UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE TECNOLOGIA
DEPARTAMENTO DE ENGENHARIA QUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

TATIANE CAVALCANTE MACIEL

VALORIZAÇÃO DA PODA DA VIDE E DA CASCA DA CASTANHA: PRÉ-TRATAMENTO E HIDRÓLISE ENZIMÁTICA DA CELULOSE

FORTALEZA

2017

TATIANE CAVALCANTE MACIEL

VALORIZAÇÃO DA PODA DA VIDE E DA CASCA DA CASTANHA: PRÉ-TRATAMENTO E HIDRÓLISE ENZIMÁTICA DA CELULOSE

Tese apresentada ao Programa de Pós-graduação em Engenharia Química, da Universidade Federal do Ceará, como requisito parcial para obtenção do título de doutor em Engenharia Química. Àrea de concentração: Processos Químicos e Bioquímicos.

Orientadora:Prof[a]. Dra. Sueli Rodrigues.
Coorientadora:Prof[a]. Dra. Lígia Rodrigues

FORTALEZA
2017

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

M139v Maciel, Tatiane.
Valorização da poda da vide e da casca da castanha: pré-tratamento e hidrólise enzimática da celulose / Tatiane Maciel. – 2020.
88 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Tecnologia, Programa de Pós-Graduação em Engenharia Química, Fortaleza, 2020.
Orientação: Profa. Dra. Sueli Rodrigues.
Coorientação: Profa. Dra. Lígia Rodrigues.

1. Poda da vide. 2. Casca da castanha (Sativa Miller). 3. Pré-tratamento ácido. 4. Pré-tratamento alcalino. 5. Hidrólise enzimática. I. Título.

CDD 660

TATIANE CAVALCANTE MACIEL

VALORIZAÇÃO DA PODA DA VIDE E DA CASCA DA CASTANHA: PRÉ-TRATAMENTO E HIDRÓLISE ENZIMÁTICA DA CELULOSE

Tese apresentada ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal do Ceará, como requisito parcial à obtenção do título de doutor em Engenharia Química. Área de concentração: Processos Químicos e Bioquímicos

Aprovada em: 17/10/2017.

BANCA EXAMINADORA

_____

Prof[a] Dra. Sueli Rodrigues (Orientadora)
Universidade Federal do Ceará (UFC)

_____

Prof[a] Dra Luciana Rocha Barros Gonçalves
Universidade Federal do Ceará (UFC)

_____

Prof[a] Dra Maria Valderez Ponte Rocha
Universidade Federal do Ceará (UFC)

_____

Dra Thatyane Vidal Fonteles
Universidade Federal do Ceará (UFC)

_____

Prof[a] Dra Tigressa Helena Soares Rodrigues
Universidade Estadual Vale do Acaraú (UVA)

A minha mãe, meu pai, minha irmã e meu irmão.

Dedico

AGRADECIMENTOS

À Deus por seu infinito amor e cuidado com a minha vida.

À minha mãe, Jeanne Maria, por todo seu amor, dedicação, compreensão e companheirismo. Obrigada por compreender minhas ausências.

À minha irmã, Amanda Cavalcante, por seu companheirismo, amor e paciência. Obrigada por estar sempre ao meu lado e me incentivar.

Ao meu pai, Francisco Nerício, por seu amor e cuidado.

Ao meu irmão, Gabriel Levi, por me ensinar o valor das pequenas coisas.

Aos meus queridos amigos do Labiotec, Cristiane, Niédila, Simone, Lívia, Dayana, Rhonyele, Ronnyele, Priscila, Iane por todo o companherismo, apoio e amizade. Obrigada por tentarem me alegrar especialmente quando tudo dava errado. Sentirei muitas saudades desses momentos, em especial das conversas no café.

À Soraya Sancho, por sua amizade, paciência, disponibilidade e constante incentivo.

À Ana Raquel, por sua amizade, ajuda valiosa e tanto conhecimento compartilhado.

À Fátima e a Priscila Sales, duas amigas que ganhei durante o período do intercâmbio. Muito obrigada por todo carinho, paciência, incentivos e conselhos.

À todos os meus amigos do Laboratório de Fermentações (LF) da Universidade do Minho, obrigada pelo acolhimento e compreensão. Trago em meu coração lembranças maravilhosas de todos vocês.

À Profª. Drª. Sueli Rodrigues, obrigada por sua dedicação, compreensão e profissionalismo. Obrigada por sua preciosa contribuição para minha formação acadêmica. Foram 8 anos de muito aprendizado. Especialmente, agradeço pelo incentivo à realização do doutorado sanduiche, sem seu encorajamento, paciência e compreensão provavelmente eu não teria vivido essa experiência fantástica em minha vida. Muito obrigada!

À Profª. Drª Lígia Rodrigues, por me receber em seu País e em seu laboratório. Seu cuidado e suas orientações foram valiosos durante todo o período do doutorado sanduiche. Obrigada por sua participação em minha Qualificação, sua correção sempre minuciosa contribuiu sobremaneira para melhoria deste trabalho.

À Profª. Drª. Luciana Gonçalves, obrigada por aceitar meu convite para participar de minha Qualificação, suas sugestões e correções enriqueceram sobremaneira este trabalho.

Obrigada por disponibilizar seu tempo no aprimoramento deste trabalho e por aceitar nosso convite para participar também da defesa.

À Profª. Drª. Valderez, obrigada por sua disponibilidade em participar da defesa desta Tese. Suas correções e sugestões foram extremamente importantes para a melhoria desse trabalho.

À Profª. Drª. Tigressa, obrigada por aceitar meu convite para participar da defesa dessa Tese. Sua ampla experiência nesse tema certamente contribui para a melhoria desse trabalho.

À Drª. Thatyane Vidal, obrigada por disponibilizar seu tempo na correção desse trabalho.

À Central Analítica da Universidade Federal do Ceará e ao Prof. Dr. Emílio Miguel pela realização das análises microscópicas.

Aos Departamentos de Engenharia Química e Tecnologia de Alimentos da Universidade Federal do Ceará pela minha formação acadêmica e em especial, pela oportunidade de realizar esse trabalho.

Ao Departamento de Engenharia Biológica da Universidade do Minho (Braga-PT) pela oportunidade de realizar uma parte desse trabalho durante o período do Doutorado Sanduiche.

O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Muito obrigada!

“E sobre tudo isto, revesti-vos de amor, que é o vínculo perfeito.”

(Colossenses 3:14)

RESUMO

A biomassa lignocelulósica, fonte de carbono renovável mais abundante no mundo, é constituída majoritariamente por polímeros de lignina, hemicelulose e celulose. Esses materiais são resíduos de outros processos de produção e são ricos em açucares. Por isso, vários estudos sobre o reaproveitamento desses materiais em bioprocessos têm sido realizados no mundo todo. O objetivo do presente estudo foi avaliar as melhores condições para fracionar a poda da vide e a casca da castanha com o objetivo de permitir o acesso da celulose a ação de enzimas celulases. Os materiais tratados foram submetidos à hidrólise enzimática com as enzimas Cellic® CTec2 e Novozyme 188 a fim de avaliar a eficácia dos processos. O pré-tratamento da poda da vide foi realizado em duas etapas, a primeira com ácido sulfúrico diluído e a segunda com NaOH. No tratamento com ácido (1,5% de H_2SO_4) realizado a 120 ºC, durante 30 minutos, a maior quantidade de hemicelulose foi solubilizada. Essa condição foi selecionada para a realização da próxima etapa. Na deslignificação, dentre as condições testadas, a maior remoção de lignina (63,8%) ocorreu no tratamento com 3,0% de NaOH e 120 ºC (sem agitação durante 60 minutos). No entanto, o maior rendimento em glicose obtido a partir da hidrólise enzimática (com as enzimas comerciais) ocorreu no material tratado com 2,0% de NaOH a 100 ºC sem agitação. Sendo possível concluir que nem sempre o material com menor conteúdo de lignina, resultará nos maiores rendimento na hidrólise enzimática. O outro resíduo utilizado nesse estudo, a casca da castanha, foi submetido apenas à deslignificação com NaOH em diferentes combinações de temperatura e concentração do álcali. No ensaio realizado à temperatura de 100 ºC com 2,0% de NaOH sob agitação, a lignina foi quase completamente solubilizada (94,3%). O menor teor de lignina removida foi 75% e ocorreu quando o material foi tratado com 1,0% de NaOH, 60 ºC durante 1 hora (condição mais branda dentre aquelas que foram testadas). Por isso, os materiais obtidos em todas as condições de tratamento testadas foram submetidos à hidrólise enzimática com enzimas comerciais. Os valores dos rendimentos em glicose obtidos após 72 horas de hidrólise não apresentaram diferença significativa entre si segundo o teste de Tukey. Deste modo, o tratamento realizado com 1,0% de NaOH a 60 ºC foi selecionado e nessas condições foi possível obter 86% de glicose. Com esses resultados foi possível observar que nem sempre as condições de tratamento que removem mais hemicelulose e/ou lignina serão as mais interessantes para o fim que se deseja. Dependendo da mudança ocorrida no material e das enzimas empregadas, condições mais brandas de tratamento podem proporcionar

resultados satisfatórios. Portanto, ficou clara a importância de avaliar em conjunto os dados da hidrólise da celulose e do pré-tratamento na seleção das melhores condições de tratamento. Os resultados obtidos pela análise clássica recomendada pelo NREL para ambos os materiais foram corroborados por MEV em que se mostrou claramente a remoção de lignina e a exposição da estrutura celular interna na biomassa.

