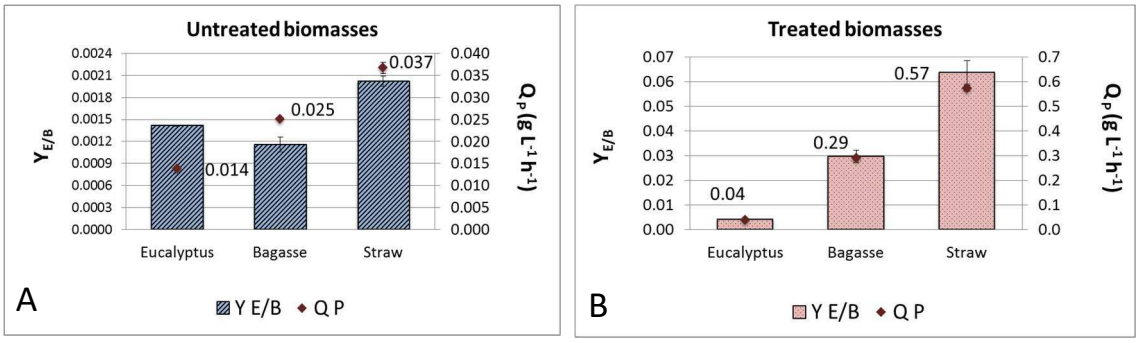

Figure 6 - Column graphic showing the ethanol yield ($Y_{E/B}$) and scatter graphic showing the volumetric productivity of ethanol (Q_P) after 12 hours of presaccharification and 10 hours of simultaneous saccharification and fermentation for untreated biomasses (**A**) and for biomasses pretreated using the optimal conditions for cold alkaline extraction (**B**).

4. Conclusions

- Pseudo-extractives were generated in eucalyptus during the CAE;

- The optimal conditions for cold alkaline extraction were: 40ºC, 60 min and 70 g L^{-1} NaOH for eucalyptus; 33ºC, 60 min and 110 g L^{-1} NaOH for bagasse; and 31ºC, 55 min and 110 g L^{-1} NaOH for straw;
- A 46%, 52% and 61% of xylan (based on xylose) and 15%, 37% and 45% of lignin were removed from eucalyptus, bagasse and straw, respectively. The highest lignin removal for bagasse and straw most likely was favored by the higher solubility of grass lignin in alkaline conditions (due to higher amount of free phenolic groups and ester bonds present in grass lignin compared to wood lignin) and
- The highest ethanol yield (0.064 $g_{ethanol}/g_{biomass}$), ethanol concentration (5.74 g L^{-1}) and volumetric productivity of ethanol (0.57 g L^{-1} h^{-1}) were obtained for sugarcane straw.

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Appendix

Appendix A - Results of solid yield, extraction yield, xylose content, xylose retained and xylose extracted for eucalyptus (A1), sugarcane bagasse (A2) and sugarcane straw (A3) treated by cold alkaline extraction.