Palavras-chave: Pré-tratamento ácido; deslignificação; Poda da vide; Casca da castanha (Sativa Miller)

ABSTRACT

The lignocellulosic biomass represents the carbon source renewable most abundant in the world, being constituted by polymers of lignin, hemicellulose and cellulose. In the last yeares, these materials has received more attention because are rich in sugars and most part they are residues from other production processes. For this reason, the numbers of researches with the objective of fractionate them and apply their constituents in the production of products high added value has grown in around the world. The sugars released from biomass can be employed as carbon source, in the production of antibiotics, amino acids, enzymes and biochemicals as biofuels.The fractionate of the biomass occurs through pretreatments that can to be chemical, phisical, phisical-chemical and biological. Because of complexity these materials there is no single pretreatment that are equally efficient for all them. Therefore, studies to check which the treatment most indicated for the each material or for a specific application must be performed. In this work, two residues, the vine pruning and chestnut shell were selected. The objective was to define the best conditions for to remove hemicellulose and lignin in these materials, and the residues treated were hydrolyze with commercial enzymes Cellic® CTec2 e Novozyme 188 in order to assess the efficacy of the processes. In the delignification, among conditions tested, the higher removal of lignin (63.8%) occurred in the material treated with 3.0% of NaOH and 120 ºC (without agitation during 60 minutes). However, the most yield in glucose was obtained from enzymatic hydrolysis of the vine pruning treated with 2.0% of NaOH and 100 ºC (without agitation during 60 minutes). It could be concluded that do not always the material with lower content of lignin result in higher yields in glucose. The chestnut shell was the other residue utilized in this work. It was subbmited only the delignification with NaOH in differents combinations of temperature and concentration. In the assay performed at 100 ºC with 2.0% of NaOH under agitation, the lignin was almost completely removed (94.3%). The lower amount of lignin (75%) removal with treatments occured when the material was subbmited at 1.0% NaOH, 60 ºC during 1 hour. Therefore, the materials obtained in all conditions were subjected at enzymatic hydrolysis with commercial enzymes. The yields quantified in glucose after 72 hours of hydrolysis not showed difference significative in accordance Tukey's test. For this reason, the treatment performed with 1.0% of NaOH and 60 ºC was chosen. With these results was to possible that confirm the importance of the enzymatic hydrolysis data in the evaluation of efficiency of lignocellulosic material pretreatment, because not always the conditions that

removed the highest quantify of lignin or hemicellulose are the most interesting. Depending on the change occured in material and the enzymes employed, the mild conditions may provide satisfactory performance. The results obtained by the classical analysis recommended by NREL were corroborated by eletronic microscopy in which it is clearly shown the lignin removal and the exposure of the internal vegetal cell structure in the biomass.

Keywords: Acid pretreatment; Alkaline pretreatment; Vine pruning; Chestnut shell (Sativa Miller).

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO

Os materiais lignocelulósicos representam a fonte de carbono renovável mais abundante no mundo (BEHERA; RAY, 2016), sendo formados, basicamente por lignina, hemicelulose e celulose que juntos formam uma estrutura complexa e resistente à ação de enzimas e micro-organismos (NARRA; JAMES; BALASUBRAMANIAN, 2015). A palha de trigo e milho, casca de amendoim, palha e bagaço de cana, bagaço de caju, espiga de milho, podas de madeira, polpas de celulose, resíduos de frutas e vegetais são alguns exemplos desse tipo de material (ARORA; BEHERA; KUMAR, 2015; ROCHA et al., 2014).

Nos últimos anos, esses materiais têm recebido maior atenção, pois, além de ser uma fonte de carbono renovável, em sua maioria são resíduos de outros processos e, portanto, não apresentam custos diretos de produção (CHIEH-LUN CHENG; JO-SHU CHANG, 2011). Por isso cresce o número de pesquisas no mundo todo com o objetivo de fracionar esses materiais e aplicar seus constituintes na produção de produtos de valor agregado. Seu aproveitamento integral está inserido no conceito de biorrefinaria, que consiste em utilizar todas as frações da biomassa como matéria-prima em bioprocessos (VAZ DE ARRUDA et al., 2017; VECINO et al., 2017). Atualmente, algumas biorrefinarias estão sendo instaladas com o objetivo de integrar os processos de conversão da biomassa em diversos produtos e otimizar o uso dos recursos naturais, diminuindo o desperdício. Em países como Estados Unidos, Brasil e Alemanha, biorrefinarias baseadas na produção de etanol e biodiesel a partir de milho, cana-de-açúcar e soja estão consolidadas. O desafio agora consiste em utilizar matérias-primas alternativas não comestíveis, como os resíduos anteriormente mencionados (BRASIL; SILVA; SIQUEIRA, 2017).

Inúmeras são as aplicações possíveis para os constituintes dos materiais lignocelulósicos, por exemplo: a xilose, um dos principais açúcares que compõem a hemicelulose, pode ser empregada na produção de xilitol, um adoçante amplamente comercializado e que apresenta também diversas outras aplicações nas indústrias de alimentos e farmacêutica (ROCHA et al., 2014; VAZ DE ARRUDA et al., 2017; VECINO et al., 2017). A glicose, presente em sua maior parte na celulose, pode ser empregada na produção de biocombustíveis e outros produtos químicos (DUTTA; PAL, 2014; YAN et al., 2015) e a lignina, na produção de polímeros aromáticos, como fenol,

vanilina, siringaldeído que são matérias-primas importantes para a indústria de aromas e fragrâncias (MOTA et al., 2016).

Diversos países na Europa, em particular Portugal, possuem uma longa tradição na produção de vinhos (BRITO; OLIVEIRA; RODRIGUES, 2014). França, Itália, Espanha, Alemanha e Portugal são os principais produtores de vinho na Europa e em 2016 foram responsáveis por 54% da produção mundial dessa bebida (26 bilhões de litros). Dentre esses países, Portugal ocupa a 5º posição no ranking e teve uma produção de 600 milhões de litros em 2016 (IVV, 2017). No Brasil, a produção de uvas e vinhos concentra-se especialmente no estado do Rio Grande do Sul, com grande destaque para as regiões da Serra Gaúcha e Campanha (BELMIRO; PEREIRA; PAIM, 2017). Em 2015, esse estado foi responsável pela produção de 250 milhões de litros de vinho (UVIBRA, 2017). Anualmente, a árvore produtora de uvas, a videira, precisa ser podada após a colheita, com o objetivo de aumentar sua produtividade e a qualidade dos seus frutos na próxima safra (MILLER et al., 2001). Os resíduos gerados nesse processo normalmente são queimados no próprio campo causando problemas ambientais especialmente relacionados à combustão da lignina (BUSTOS et al., 2004). Numa amostra de poda da vide, obtida do cultivo de uvas na região do Minho, em Portugal, foi quantificado 36% de celulose, 21,8% de hemicelulose e 29,6% de lignina (dados obtidos no presente estudo).

A castanha-portuguesa ou castanheiro-bravo ou castiro ou pinhão doce tem o nome científico de *Castanea sativa Miller* e é uma das 12 espécies diferentes de castanha existentes em todo o mundo e na Europa é uma das principais cultivadas (LI et al., 2016). Na Europa, cerca de 108.00 toneladas de castanha são produzidas por ano, sendo a Itália o maior produtor (50.000 t/a), seguido de Portugal (31.051 t/a) e Espanha (20.000 t/a) (VELOSO, 2008). A casca da castanha representa cerca de 10% do peso do fruto (LEE et al., 2016), isso significa que cerca de 10 toneladas de casca de castanha são liberadas por ano na Europa. Esse resíduo é uma fonte natural de antioxidantes e corantes (ZHAO; FENG; WANG, 2014) e sua composição varia de acordo com a área na qual o fruto foi cultivado. No presente estudo, uma amostra de casca de castanha da região de Trás-os-Montes's, em Portugal, apresentou 27,3% de celulose, 12,6% de hemicelulose e 43,6% de lignina. Nesse contexto, a casca da castanha é um material lignocelulósico abundante que pode ser reaproveitado na produção de produtos de elevado valor agregado.

Entretanto, a primeira limitação encontrada para a reutilização desses materiais é a dificuldade de transformar seus polissacarídeos estruturais em açúcares simples (MISHRA et al., 2017) e isso ocorre, principalmente, devido a sua natureza recalcitrante, causada em grande parte pela lignina incorporada às fibrilas de celulose (MISHRA et al., 2017; SARATALE et al., 2017). Por isso, normalmente, a primeira etapa no processo de transformação desses materiais consiste num pré-tratamento para abertura da estrutura do material e separação das frações: lignina, hemicelulose e celulose (LIM et al., 2012; SARKAR et al., 2012).

Em relação ao objetivo deste trabalho, vale registrar que inicialmente era estudar de forma detalhada a hidrólise enzimática dos resíduos com uma enzima produzida por nosso grupo de pesquisa, e, portanto, ainda não disponível comercialmente. Essa enzima é produzida a partir da fermentação em estados sólido do bagaço de cana utilizando o micro-organismo *Melanoporia* sp. CCT 7736 isolado por nosso grupo de pesquisa. A otimização do processo de produção da mesma ocorreu durante a realização do meu mestrado (MACIEL, 2013). Entretanto, devido a inúmeras interrupções, não avisadas, no fornecimento de energia no Campus do Pici, diversas fermentações foram perdidas, além da própria cepa do micro-organismo. Muito tempo foi investido na tentativa de reativar estoques antigos para que o mesmo voltasse a produzir a enzima de interesse, sempre sem sucesso. Por causa disso, se fez necessário mudar o enfoque do trabalho que passou a ter como objetivos definir as melhores condições de pré-tratamento da casca da castanha (*Castanea sativa Miller*) e da poda da vide com o objetivo de remover a hemicelulose e a lignina presentes nos materiais, possibilitando assim a hidrólise enzimática da celulose através de enzimas comerciais.

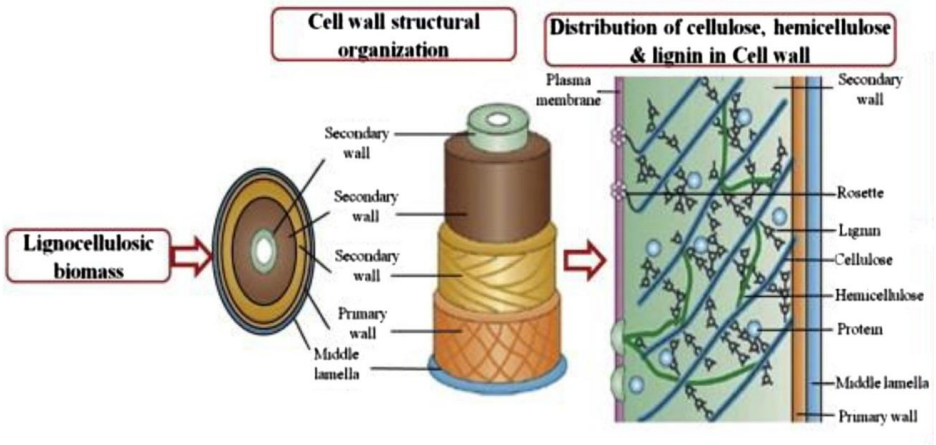
O trabalho apresenta-se dividido em três capítulos. No Capítulo 1 é apresentada uma revisão bibliográfica versando sobre a composição da biomassa lignocelulósica, possibilidades de reaproveitamento e os pré-tratamentos empregados no presente trabalho. Os Capítulos 2 e 3 apresentam o estudo do pré-tratamento da poda da vide e da casca da castanha, respectivamente, bem como a hidrólise enzimática desses materiais utilizando enzimas comerciais.

2 REVISÃO BIBLIOGRÁFICA

2.1 Materiais lignocelulósicos

Os materiais lignocelulósicos representam a fonte de carbono renovável mais abundante no mundo (BEHERA; RAY, 2016). Dentro desse grupo encontra-se a biomassa vegetal que compreende cerca de 60% da biomassa total e inclui resíduos de madeira, gramíneas, resíduos da indústrial do papel, resíduos agroindustriais como caules, palhas, cascas e sementes, resíduos domésticos e resíduos urbanos (BILAL *et al.*, 2017). Esses materiais são formados, basicamente por lignina, hemicelulose e celulose em uma estrutura complexa e de difícil decomposição (Figura 1). A composição de cada uma dessas frações é variável de acordo com o material (NARRA; JAMES; BALASUBRAMANIAN, 2015).

Figura 1 – Componentes estruturais da biomassa lignocelulósica



Fonte: (Bilal *et al*., 2017)

2.1.1 Celulose

A celulose é o polissacarídeo mais importante da parede celular das plantas sendo produzido continuamente através do processo de fotossíntese (BEHERA; RAY, 2016). Ela é um polímero linear com alto peso molecular e altamente ordenado, sendo formada por moléculas de D-glicose unidas entre si através de ligações β-(1,4)-glicosídicas com grau de polimerização (DP) que pode chegar a 10.000 (LE FLOCH; JOURDES; TEISSEDRE, 2015). Moléculas de celulose associam-se formando

microfibrilas de celulose (MOOD *et al.*, 2013) que são feixes dessas moléculas estabilizadas por pontes de hidrogênio entre seu grupo hidroxila e o oxigênio de moléculas adjacentes (JÖNSSON; MARTÍN, (2016)). Esse polissacarídeo apresenta regiões de elevada organização molecular (celulose cristalina), intercaladas com regiões altamente desorganizadas (celulose amorfa) (NEVES; PITARELO; RAMOS, 2016) sendo por isso, insolúvel em água e na maioria dos solventes orgânicos (MOOD *et al.*, 2013). A partir da glicose é possível produzir diversos bioprodutos como bioetanol (BILAL et al., 2017), biosurfactantes (VECINO et al., 2017), enzimas (IDRIS et al., 2017) e outros.

2.1.2 Hemicelulose

A hemicelulose é um constituinte das paredes celulares secundárias das plantas e pode representar de 20-30% da composição de biomassa de madeiras e pantas herbáceas (VAZ DE ARRUDA et al., 2017). Ela é um heteropolissacarídeo que pode conter pentoses (xilose e arabinose), hexoses (glicose, manose e galactose) e resíduos de ácidos urônicos (como por exemplo, glucurônicos e 4-O-metil-glucurônico) (NEVES; PITARELO; RAMOS, 2016). Na biomassa lignocelulósica, as fibrilas de hemicelulose encontram-se recobrindo a celulose (MOOD *et al.*, 2013).

A composição da hemicelulose depende do tipo de material, por exemplo, em madeiras duras e herbáceas, ela é composta essencialmente por xilano (ZHUANG *et al.*, 2016). No bagaço de cana, poda da vide e casca da castanha, o xilano é o principal componente estrutural e representa 25%, 22% e 13% da composição total dos materiais, respectivamente. Ele é constituído por uma cadeia principal linear formada por unidades de xilanopiranose unidas entre si através de ligações do tipo β-1-4, tais unidades, podem ser substituídas por α-L-arabinofuranosil, grupos acetil ou glucuronopiranosil e 4-O-metil-glucuronopiranosil. Devido a essa heterogeneidade, a hidrólise desse polímero requer o efeito conjunto de diversas enzimas, como as endoxilanases (atuam sobre o xilano e liberam xilo-oligossacarídeos (XOS - xilobiose e xilotriose), glucoronidases, arabinofuranosidases, acetil esterases e β-xilosidases (que hidrolisam os XOS a xilose) (XIN *et al.*, 2015). Por outro lado, em madeiras moles, os componentes majoritários são galactomananos e glucomananos (ZHUANG *et al.*, 2016).

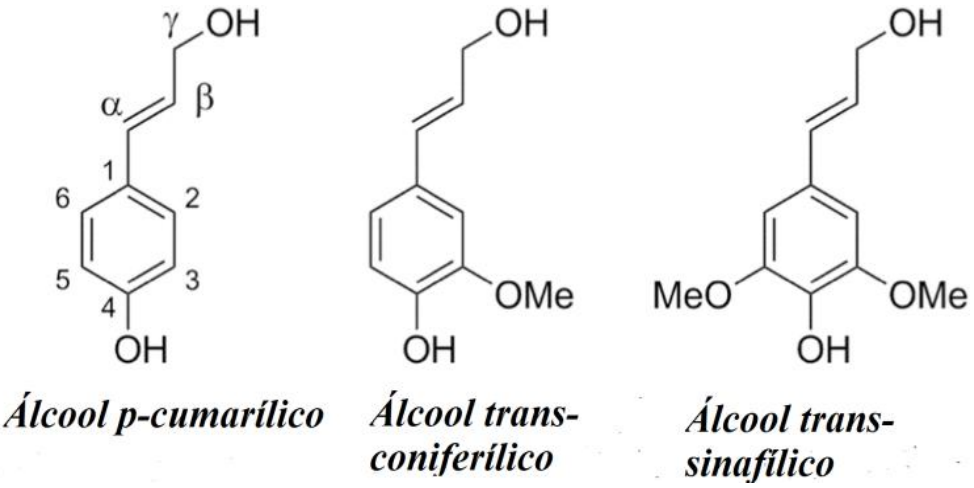
A xilose liberada a partir da hidrólise da hemicelulose pode ser utilizada em diversos bioprocessos, como por exemplo, na produção de xilitol (VAZ DE ARRUDA

et al., 2017), surfactantes (MARTINS et al., 2017) e ácido succínico (PATERAKI et al., 2016), dentre outros.

2.1.3 Lignina

A lignina é um polímero heterogêneo formado por unidades fenilpropanóides (álcool p-cumárico, álcool trans-coniferílico, álcool trans-sinafílico) (Figura 2). Em madeiras moles, a lignina é formada principalmente por álcool coniferílico. Por outro lado, em madeiras duras, os alcoóis que estão presentes são coniferílico e sinafílico, sendo a proporção dessas unidades variável de acordo com a espécie (CHEN; WAN, 2017).

Figura 2 - Unidades fenilpropanóides (álcool p-cumárico, álcool trans-coniferílico, álcool trans-sinafílico) presentes no polímero lignina

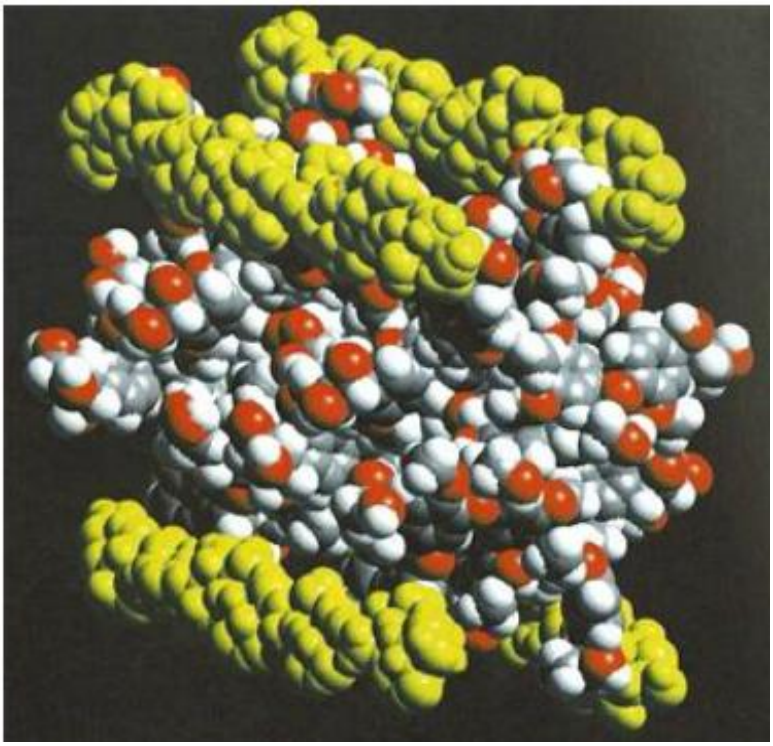


Fonte: (FORSS; FREMER, 2006).

As unidades fenilpropanóides presentes na lignina encontram-se ligadas entre si através de uma complexa rede de ligações éter e carbono-carbono. Os ácidos cumarílico, ferúlico e diferúlico são compostos fenólicos que embora não façam parte da lignina estão envolvidos em sua ligação com a hemicelulose (JÖNSSON; MARTÍN, 2016). Tanto a composição desse polímero como suas ligações com os carboidratos dificultam a hidrólise da celulose presente na biomassa lignocelulósica (CARVALHO *et al.*, 2015). Na Figura 3 encontra-se uma representação de como a hemicelulose e a lignina estão ligadas.

Devido as suas propriedades químicas e complexidade de sua estrutura esse polímero é de grande interesse, especialmente, na produção de polímeros aromáticos, como fenol, vanilina, siringaldeído que são matérias-primas importantes para a indústria de aromas e fragâncias (MOTA et al., 2016). Por isso alguns estudos tem se concentrado em desenvolver estratégias para uma conversão eficiente da lignina em produtos químicos de elevado valor agregado (BILAL *et al.*, 2017).

Figura 3 – Estrutura tridimensional de um fragmento de lignina (estrutura em vermelho e branco) composto de quatro unidades repetidas, sendo cada uma delas ligada a uma cadeia de xilano (em amarelo)



Fonte: (FORSS; FREMER, 2006)

2.1.4 Extrativos

Os extrativos presentes na biomassa são um grupo heterogêneo de componentes que podem ser extraídos através de solventes polares e apolares. Eles englobam terpenos, gorduras, graxas e compostos fenólicos e seu conteúdo e composição variam de acordo com a espécie. Estão presentes em pequenas quantidades

e são responsáveis pela cor, odor e proteção da biomassa contra parasitas (JÖNSSON; MARTÍN, 2016).

2.2 Aproveitamento dos materiais lignocelulósicos

Atualmente, cerca de 220 bilhões de toneladas de biomassa são geradas como resíduo de operações agrícolas em todo o mundo (SARATALE *et al.*, 2017). Os elevados volumes de resíduos e subprodutos inerentes a todos os setores produtivos aliados ao aumento da consciência ecológica tem motivado a criação de projetos que promovam a sustentabilidade dos sistemas de produção (SANTOS DOS *et al.*, 2011). O que tem motivado o desenvolvimento de muitos estudos no âmbito do desenvolvimento de estratégias para tornar viável o uso integral da biomassa na produção de produtos com elevado valor agregado (VAZ DE ARRUDA et al., 2017), tais como, produtos químicos, celulose e papel, rações animais, biocatalisadores e biocombustíveis (BILAL *et al.*, 2017).