A1	**Eucalyptus**				
Normalized values[a] of temp. (X_T), reaction time (X_t) and alkaline charge (X_C)	Solid yield (%)	Extraction yield (%)	Xylose content (%)	Xylose retained (%)	Xylose extracted (%)
0 0 0	96.42	3.58	10.00	80.35	19.65
0 0 0	96.40	3.60	10.05	80.74	19.26
1 1 1	95.38	4.62	8.83	70.18	29.82
1 1 -1	95.51	4.49	8.41	66.94	33.06
1 -1 1	95.95	4.05	9.09	72.68	27.32
1 -1 -1	95.99	4.01	9.30	74.39	25.61
-1 1 1	96.66	3.34	9.07	73.06	26.94
-1 1 -1	96.53	3.47	9.21	74.09	25.91
-1 -1 1	95.00	5.00	9.55	75.60	24.40
-1 -1 -1	95.32	4.68	10.05	79.83	20.17
1 0 0	96.43	3.57	9.65	77.55	22.45
-1 0 0	96.65	3.35	10.03	80.78	19.22
0 1 0	96.77	3.23	9.45	76.21	23.79
0 -1 0	96.83	3.17	10.33	83.35	16.65
0 0 1	95.40	4.60	9.34	74.25	25.75
0 0 -1	95.44	4.56	9.35	74.36	25.64
A2	**Sugarcane bagasse**				
Normalized values[a] of temp. (X_T), reaction time (X_t) and alkaline charge (X_C)	Solid yield (%)	Extraction yield (%)	Xylose content (%)	Xylose retained (%)	Xylose extracted (%)
0 0 0	75.80	24.20	20.21	61.77	38.23
0 0 0	75.60	24.40	20.22	61.64	38.36
1 1 1	73.61	26.39	18.10	53.72	46.28
1 1 -1	79.07	20.93	19.25	61.37	38.63
1 -1 1	81.01	18.99	18.05	58.96	41.04
1 -1 -1	86.60	13.40	19.15	66.87	33.13
-1 1 1	76.61	23.39	18.25	56.38	43.62
-1 1 -1	80.06	19.94	19.90	64.24	35.76
-1 -1 1	82.79	17.21	19.35	64.60	35.40
-1 -1 -1	86.04	13.96	21.00	72.86	27.14
1 0 0	77.17	22.83	19.85	61.77	38.23
-1 0 0	79.46	20.54	20.67	66.23	33.77
0 1 0	74.37	25.63	18.70	56.08	43.92
0 -1 0	80.62	19.38	19.26	62.61	37.39
0 0 1	73.40	26.60	19.86	58.78	41.22
0 0 -1	77.90	22.10	20.94	65.78	34.22
A3	**Sugarcane straw**				
Normalized values[a] of temp. (X_T), reaction time (X_t) and alkaline charge (X_C)	Solid yield (%)	Extraction yield (%)	Xylose content (%)	Xylose retained (%)	Xylose extracted (%)
0 0 0	68.95	31.05	18.61	49.30	50.70
0 0 0	69.05	30.95	18.56	49.23	55.77
1 1 1	69.18	30.82	18.55	49.30	50.70
1 1 -1	76.87	23.13	20.45	60.39	39.61
1 -1 1	89.44	10.56	20.50	70.44	29.56
1 -1 -1	88.30	11.70	21.65	73.44	26.56
-1 1 1	71.38	28.62	19.15	52.51	47.49
-1 1 -1	77.17	22.83	21.50	63.74	36.26
-1 -1 1	88.50	11.50	20.85	70.89	29.11
-1 -1 -1	88.13	11.87	23.10	78.21	21.79
1 0 0	76.88	23.12	20.25	59.81	40.19
-1 0 0	79.08	20.92	21.45	65.17	34.83
0 1 0	66.27	33.73	18.40	46.84	53.16
0 -1 0	80.32	19.68	20.45	63.10	36.90
0 0 1	65.60	34.40	16.54	41.68	28.32
0 0 -1	71.08	28.92	18.15	49.56	50.44

[a] Meaning of normalized values for each independent variable: - 1 → 20ºC, 10 min and 70 g L^{-1}; 0 → 30ºC, 35 min and 90 g L^{-1}; and 1 → 40ºC, 60 min and 110 g L^{-1}.

Appendix B - Chemical composition (lignin, anhydrosugar, ashes and extractives/pseudo-extractives) of pretreated biomasses produced under optimal conditions for cold alkaline extraction based on biomass with extractives and based on the complete mass balance[a].

Biomasses[b]	Klason Lignin %	Soluble Lignin %	Glucose %	Xylose %	Galactose %	Mannose %	Arabinose %	Ash %	Extractives[c] %
Biomass with extractives									
Eucalyptus	23.9	3.84	49.5	8.38	1.16	1.23	0.21	2.21	7.01
Bagasse	17.3	1.07	51.4	17.3	0.43	0.00	1.93	3.40	8.26
Straw	13.0	0.77	50.9	14.5	0.55	0.00	2.60	6.35	10.1
Complete mass balance[a]									
Eucalyptus	21.8	3.49	53.6	9.08	1.26	1.33	0.23	2.21	7.01
Bagasse	15.4	0.95	52.1	17.5	0.44	0.00	1.95	3.40	8.26
Straw	11.4	0.68	53.1	15.1	0.57	0.00	2.71	6.35	10.1

[a] Calculated from average of chemical components.