Os resíduos lignocelulósicos têm sido considerados uma fonte sustentável de energia não apenas para a produção de eletricidade (principal emprego atualmente), mas especialmente para aplicação em processos de biorrefinaria na produção de biogás e biocombustíveis de segunda geração (NYGAARD et al., 2016). Isso porque esses materiais são ricos em açúcares e/ou proteínas (SALIM *et al.*, 2017). Estima-se que até 2050, aproximadamente 38% do combustível do mundo e 17% da eletricidade serão fornecidos por esses materiais (FENG; LIN, 2017).

Entretanto, a primeira limitação encontrada na biorrefinaria desses materiais, se refere à dificuldade de transformar seus polissacarídeos estruturais em açúcares simples (MISHRA *et al.*, 2017). Por isso, no processo de transformação desses materiais, a primeira etapa consiste num pré-tratamento para a abertura da estrutura do material e separação das frações: lignina, hemicelulose e celulose (LIM et al. (2012); SARKAR et al. (2012)).

2.3 Pré-tratamento dos materiais lignocelulósicos

O pré-tratamento da biomassa lignocelulósica tem como principais objetivos a abertura da cadeia do material e a remoção parcial ou completa da hemicelulose e da lignina (XIN et al., 2015). Um pré-tratamento adequado deve ser capaz de desfazer as

pontes de hidrogênio na estrutura cristalina da celulose, quebrar as ligações da matriz hemicelulose-lignina e aumentar a porosidade e área superficial da celulose (MOOD *et al.*, 2013).

No contexto da produção de enzimas e biocombustíveis, por exemplo, diversos métodos de pré-tratamento de resíduos lignocelulósicos têm sido estudados (CHEN; TU; SHEEN, 2011), tais como, métodos físicos (mecânico e extrusão), químicos (com ácidos, álcalis, ozônio, solventes orgânicos ou líquidos iônicos) e físico-químicos (explosão a vapor, AFEX-ammonia fiber explosion, água quente (LHW), microondas e ultrasson) (ZHUANG *et al.*, 2016). Nas plantas de demonstração, atualmente em operação, os pré-tratamentos mais frequentemente empregados têm sido aqueles a base de ácido diluído, álcali, por imersão em amônia líquida e por explosão a vapor (ZHUANG *et al.*, 2016).

É importante considerar que independentemente do método de pré-tratamento utilizado, durante o processo, alguns inibidores podem ser formados, os quais podem apresentar efeito negativo sobre enzimas e micro-organismos que serão utilizados etapas subsequentes. Os três principais grupos de inibidores são: ácidos fracos como o levulínico, acético e fórmico; derivados de furanos como furfural e hidroximetil-furfural (HMF) e compostos fenólicos (MOOD *et al.*, 2013).

Sabe-se que a eficiência do pré-tratamento de um resíduo lignocelulósico está diretamente relacionada com a composição do material, por exemplo, seu conteúdo de lignina e grupos acetil, ou ainda, fatores como área superficial específica, índice de cristalinidade da celulose (CrI), grau de polimerização (Dp), revestimento da fração celulósica pela hemicelulose, dentre outros (MOOD et al., 2013). A seguir encontram-se descritos os pré-tratamentos que foram aplicados aos materiais lignocelulósicos utilizados nesse trabalho.

2.3.1 Ácido diluído

O pré-tratamento da biomassa lignocelulósica com ácido diluído é considerado um dos mais promissores. Nele o material é submetido a uma combinação de pH ácido calor e pressão. O processo promove a despolimerização da hemicelulose (CARVALHO *et al.*, 2015) com liberação de seus açúcares constituintes, tais como, xilose, glucose, manose, galactose e arabinose (CHEN; TU; SHEEN, 2011).

O processo que emprega ácido sulfúrico é o mais utilizado, podendo ser realizado com alta concentração de ácido e baixa temperatura ou baixa concentração de ácido e alta temperatura. A utilização de ácido concentrado apresenta como desvantagens: elevada toxicidade, corrosividade de equipamentos, dificuldade na recuperação do ácido após o processo e produção de inibidores a partir da degradação dos monossacarídeos liberados. Industrialmente, o pré-tratamento que emprega ácido diluído é o mais utilizado, pois gera menor quantidade de inibidores (HAGHIGHI MOOD *et al*., 2013).

A hemicelulose apresenta uma estrutura predominantemente amorfa (região com baixa organização macromolecular) o que a torna mais susceptível à hidrólise ácida que a celulose (NEVES; PITARELO; RAMOS, 2016). Devido à elevada sensibilidade da hemicelulose as condições de tratamento, parâmetros como temperatura e tempo precisam ser bem controlados a fim de minimizar a formação de furfural, HMF e pseudo-lignina que são substâncias com efeito inibidor nos processos subsequentes de emprego da biomassa. Quando o tratamento é realizado em altas temperaturas, os inibidores, como o furfural, podem se degradar formando o ácido fórmico e levulínico (MOOD *et al*., 2013).

Vários estudos reportam que o conteúdo de lignina (lignina de Klason) presente no material após o tratamento ácido é maior que aquele presente na matéria-prima, esse fenômeno é explicado, em parte, devido à formação de pseudo-lignina. Grahi et al. (2011) acompanharam a formação dessa estrutura em diferentes condições de tratamento do Álamo *(Populus trichocarpa x deltoides)* e concluíram que a pseudo-lignina é um material aromático que pode ser quantificada como lignina de Klason, e que inclusive assemelha-se a aparência física da lignina nativa, não sendo, contudo derivada desta, podendo ser formada exclusivamente pela degradação dos carboidratos presentes na biomassa. Portanto, em condições de tratamento severas, a degradação dos carboidratos além de gerar os inibidores anteriormente mencionados, produz também pseudo-lignina que assim como a própria lignina pode formar ligações não produtivas com enzimas utilizadas na hidrólise da celulose, por exemplo. Por isso, condições severas de pré-tratamento ácido devem sempre ser evitadas (CARVALHO *et al.*, 2015).

Em madeiras duras, o xilano é o principal componente da hemicelulose e juntamente com os grupos acetil nela presentes aumenta a recalcitrância desses materiais. Os grupos acetil e urônicos removidos durante esse pré-tratamento, catalisam a hidrólise da hemicelulose e dos oligossacarídeos devido à liberação de íons hidrônio

para o meio reacional. A fração amorfa da celulose também pode ser hidrolisada nesse processo, liberando glicose. A fração cristalina da celulose, entretanto, permanece no material sólido recuperado após o tratamento, pois, para hidrolisá-la seriam necessárias condições de tratamento mais severas (combinações de altas concentrações de ácido e temperatura elevada) (CARVALHO *et al.*, 2015).

2.3.2 Alcalino (deslignificação)

A lignina presente nos materiais lignocelulósicos atua como uma barreira física dificultando o acesso das enzimas e/ou microorganismos a celulose e a hemicelulose (ALVIRA *et al.*, 2010). Além disso, β-glucosidases e endoglucanases, por exemplo, podem ter mais afinidade pela lignina do que pela própria celulose, formando ligações não-produtivas com esse polímero, o que reduz a eficiência do processo de hidrólise enzimática. Por isso, convém que esses materiais sejam primeiramente submetidos a um processo de tratamento para remover parcialmente ou totalmente a lignina (NARRA; JAMES; BALASUBRAMANIAN, 2015).

O tratamento que tem como finalidade remover a lignina dos materiais lignocelulósicos é denominado de deslignificação. Como resultado desse processo, a acessibilidade e a digestibilidade dos polissacarídeos presentes nesses materiais aumenta, uma vez que os carboidratos ficarão mais disponíveis para a ação de enzimas e/ou microorganismos. Os açúcares liberados poderão ser empregados em diversos processos biotecnológicos (RABEMANOLONTSOA; SAKA, 2015).

Os álcalis são os reagentes preferencialmente utilizados, e dentre eles os principais são a amônia, o hidróxido de cálcio e o hidróxido de sódio, sendo este o mais estudado. Em comparação com o pré-tratamento ácido, por exemplo, a deslignificação apresenta como vantagem o fato de ocorrer em temperatura e pressão menores e, consequentemente, provocar menor degradação dos açúcares. Em contrapartida, nesse tipo de pré-tratamento podem ser necessários tempos de reação maiores (BALI *et al.*, 2015). Esse tipo de tratamento é muito eficaz para resíduos agrícolas e culturas herbáceas (NARRA; JAMES; BALASUBRAMANIAN, 2015).

O álcali provoca o intumescimento da parede celular e o aumento na sua superfície interna. Ocorre então a saponificação das ligações éster de ácido 4-O-metil-D-glucurônico (ácido urônico) ligados ao xilano. Um grupo carboxílico é produzido e cliva as ligações entre a lignina e a hemicelulose. A matriz celulose-hemicelulose-

lignina é desfeita e ocorre o rompimento das pontes de hidrogênio com a celulose (RABEMANOLONTSOA; SAKA, 2015). Ocorre também a quebra das ligações internas na molécula de lignina (ligações éter, fenil glicosídicas e acetil) (KOYAMA et al., 2017).

A lignina nos materiais lignocelulósicos é formada basicamente por unidades guaiacil e siringil. Ela está ligada principalmente via carbono-carbono e ligações carbono-oxigênio com a ligação aril-éter. A eficiência do processo de deslignificação é diretamente influenciada por fatores como a concentração alcalina, tempo e temperatura do processo e especialmente, pela composição da lignina do material, por exemplo, é sabido que a lignina do tipo siringil é mais facilmente clivada que a do tipo guaiacil (SANTOS *et al.*, 2011). Por isso, a relação entre os grupos Siringil e Guaiacil (S/G) funciona como um indicador da reatividade desse polímero ao tratamento químico empregado. Por exemplo, para eucalipto a relação S/G é maior que para bagaço e palha de cana, o que indica que a reatividade da lignina do eucalipto é maior quando comparada com a dos demais resíduos mencionados (CARVALHO *et al.*, 2015). Portanto, a escolha de um método de pré-tratamento adequado é importante para a eficiência e competitividade do bioprocesso.

2.4 Hidrólise enzimática da celulose

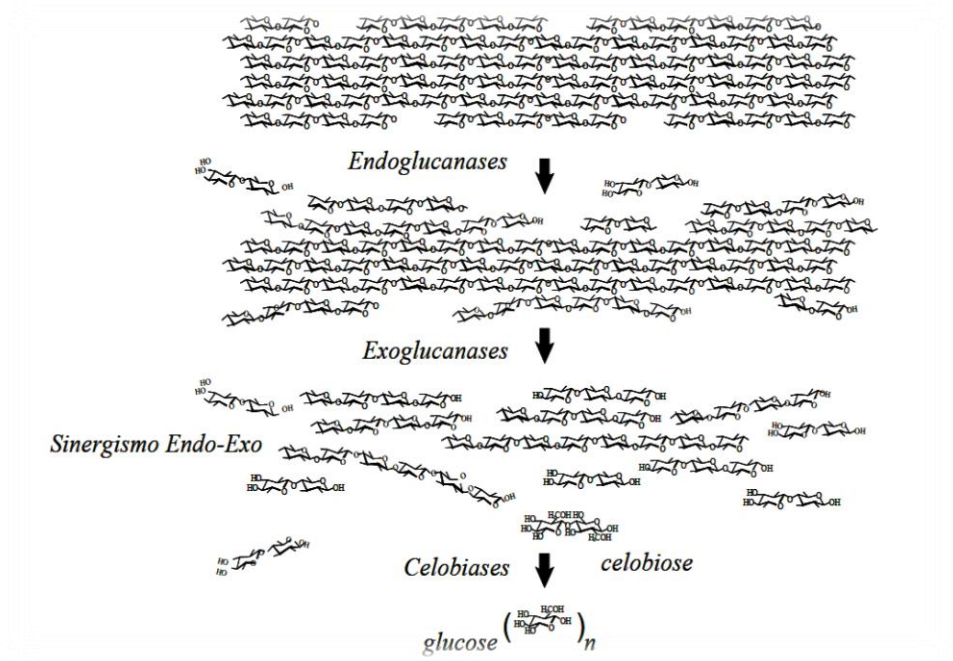
Após a remoção de hemicelulose e lignina do material lignocelulósico, a celulose, presente na fração sólida, precisa então ser hidrolisada (por ácidos ou enzimas) até glicose, que conforme mencionado anterirormente, pode ser empregada em diversos processos. Dentre as várias possibilidades de aplicação para esse açúcar, no cenário atual, aquela que mais tem se destacado, é a produção de bioetanol. Nesse processo, após a etapa de deslignificação, o material sólido é submetido a hidrólise enzimática com celulases, sendo essa etapa um dos principais gargalos nesse biorocesso, especialmente devido ao custo elevado dos biocatalisadores (LI *et al.*, 2017). Uma vez que, o emprego dessas enzimas pode representar até 40% do custo total do processo é necessário que processos de produção menos dispendiosos sejam desenvolvidos (BEHERA; RAY, 2016). A glicose liberada, poderá então ser convertida em bioetanol (LI *et al.*, 2017).

Celulases são um grupo de enzimas (endoglucanases, exoglucanases e β-glucosidases) que atuam sinergisticamente na hidrólise completa da celulose, (IDRIS *et*

al., 2017). Endoglucanases ou carboximetilcelulases são responsáveis por iniciar a hidrólise enzimática da celulose. Elas atum clivando, de forma aleatória, as ligações intramoleculares na fibra, gerando oligossacarídeos de vários tamanhos e consequentemente novas cadeias terminais. Exoglucanases ou celobiohidrolases hidrolisam as extremidades redutoras e não-redutoras da cadeia de celulose liberando celobiose ou glucose (BANSAL *et al.*, 2012). Esta enzima sofre inibição por seu produto de hidrólise (CASTRO; DE; PEREIRA, 2010). Por fim, a β-glucosidase também conhecida como celobiase é responsável pela hidrólise da celobiose e dos oligossacarídeos solúveis em glicose (BANSAL *et al.*, 2012), eliminando desta forma a inibição pela celobiose (PERCIVAL ZHANG; HIMMEL; MIELENZ, 2006).

Essas enzimas agem em sinergia e, portanto, a presença de todas é necessária para uma eficiente hidrólise da celulose em açúcares solúveis, como pode ser observado na Figura 4 (IDRIS *et al.*, 2017). Basicamente, a diferença entre essas enzimas está relacionada ao seu local de atuação na cadeia celulósica (CASTRO, DE E PEREIRA, 2010).

Figura 4 – Representação esquemática do efeito sinérgico das enzimas que compõe o complexo celulolítico



Fonte: MARTINS (2005).

Esse grupo de biocatalisadores é empregado nas indústrias de produção de medicamentos, perfumes, resinas, produção de amido, fermento, tratamento de resíduos e produção de etanol de segunda geração a partir de materiais lignocelulósicos (BEHERA; RAY, 2016). Atualmente, a nível comercial, elas são produzidas em sua maior parte através de fermentação submersa empregando-se substratos puros (CERDA *et al.*, 2017). Os principais micro-organismos produtores de celulases são os fungos filamentosos, com destaque para *Trichoderma reesei* (ZHENG *et al.*, 2017). Os processos industriais de produção de enzimas são de alto custo, o que ainda constitui um fator limitante ao amplo emprego desses biocatalisadores em processos industriais, por isso, essa produção precisa ser otimizada com o objetivo de reduzir tais custos (CERDA *et al.*, 2017).

2.5 Referências

ALVIRA, P.; TOMÁS-PEJÓ, E.; BALLESTEROS, M.; NEGRO, M. J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. **Bioresource Technology**, v. 101, n. 13, p. 4851–4861, 2010.

BALI, G.; MENG, X.; DENEFF, J. I.; SUN, Q.; RAGAUSKAS, A. J. The effect of alkaline pretreatment methods on cellulose structure and accessibility. **ChemSusChem**, v. 8, n. 2, p. 275–279, 2015.

BANSAL, N.; TEWARI, R.; SONI, R.; SONI, S. K. Production of cellulases from Aspergillus niger NS-2 in solid state fermentation on agricultural and kitchen waste residues. **Waste Management**, v. 32, n. 7, p. 1341–1346, 2012.

BEHERA, S. S.; RAY, R. C. Solid State Fermentation for Production of Microbial Cellulases: Recent Advances and Improvement Strategies. **International Journal of Biological Macromolecules**, v. 86, p. 656–669, 2016.

BILAL, M.; ASGHER, M.; IQBAL, H. M. N.; HU, H,; ZHANG, X. Biotransformation of lignocellulosic materials into value-added products − A review. **International Journal of Biological Macromolecules**, v. 98, p. 447–458, 2017.

CERDA, A.; GEA, T.; VERGAS-GARCIA, M. C.; SANCHEZ, A. Towards a competitive solid state fermentation: Cellulases production from coffee husk by sequential batch operation and role of microbial diversity. **Science of The Total Environment**, v. 589, p. 56–65, 2017.

CHEN, W. H.; TU, Y. J.; SHEEN, H. K. Disruption of sugarcane bagasse lignocellulosic structure by means of dilute sulfuric acid pretreatment with microwave-assisted heating. **Applied Energy**, v. 88, n. 8, p. 2726–2734, 2011.

CHEN, Z.; WAN, C. Biological valorization strategies for converting lignin into fuels and chemicals. **Renewable and Sustainable Energy Reviews**, v. 73, n. March 2016, p.

610–621, 2017.

CARVALHO, D. M.; SEVASTYANOVA, O.; PENNA, L. S.; DA SILVA, B. P.; LINDSTROM, M. E.; COLODETTE, J. L. Assessment of chemical transformations in eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid, and alkaline pretreatments. **Industrial Crops and Products**, v. 73, p. 118–126, 2015.

CASTRO, A. M.; PEREIRA, N. Produção, propriedades e aplicação de celulases na hidrólise de resíduos agroindustriais. **Quimica Nova**, v. 33, n. 1, p. 181–188, 2010.

FENG, Q.; LIN, Y. Integrated processes of anaerobic digestion and pyrolysis for higher bioenergy recovery from lignocellulosic biomass: A brief review. **Renewable and Sustainable Energy Reviews**, n. November 2016, p. 1–16, 2017.

FORSS, K. G.; FREMER, K. E. Spruce and birch wood lignins – a comparasion. **Cellulase Chemistry and Technology**, v.40, p. 739-748, 2006.

IDRIS, A. S. O.; PANDEY, A.; RAO, S. S.; SUKUMARAN, R. K. Cellulase production through solid-state tray fermentation, and its use for bioethanol from sorghum stover. **Bioresource Technology**, 2017.

JÖNSSON, L. J.; MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource Technology**, v. 199, p. 103–112, 2016.

KOYAMA, M.; WATANABE, K.; KUROSAWA, N.; ISHIKAWA, K.; BAN, S.; TODA, T. Effect of alkaline pretreatment on mesophilic and thermophilic anaerobic digestion of a submerged macrophyte: inhibition and recovery against dissolved lignin during semi-continuous operation. **Bioresource Technology**, v. 238, p. 666–674, 2017.

LE FLOCH, A.; JOURDES, M.; TEISSEDRE, P. L. Polysaccharides and lignin from oak wood used in cooperage: Composition, interest, assays: A review. **Carbohydrate Research**, v. 417, p. 94–102, 2015.

LI, Y.; ZHANG, X.; XIONG, L.; MEHMOOD, M. A.; ZHAO, X.; BAI, F. On-site cellulase production and efficient saccharification of corn stover em- ploying cbh2 overexpressing Trichoderma reesei with novel induction system. **Bioresource Technology**, v. 238, p. 643–649, 2017.

LIM, J. S.; MANAN, Z. A.; ALWI, S. R. W.; HASHIN, H. A review on utilisation of biomass from rice industry as a source of renewable energy. **Renewable and Sustainable Energy Reviews**, v. 16, n. 5, p. 3084–3094, 2012.

MARTINS, G. M.; BOCCHINI-MARTINS, D. A.; BEZZERRA-BUSSOLI, C.; PAGNOCCA, F. C.; BOSCOLO, M.; MONTEIRO, D. A.; SILVA, R.; GOMES, E. The isolation of pentose-assimilating yeasts and their xylose fermentation potential. **Brazilian Journal of Microbiology**, p. 1–7, 2017.

MISHRA, V.; JANA, A. K.; JANA, M. J.; GUPTA, A. Bioresource Technology Enhancement in multiple lignolytic enzymes production for optimized lignin

degradation and selectivity in fungal pretreatment of sweet sorghum bagasse. **Bioresource Technology**, v. 236, p. 49–59, 2017.

MOOD, H. S; GOLFESHAN, A. H.; TABATATABAEI, M.; JOUZANI, G. S.; NAJAFI, G. H.; GHOLAMI, M.; ARDJMAND, M. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. **Renewable and Sustainable Energy Reviews**, v. 27, p. 77–93, 2013.

NARRA, M.; JAMES, J. P.; BALASUBRAMANIAN, V. Simultaneous saccharification and fermentation of delignified lignocellulosic biomass at high solid loadings by a newly isolated thermotolerant Kluyveromyces sp. for ethanol production. **Bioresource Technology**, v. 179, p. 331–338, 2015.

NEVES, P. V.; PITARELO, A. P.; RAMOS, L. P. Production of cellulosic ethanol from sugarcane bagasse by steam explosion: Effect of extractives content, acid catalysis and different fermentation technologies. **Bioresource Technology**, v. 208, p. 184–194, 2016.