[b] Optimized condition: 40ºC, 60 min and 70 g L^{-1} NaOH for eucalyptus; 33 ºC, 60 min and 110 g L^{-1} NaOH for sugarcane bagasse; and 31ºC, 55 min and 110 g L^{-1} NaOH for sugarcane straw.

[c] Structures which were removed by extraction with toluene, ethanol and hot water according to TAPPI T 264 cm-07 method (see experimental).

FINAL CONCLUSIONS

- During the pretreatments (hydrothermal, acid, alkaline and cold alkaline extraction) the formation of pseudo-extractives in eucalyptus and pseudo-lignin in sugarcane bagasse and straw was observed, and their formation was only identified using the complete mass balance approach. This new approach, which takes into account the ash and extractives content, proved to be an appropriate way to compare the chemical composition of original and pretreated biomasses and the chemical transformations performed in biomasses during pretreatments, in addition to allow comparisons between various biomasses;
- Eucalyptus, sugarcane bagasse and sugarcane straw proved to be chemically different. Eucalyptus presented higher amount of lignin and cellulose than sugarcane bagasse and straw. In the other hand, sugarcane bagasse and straw presented higher amounts of ash, extractives and hemicelluloses, such as xylan;
- The xylan is the more abundant hemicellulose from eucalyptus, sugarcane bagasse and sugarcane straw, but with different chemical structure (amounts of acetyl groups and substitutes) among such biomasses;
- Eucalyptus xylan was a 4-O-methylglucuronoxylan type and it was observed a molar ratio of xylose units to branches of 4-O-methylglucuronic acid of 10:1.1 and a degree of acetylation of 0.39. The 4-O-methylglucuronic acid were attached at position O-2 of acetylated xylose (acetyl group at position O-3). The acetyl groups were attached to the xylose unit at positions O-3 (64%) (including those in xylose substituted by 4-O-methylglucuronic acid), O-2 (26%) and O-2,3 (10%);
- Bagasse xylan was a arabinoxylan type and it was observed a molar ratio of xylose units to branches of arabinose 10:0.5 and a degree of acetylation of 0.29. The acetyl groups were attached to xylose unit at positions O-3 (60%), O-2 (13%) and O-2,3 (27%);
- Straw xylan was a arabinoxylan type and it was observed a molar ratio of xylose units to branches of arabinose 10:0.6 and a degree of acetylation of 0.08. The acetyl groups were attached to xylose unit at positions O-3 (67%) and O-2 (33%);
- In xylan from bagasse and straw the arabinose substitution happened in higher frequency at position O-3 in xylose unit than at position O-2. Xylan from bagasse and straw was definitely less acid than xylan from eucalyptus;

- Glucose release during presaccharification was negatively affected by the residual amount of hemicelluloses and lignin and potentially harmed by the presence of pseudo-lignin and pseudo-extractives in pretreated biomasses;
- The eucalyptus hydrothermally pretreated presented the highest ethanol yield (0.0168 $g_{ethanol}/g_{biomass}$), yield ethanol concentration (1.6 g L^{-1}) and volumetric productivity of ethanol (0.16 g L^{-1} h^{-1}) among biomasses pretreated in the same conditions (Table 1), probably because the eucalyptus xylan was a more abundant source of acid for pretreatment than bagasse xylan or straw xylan;
- For bagasse and straw, the acid doses used during acid pretreatments at the optimal condition was significant higher (4.5% w/w H_2SO_4) than that for eucalyptus (1.5% w/w H_2SO_4), most likely due to the lower acetyl groups content in these xylans compared to that from eucalyptus. Acid doses over than 1.5% H_2SO_4 did not change chemical composition of pretreated eucalyptus;
- The acid pretreatments were more effective to pretreat biomasses than the hydrothermal pretreatment, achieving higher values of ethanol yield, ethanol concentration and volumetric productivity of ethanol. The exception was the eucalyptus, most likely due to the highest formation of pseudo-extractives (a potential SSF inhibitor) in acid pretreatment than in hydrothermal.
- After acid pretreatment the straw provided the highest ethanol yield (0.056 $g_{ethanol}/g_{biomass}$), ethanol concentration (5.1 g L^{-1}) and volumetric productivity of ethanol (0.51 g L^{-1} h^{-1}) (Table 1);
- During alkaline pretreatment, the amount of glucose was little changed for eucalyptus and bagasse, irrespectively to the alkaline charge. Unlike to straw, for which a substantial glucose removal was observed in higher alkaline charge. After alkaline pretreatment, the residual lignin and hemicelluloses negatively affect the glucose releasing during presaccharification, whereas for straw the glucose removal during pretreatment impaired the glucose releasing during presaccharification;
- The optimal condition for alkaline pretreatment were 10% NaOH for eucalyptus, 15% NaOH for bagasse and 5% NaOH for straw;
- After alkaline pretreatment the bagasse provided the highest ethanol yield (0.101 $g_{ethanol}/g_{biomass}$), ethanol concentration (8.8 g L^{-1}) and volumetric productivity of ethanol (0.88 g L^{-1} h^{-1}) (Table 1);