NYGAARD, I.; DEMBELE, F.; DAOU, I.; MARIKO, A.; KAMISSOKO, F.; COULIBALY, N.; BORGSTROM, R.; BRUUN, T. B. Lignocellulosic residues for production of electricity, biogas or second generation biofuel: A case study of technical and sustainable potential of rice straw in Mali. **Renewable and Sustainable Energy Reviews**, v. 61, p. 202–212, 2016.

PATERAKI, C.; ALMQVIST, H.; LADAKIS, D.; LIDEN, G.; KOUTINAS, A. A.; VLYSIDIS, A. Modelling succinic acid fermentation using a xylose based substrate. **Biochemical Engineering Journal**, v. 114, p. 26–41, 2016.

PERCIVAL ZHANG, Y. H.; HIMMEL, M. E.; MIELENZ, J. R. Outlook for cellulase improvement: Screening and selection strategies. **Biotechnology Advances**, v. 24, n. 5, p. 452–481, 2006.

RABEMANOLONTSOA, H.; SAKA, S. Various pretreatments of lignocellulosics. **Bioresource Technology**, v. 199, p. 83–91, 2015.

SALIM, A. A.; GRBAVC IC, S.; SEKULJICA, N.; STEFANOVIC, A.; TANASKOVIC, S. J.; LUKOVIC, N.; KNEZ EVIC-JUGOVIC, Z. Production of enzymes by a newly isolated Bacillus sp . TMF-1 in solid state fermentation on agricultural by-products : The evaluation of substrate pretreatment methods **Bioresource Technology**, v. 228, p. 193–200, 2017.

SANTOS, R. B.; CAPANEMA, E. A.; BALAKSHIN, M. Y.; CHANG, H-M.; JANEEL, H. Effect of hardwoods characteristics on kraft pulping process: Emphasis on lignin structure. **BioResources**, v. 6, n. 4, p. 3623–3637, 2011.

SARATALE, G. D.; SARATELE, R. G.; GHODAKE, G. S.; JIANG, Y-Y.; CHANG,J-S.; SHIN, H-S.; KUMAR,G. Characterization and its application in the saccharification of rice waste biomass for ethanol production : An integrated biotechnological approach. **Journal of the Taiwan Institute of Chemical Engineers**, v. 0, p. 1–8, 2017.

SARKAR, N.; GHOSH, S. K.; BANNERJEE, S.; AIKAT, K. Bioethanol production from agricultural wastes: An overview. **Renewable Energy**, v. 37, n. 1, p. 19–27, 2012.

VAZ DE ARRUDA, P.; SANTOS, J. C.; RODRIGUES, R. C. L. B.; SILVA, D. D. V.; YAMAKAWA, C. K.; ROCHA, G. J. M.; JUNIOR, J. N.; PRADELLA, J. G. C.; ROSSELL, C. E. V.; FELIPE, M. G. A. Scale up of xylitol production from sugarcane bagasse hemicellulosic hydrolysate by Candida guilliermondii FTI 20037. **Journal of Industrial and Engineering Chemistry**, v. 47, p. 297–302, 2017.

VECINO, X.; RODRIGUEZ-LOPEZ, L.; GUDINA, E. J.; CRUZ, J. M.; MOLDES, A. B.; RODRIGUES, L. R. Vineyard pruning waste as an alternative carbon source to produce novel biosurfactants by Lactobacillus paracasei. **Journal of Industrial and Engineering Chemistry**, v. 55, p. 40–49, 2017.

XIN, D.; SUN, Z.; VIIKARI, L.; ZHANG, J. Role of hemicellulases in production of fermentable sugars from corn stover. **Industrial Crops and Products**, v. 74, p. 209–217, 2015.

ZHENG, W.; ZHENG, Q.; XUE, Y.; HU, J.; GAO, M-T. Influence of rice straw polyphenols on cellulase production by Trichoderma reesei. **Journal of Bioscience and Bioengineering**, v. 123, n. 6, p. 731–738, 2017.

ZHUANG, X.; WANG, W.; YU, Q.; QI, W.; WANG, Q.; TAN, X.; ZHOU, G.; YUAN, Z. Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products. **Bioresource Technology**, v. 199, p. 68–75, 2016.

3 VINE PRUNING COMBINED PRETREATMENTS AND ENZYME HYDROLYSIS

T. C. MACIEL[1], E. GUDIÑA[2], E. C. MIGUEL[3], L. R. RODRIGUES[2], S. RODRIGUES[4]

[1] Federal University of Ceará, Centre of Technology, Department of Chemical Engineering
[2] CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[3] Federal University of Ceará, Physical Department/Central analytical
[4] Federal University of Ceará, Centre of Agricultural Sciences, Department of Technology

3.1 Abstract

The lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin, forming a structure difficult for microbiological and enzymatic degradation. Due to the complexity of the lignocellulosic materials, this biomass needs a pretreatment to remove the lignin and the hemicellulose, exposing the cellulose to the enzyme action. For this reason, this work aimed to study the vine pruning pretreatment (acid and alkali pretreatment) followed by the enzymatic hydrolysis with commercial cellulases. The influence of acid pretreatment of a residue was evaluated through a central composite design and the material recovered from the best conditions was further used in an alkaline hydrolysis. The commercial enzymes Cellic® CTec2 and Novozyme 188 were used to hydrolyze the pretreated biomass. The vine pruning composition was: cellulose 36%, hemicellulose 22% and lignin 30%. Regarding the acid pretreatment, the best condition was: 1.5% (v/w) H_2SO_4, 120 ºC and 30 minutes, which removed 68.7% of hemicellulose recovering 95.8% cellulose. This material was then submitted to an alkaline pretreatment using different conditions, and the greatest removal of lignin occurred using 3.0% (w/v) of NaOH at 120 ºC without agitation for 60 minutes. Under this condition, it was possible to obtain a material with 75.0% of cellulose and 25.0% of lignin. However, the highest glucose yield was obtained with the enzymatic hydrolysis of the vine pruning delignified at 100 ºC, without agitation and 2.0% of NaOH, which confirms the importance of taking into account the enzymatic hydrolysis results to select the pretreatment for a lignocellulosic residue.

Keywords: Cellulase; Enzymatic hydrolysis; chestnut shell; vine pruning.

3.2 Introduction

Currently, the interest in lignocelulosic biomass has been growing because of its potential to be used as raw material in the biofuels and biomaterials production (YOO et al., 2017). According to the biorefinery concept, the lignocellulosic biomass can be used as a raw material in different industrial processes. Thus, the use of all fractions of these materials (sugars from hemicellulose, cellulose, and lignin) in the production of value-added products would make the transformation of biomass viable and of economical interest (VAZ DE ARRUDA et al., 2017).

The lignocellulosic materials are composed basically of an aromatic polymer (lignin) and carbohydrates polymers such as cellulose and hemicellulose, whose structural units are fermentable sugars (YANG et al., 2015). Due to its composition, lignocellulosic materials are highly resistant to degradation (SU et al., 2012). Therefore, the first step of the biomass transformation process is a pretreatment of the residue that results in the chain opening and the lignin and hemicellulose release (LIM et al., 2012). The pretreatment also reduces the cellulose crystallinity increasing the amorphous cellulose fraction and making the cellulose available for enzymatic attack (SARKAR et al., 2012). Because of the complexity of the lignocellulosic materials, there is not a single pretreatment that is equally effective for all of them (NARRA; JAMES; BALASUBRAMANIAN, 2015). Whenever selecting the pretreatment methods, it is important to consider some factors, such as digestibility of the treated material, sugars recovery, the presence of inhibitors and energy consumption (SARATALE; JUNG; OH, 2016). The pretreatment step is crucial for the bioprocess development, as it can comprise 20% of the total biofuels production coast (BANERJEE et al., 2016). The acid hydrolysis removes the hemicellulose and can solubilize a small fraction of lignin. Nonetheless, the majority of this polymer remains in the solid fraction; therefore, another treatment step is necessary to remove the remaining lignin (NARRA; JAMES; BALASUBRAMANIAN, 2015). Afterwards, the resultant material is subjected to enzymatic hydrolysis to release the monomeric sugars, which can be to use in fermentation processes (YANG et al., 2015).

Some European countries, in particular, Portugal, have a long tradition of wine production (BRITO; OLIVEIRA; RODRIGUES, 2014). In 2015, France, Italy, Spain, Germany, and Portugal were responsible for 70% of the World wine production (26 billions of liters). Among these countries, Portugal is the fifth largest producer with

a production of 705 millions of liters in 2015 (IVV, 2017). Annually, the vineyard trees need to be pruned to increase the productivity and the grape quality in the next harvest (MILLER et al., 2001). In the pruning process, the cut thin branches are often burned (VECINO et al., 2017), causing environmental problems related to the lignin combustion (BUSTOS et al., 2004). Therefore, the development of technologies that can add value to this residue is industrially relevant (BIAGINI; BARONTINI; TOGNOTTI, 2015). Some studies regarding the application of vine pruning has already been done such as: the production of wood-based panels (NTALOS; GRIGORIOU, 2002), the vine pruning gasification (BIAGINI; BARONTINI; TOGNOTTI, 2015), lactic acid production by fermentation of the hemicellulose sugars (BUSTOS et al. (2005), MOLDES et al. (2007)), biosurfactants production by fermentation of the cellulose sugars (VECINO et al., 2017). In this way, this work aimed to study two steps of pretreatment to remove hemicellulose and lignin from the vine pruning. The resultant cellulosic fraction in the solid material was submitted to enzymatic hydrolysis, and the concentration of glucose released in this process was also taken into account in the selection of the better pretreatment conditions.

3.3 Materials and methods

3.3.1 Vine pruning raw material

The vine pruning used in this work was kindly provided by a local farm in the Minho's province in Portugal. The residue was ground in a nife mill and dried in an oven at 60 ºC for 24 hours.

3.3.2 Acid pretreatment of the vine pruning residue

A pretreatment with diluted sulphuric acid (H_2SO_4) was applied to the vine pruning residue according to a central composite design (CCD) that enabled the definition of the adequate experiments covering the relevant experimental domain. The studied variables were temperature (100 ºC to 120 ºC), time (15 to 45 minutes) and acid concentration (0.5% to 2.5% (v/v)). The biomass was impregnated with the alkali liquor at a solid/liquor ratio of 1:20 (w/v). These values were selected based on the acid

pretreatment of olive tree pruning (MATEO et al., 2014), almond tree pruning (CUEVAS; GARCÍA; SÁNCHEZ, 2014) and vine pruning (BUSTOS et al., 2004).

After each treatment, the solid and the liquid fractions were separated by vacuum filtration, in a Buchner funnel with a soft cloth, and the solid fraction was washed until neutral pH, weighted and represented as total mass recovery (TMR), calculated according to Eq.(1). The calculations were performed considering the humidity on dry basis. The wet material was stored at 10 ºC until it was used in the assays of enzymatic hydrolysis.

$$\text{TMR } (\%) = \frac{\text{mass of the solid (insoluble) fraction (g)}}{\text{initial mass (g)}} \times 100 \quad (1)$$

where the "mass of the solid (insoluble) fraction" corresponds to the mass recovered after the filtration and successive washings, and the "initial mass" are the mass of the residue before treatment.

The percentages of cellulose, hemicellulose and lignin which were solubilized with the treatment were calculated on the basis in the fraction present in the treated biomass and in the untreated biomass and TMR (%), according to the Equations (2), (3) and (4).

$$CS\ (\%) = 100 - \frac{\text{TMR\%} * \text{CTB\%}}{\text{CUB\%}} \quad (2)$$

CS is the percentage of cellulose which was solubilized with treatment, CTB is the percentage of cellulose in the treated biomass and CUB is the percentage of cellulose in the untreated biomass.

$$HS\ (\%) = 100 - \frac{\text{TMR\%} * \text{HTB\%}}{\text{HUB\%}} \quad (3)$$

HS is the percentage of hemicellulose which was solubilized with treatment, HTB is the percentage of hemicellulose in the treated biomass and HUB is the percentage of hemicellulose in the untreated biomass.

$$LS\ (\%) = 100 - \frac{\text{TMR\%} * \text{LTB\%}}{\text{LUB\%}} \quad (4)$$

LS is the percentage of cellulose which was solubilized with treatment, LTB is the percentage of lignin in the treated biomass and LUB is the percentage of lignin in the untreated biomass.

The best conditions from the experimental design, the condition that removed the higher percentage of hemicellulose with minimum solubilization of cellulose, were selected for the alkaline pretreatment).

3.3.3 Alkaline pretreatment of the residue

A pretreatment with sodium hydroxide was carried out on the residue obtained from the best acid pretreatment (determined in section 2.2) to promote the residue delignification. Three NaOH concentrations (1.0%, 2.0% and 3.0% w/v) were used at 120 ºC without agitation (autoclave) and 100 ºC with agitation in a stirred-heating plate (MS7-H550-S LAB 1000). All the experiments were conducted with a substrate to alkali solution ratio (w/v) of 1:20 during 60 minutes. After each treatment, the solid and liquid fractions were separated by filtration in a fabric cloth under vacuum and the solid fraction was washed until pH 7.0 and stored at 4 ºC. The chemical composition of the treated material (percentage of cellulose, hemicellulose, and lignin) was determined as described in section 2.5 and the TMR (%) was calculated as described in section 2.2. All samples obtained in this step were submitted to enzymatic hydrolysis for the selection of the most appropriate condition.

3.3.4 Enzymatic hydrolysis

The enzymatic hydrolysis was performed with the commercial enzyme Cellic® CTec2 (30 FPU/ g of biomass) and Novozyme 188 (15 CBU/ g of biomass). The assays were performed in Erlenmeyers of 100 mL with a reaction volume of 40 mL of a mixture containing sodium citrate (pH 5.0), sodium azide (0.1%, w/v), the enzyme and 5% (w/v) of biomass. The assays were performed in triplicate and carried out in a rotatory shaker (SOLAB) at 200 rpm, 50 °C for 96 hours (FERREIRA et al. (2009), MAEDA et al. (2013)). The samples (1.0 mL of each Erlenmeyer) were collected every 24 hours and were kept in boiling water for 5 min to inactive the enzyme. Then, the samples were centrifuged at 12000 g for 20 minutes to separate the insoluble fractions. The soluble fraction was filtered through a 0.2 μm membrane filter and stored frozen to

further determine the hydrolysis products. The untreated vine pruning was also submitted to the hydrolysis. The control experiments were carried out without adding the enzyme. The hydrolysis was performed in triplicate. The enzymatic hydrolysis yield (%) was calculated according to Eq. (2) (DOWE; NREL, 2008):

$$Enzymatic\ hydrolysis\ Yield\ (\%) = \left(\frac{[glucose]+1.05\,[cellobiose]}{1.11\,f\,[biomass]}\right) * 100 \qquad (2)$$

where [glucose] is the glucose concentration (g/L), [cellobiose] is the cellobiose concentration (g/L), [biomass] is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L), f is the cellulose fraction in dry biomass (g/g), 1.05 is the factor that converts cellobiose to equivalent glucose and 1.11 is the factor that converts cellulose to equivalent glucose.

3.3.5 Analytical methods

The untreated and treated vine pruning residue chemical composition of was determined according to the analytical protocol described by the National Renewable Energy Laboratory (NREL).

The sugars and lignin were determined through biomass fractionation through of the acid hydrolysis. Firstly, the biomass was submitted to acid hydrolysis with sulfuric acid at 72% (v/v) and 30 ºC during 1 hour. Hereafter, the mixture was diluted with water up to 4.0% (v/v) and autoclaved at 120 ºC for 60 minutes. Afterwards, the solid fraction containing the acid insoluble lignin (Klason lignin) was separated from the liquid fraction containing sugars and the acid soluble lignin through filtration in Buchner funnel with sintered glass porous plate nº 2. The Klason lignin was determined gravimetrically and the acid soluble lignin was determined using an UV-Visible spectrophotometer at 215 nm and 280 nm (SLUITER et al., 2011). The concentrations of cellobiose, glucose, xylose, arabinose, acetic acid, furfural and hydroxymethylfurfural (HMF) were measured by high-performance liquid chromatography (HPLC) in an Agilent 1260 infinity system, equipped with a refractive index and UV-visible detector (Wilmington, Delaware, EUA). Separation was achieved using an Aminex HPX-87H (Bio-Rad Laboratories Inc., America) column. The mobile phase was 5 mmol/L sulfuric acid, the flow rate was 0.7 mL/min and the column

temperature 60 º. Acid acetic, furfural and HMF were UV detected at 285 nm, and the sugars were detected by refraction index.

The extractives content present in the biomass were extracted by the Sohxlet method (Soxtec™ 8000), in a two-step extraction: the first with distilled water and the second with ethanol 95% (v/v). Both extractions were carried out for 6 hours at 80 ºC without agitation. The extractives mass was quantified by gravimetry (SLUITER et al., 2008a).

The ash content was determined through dry oxidation of the biomass in a muffle at 575 ºC during 4 hours. The ash was quantified by gravimetry (SLUITER et al., 2008b).

The concentration of cellobiose, glucose, xylose arabinose released from the enzymatic hydrolysis was measured by HPLC (Agilent 1260 infinity, Wilmington, Delaware, EUA) using the same conditions as described in this section.

3.3.6 Structural changes in the biomass

To visualize the structural changes on the wine pruning surface due to the treatments and the enzymatic hydrolysis, a morphological study by Scanning Electron Microscope (SEM) (Quanta 450 FEG – FEI) was performed. The samples were freeze-drying to remove humidity and fixed in a stub. Before analysis, the samples were coated with a thin layer of gold in a metalizator (Quorum QT150ES). Afterwards, the samples and raw material surface morphologies were analyzed.

3.3.7 Statistics analysis

All experiments were carried out in triplicate. Results were expressed as mean ± SD. Statistical analysis was carried out using the software Statistica 10.0 (Statsoft). F-test and ANOVA analysis were used as significant criteria for the fitted models. The tukey's test was also done using the software Statistica 10.0 (Statsoft).

3.4 Results and discussion

3.4.1 Vine pruning raw material

The composition of the vine pruning used herein was: 36.0% of cellulose, 29.6% of lignin, 21.8% of hemicellulose, which has xylose as structural monomer, 8.6% of extractives and 3.6% of ash. These values were similar to the reported by BUSTOS et al. (2005) for vine pruning from Galicia (Spain). The cellulose is the major component in this residue, making it interesting for fermentation processing such as bioethanol production because the cellulose hydrolysis product is glucose (a fermentable sugar)

3.4.2 Acid pretreatment of the vine pruning residue

The evaluation of the pretreatment with dilute sulfuric acid was performed through a central composite design (CCD) with three independent variables and three replicates at the central point. After the pretreatment, two fractions were obtained, a liquid fraction which contains all the soluble components in the acid and a solid fraction (Total mass recovery - TMR) containing the cellulose, lignin and some hemicellulose not solubilized during the treatment. The TMR after the treatments ranged from 55.4 to 80.8% (Table 1). According to the results presented, high acid concentrations led to smallest TMR values because in these assays, a greater amount of material was solubilized, independently of the temperature and processing time. In the pretreatments 1 and 3 (both performed at 100º C and 15 min), the TMR decreased from 80.8% to 68.10% with the increase in the acid concentration from 0.5% to 2.5%, respectively. The same behavior was observed in the assays 11 (0.5% H_2SO_4), 15 (1.5% H_2SO_4) and 12 (2.5% H_2SO_4) that were done at 110 ºC for 30 minutes, in TMR of 65.4%, 63.3%, and 57.8%, respectively.

The percentages of each fraction in the treated material changed according to the pretreatment conditions. In lignocellulosic material, it is well known that the diluted acid acts particularly on the hemicellulose, due to its capacity of breaking covalent bonds, hydrogen bonds and Van der Waals forces in the molecule (YANG et al., 2015). Therefore, the content of hemicellulose solubilized with the acid pretreatment was the response variable analyzed in the CCD experimental design. The linear effect of the acid concentration on the solubilization of the hemicellulose was positive and

exhibited the greatest influence in this response (Figure 5a). The positive sign of this variable on the solubilized hemicellulose percentage means that when the acid concentration increased the hemicellulose solubilization also increased. Therefore, this type of treatment tends to increase the accessibility of the enzymes to cellulose as well as to increase the pore size of the material (CARVALHO et al., 2015).

Table 1. Total mass recovery (TMR) and composition obtained for several acid pretreatment conditions according to the CCD experimental design.