- The optimal conditions for cold alkaline extraction were: 40ºC, 60 min and 70 g L^{-1} NaOH for eucalyptus; 33ºC, 60 min and 110 g L^{-1} NaOH for bagasse; and 31ºC, 55 min and 110 g L^{-1} NaOH for straw;
- After cold alkaline extraction the straw provided the highest ethanol yield (0.064 $g_{ethanol}/g_{biomass}$), ethanol concentration (5.74 g L^{-1}) and volumetric productivity of ethanol (0.57 g L^{-1} h^{-1}) (Table 1);
- The alkaline pretreatment was, among the processes investigated in this study (hydrothermal, acid, alkaline and cold alkaline extraction), the most suitable to pretreat eucalyptus, bagasse and straw, with a view to their subsequent bioconversion into ethanol. The biomasses, after alkaline pretreatment achieved the highest values for ethanol yield, ethanol concentration and volumetric productivity of ethanol (Table 1); and
- These results may open new possibilities of use for bagasse and staw, including in the sugarcane industry itself, by integrating the first and second ethanol platforms.

Table 1 – Comparative table between the parameters in ethanol production, namely: ethanol concentration, ethanol yield ($Y_{E/B}$) and volumetric productivity of ethanol (Q_P) for eucalyptus, bagasse and straw pretreated using different processes.

Parameters	Eucalyptus	Bagasse	Straw
	Hydrothermal pretreatment[a]		
Ethanol, g L^{-1}	1.6	0.8	1.4
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.0168	0.0051	0.0069
Q_P, g L^{-1} h^{-1}	0.16	0.08	0.14
	Acid pretreatment[b]		
Ethanol, g L^{-1}	1.3	3.6	5.1
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.0140	0.0393	0.0562
Q_P, g L^{-1} h^{-1}	0.13	0.36	0.51
	Alkaline pretreatment[c]		
Ethanol, g L^{-1}	3.4	8.8	6.4
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.0369	0.1011	0.0764
Q_P, g L^{-1} h^{-1}	0.34	0.88	0.64
	Cold alkaline extraction pretreatment[d]		
Ethanol, g L^{-1}	0.39	2.90	5.74
$Y_{E/B}$, $g_{ethanol}/g_{biomass}$	0.004	0.030	0.064
Q_P, g L^{-1} h^{-1}	0.04	0.29	0.57

[a] Performed at 175ºC for 15 min for eucalyptus, bagasse and straw. Presaccharification and SSF times of 24 and 10 hours, respectively.

[b] Performed at optimized acid condition for eucalyptus (175ºC, 15 min and 1.5% w/w H_2SO_4), bagasse (175ºC, 15 min and 4.5% w/w H_2SO_4) and straw (175ºC, 15 min and 4.5% w/w H_2SO_4). Presaccharification and SSF times of 24 and 10 hours, respectively.

[c] Performed at optimized alkaline condition for eucalyptus (175ºC, 15 min and 10% NaOH on dry basis), bagasse (175ºC, 15 min and 15% NaOH on dry basis) and straw (175ºC, 15 min and 5% NaOH on dry basis). Presaccharification and SSF times of 24 and 10 hours, respectively.

[d] Performed at optimized cold alkaline extraction condition for eucalyptus (40ºC, 60 min and 70 g L^{-1} NaOH), bagasse (33ºC, 60 min and 110 g L^{-1} NaOH) and straw (31ºC, 55 min and 110 g L^{-1} NaOH). Presaccharification and SSF times of 24 and 10 hours, respectively.