Assay	t(°C)	H_2SO_4 (% v/v)	Time (min)	TMR (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
1	100	0.5	15	80.8	44.6 ± 0.3	25.4 ± 0.2	30.5 ± 0.1
2	100	0.5	45	70.6	42.7 ± 0.4	25.6 ± 0.3	30.1 ± 0.0
3	100	2.5	15	68.1	48.0 ± 0.3	17.5 ± 1.0	36.5 ±0.4
4	100	2.5	45	61.3	46.0 ± 0.8	14.0 ± 0.7	37.9 ± 0.7
5	120	0.5	15	76.0	40.0 ± 0.6	18.3 ± 0.3	36.4 ± 0.9
6	120	0.5	45	71.3	42.2 ± 0.6	18.2 ± 0.6	35.4 ± 0.6
7	120	2.5	15	57.6	51.1 ± 0.5	10.4 ± 0.2	39.3 ± 1.4
8	120	2.5	45	55.4	49.8 ± 0.7	9.8 ± 0.6	43.1 ± 0.6
9	100	1.5	30	69.0	52.0 ± 0.5	21.4 ± 0.5	32.8 ± 0.6
10	120	1.5	30	62.9	54.8 ± 1.0	10.9 ± 0.4	35.8 ± 0.6
11	110	0.5	30	65.4	45.3 ± 0.2	26.8 ± 0.9	30.7 ± 0.2
12	110	2.5	30	57.8	45.6 ± 0.2	11.2 ± 0.6	40.8 ± 0.6
13	110	1.5	15	73.2	46.5 ± 0.1	24.6 ± 0.2	30.6 ± 0.4
14	110	1.5	45	66.4	52.0 ± 0.2	11.1 ± 0.1	36.7 ± 0.3
15*	110	1.5	30	62.5	46.6 ± 0.5	16.0 ± 0.4	37.4 ± 0.0
16*	110	1.5	30	65.1	45.0 ± 0.2	16.4 ± 0.2	37.1 ± 1.0
17*	110	1.5	30	62.2	44.8 ± 0.3	16.2 ± 0.0	39.1 ± 0.9

*Central points

The effect of temperature in hemicellulose solubilization was also positive since the increase in the pretreatment temperature promoted a greater solubilization of the hemicellulose (Figure 5a). The fitted model to predict the solubilized hemicellulose in function of the independent variables is given by Eq. (3).

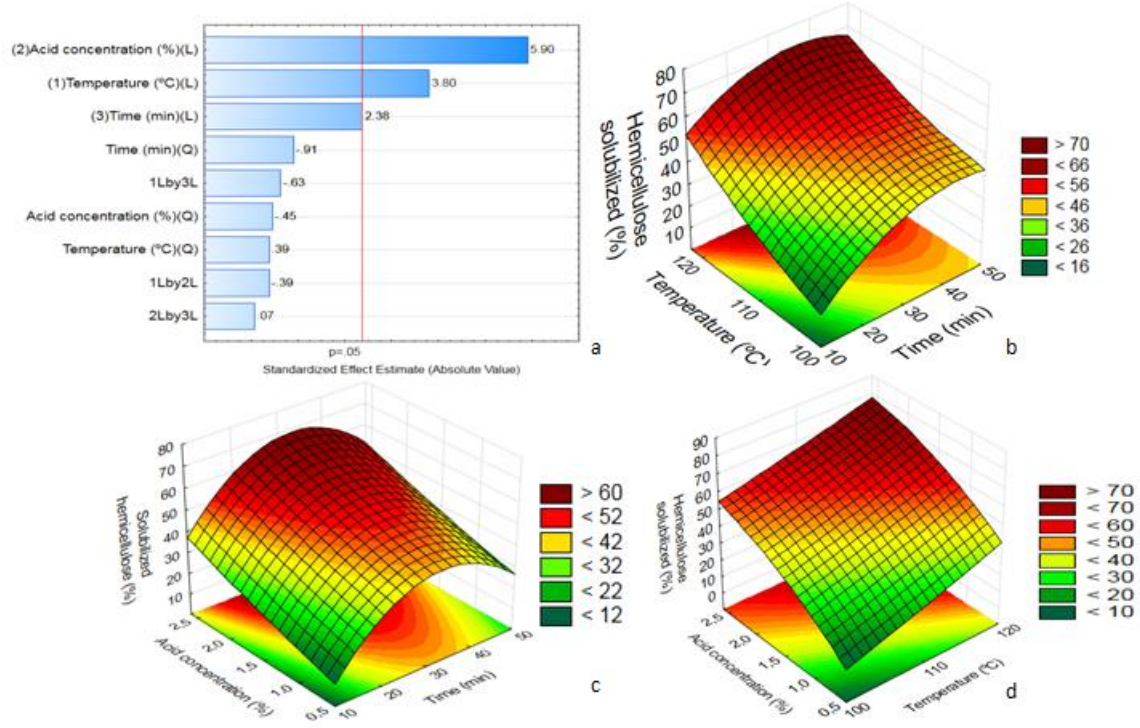
$$y = 65.5 - 3.7X_1 + 45.5\,X_2 + 3.9X_3 \quad (3)$$

where the "y" corresponds to the solubilized hemicellulose percentage and "X_1, X_2 and X_3 are the temperature, acid concentration and time of the pretreatment.

The analysis of variance (ANOVA) revealed that the calculated F value (6.35) was greater than the listed one ($F_{9.7}$ = 3.68) at 95% of confidence level and therefore, the model was statistically significant. The determination coefficient (R^2) was 0.89, which suggests that the experimental values are close to the ones estimated by the regression equation. The response surface of this model using all the possible combinations among the variables studied and the response variable is represented in Figure 1.

According to Figure 5b, temperatures greater than 110 ºC together with pretreatments times higher than 30 minutes lead to an increased solubilization of hemicellulose, confirming the estimated effects of these variables shown in Figure 1a. The response surfaces of the acid concentration with the temperature (Figure 5c) and time (Figure 5d) corroborate the same optimum region. In Figure 1, there is an optimum region at which the solubilization of hemicellulose is maximum, within the combination of the parameters studied. The higher hemicellulose solubilized occurred in the assays 7, 8 e 10 (Table 1). In this type of pretreatment, a primarily solubilization of hemicellulose occurs, but the cellulose solubilization can also (YANG et al., 2015a), which is not desirable in present study because the cellulose should remain in the solid fraction of material, to be further hydrolized. Thus the pretreatment conditions chosen for the next steps studies were 1.5% H_2SO_4, 120 ºC and 30 minutes (assay 10). At this condition, it was possible to solubilize 68.7% of hemicellulose and only 4.3% of cellulose present in the raw material (this was the less quantity of cellulose solubilized among all tested conditions -Table 2).

Figure 5. Pareto chart illustrating the estimated effects of the variables studied on the percentage of solubilized hemicellulose (response variable) (a); response surface obtained from the relation between the different variables, namely time and temperature (b); acid concentration and time (c); and acid concentration and temperature (d).



The solubilization of the hemicellulose increases the cellulose digestibility. However, the main constraints on acid treatment of lignocellulosic materials is the formation of furfural, 5-hydroxymethyl-2-furaldehyde (HMF) and acetic acid that are degradation products of the sugars released during the process as result of the conditions employed as high temperatures and low pH values. The presence of these compounds in the liquid fraction as a result from the pretreatment can compromise its utilization in bioprocess (HAFID et al., 2017), because this liquid fraction can be used as a xylose source in bioprocessing with microrganis able to ferment xylose. The xylose concentration ranged from 0 to 13.6 g/L in the hydrolisate. The higher xylose concentration was obtained in the pretreatment 8 (125 ºC/ 2.5 % H_2SO_4 and 45 minutes), which was the same condition where the greatest amount of hemicellulose (75.1 %, Table 2) was solubilized.

Table 2. Percentages of hemicellulose, cellulose and lignin which were solubilized with acid treatment.

Assay	Solubilized Cellulose (%)	Solubilized hemicellulose	Solubilized Lignin
1	0.0 ± 0.0	5.8 ± 0.2	16.7 ± 0.1
2	16.3 ± 0.4	17.1 ± 0.5	28.2 ± 0.3
3	9.2 ± 0.3	45.3 ± 0.8	16.0 ±0.4
4	21.6 ± 0.8	60.6 ± 0.7	21.5 ± 0.7
5	15.5 ± 0.4	36.2 ± 0.5	6.5 ± 0.9
6	16.5 ± 0.6	40.5 ± 0.6	14.8 ± 0.6
7	18.2 ± 0.3	72.5 ± 0.2	23.8 ± 1.4
8	23.3 ± 0.7	75.1 ± 0.6	19.3 ± 0.6
9	0.3 ± 0.5	32.3 ± 0.5	23.6 ± 0.9
10	4.3 ± 0.9	68.6 ± 0.8	24.0 ± 0.8
11	17.7 ± 0.2	19.6 ± 0.9	32.2 ± 0.6
12	26.8 ± 0.5	70.3 ± 0.6	20.4 ± 0.6
13	5.5 ± 0.4	17.4 ± 0.2	24.4 ± 0.4
14	4.1 ± 0.3	66.2 ± 0.1	17.6 ± 0.7
15	19.0 ± 0.2	54.1 ± 0.4	21.0 ± 0.5
16	18.7 ± 0.6	51.0 ± 0.2	18.4 ± 0.9
17	22.6 ± 1.0	53.8 ± 0.0	17.9 ± 0.6

*Percentages of cellulose, hemicellulose and lignin which were solubilized with acid treatment. Values were corrected multiplying each component percentage (Table 1) by TMR and divided by percentage of each component of raw material.

Table 3 shows the concentrations of acetic acid and furfural quantified in hydrolyzate resulting from acid pretreatment. For the same temperature of treatment where the increase in sulphuric acid concentration resulted in higher acetic acid. The higher acetic acid concentration was 3.6 g/L, released ate the more severe treatment (120 ºC and 2.5% H_2SO_4 during 45 minutes). The furfural concentration was practically the same in all treatments.

Table 3. Acetic acid and furfural quantified in the liquid fraction from acid pretreatment

Assays	t (ºC)	H_2SO_4 (%, v/v)	Time (min)	Xylose (g/L)	Acetic acid (g/L)	Furfural (g/L)
1	100	0.5	15	0.0	0.3	0.3
2	100	0.5	45	0.0	0.4	0.3
3	100	2.5	15	4.4	2.3	0.3
4	100	2.5	45	7.5	2.9	0.3
5	120	0.5	15	1.8	0.5	0.3
6	120	0.5	45	2.5	1.0	0.3
7	120	2.5	15	9.4	2.9	0.3
8	120	2.5	45	13.6	3.6	0.4
9	100	1.5	30	3.0	1.8	0.2
10	120	1.5	30	10.0	3.0	0.2
11	110	0.5	30	1.9	0.5	0.3
12	110	2.5	30	8.0	3.0	0.3
13	110	1.5	15	1.7	0.9	0.3
14	110	1.5	45	7.5	2.6	0.3
15	110	1.5	30	7.4	3.1	0.3
16	110	1.5	30	5.8	2.5	0.3
17	110	1.5	30	6.0	2.6	0.3

3.4.3 Alkaline pretreatment of the residue

The acid pretreatment previously performed aimed to the hemicellulose removal from the residue. It is known that the hemicellulose and lignin are strongly linked to cellulose, and therefore, their removal is crucial to increase the accessibility of the enzymes to the cellulose (SUN et al., 2015). Sodium hydroxide is extensively used in this type of pretreatment because besides the benefits before mentioned, it increases the internal surface of cellulose and decreases its crystallinity degree (ALVIRA et al., 2010). Based on this discussion, the vine pruning treated with diluted acid was further subjected to a pretreatment with NaOH at different concentrations. In all the tested conditions, the residual hemicellulose (which was not previously solubilized with the acid treatment) was now completely solubilized (Table 4). The TMR after the treatments ranged from 49.9% to 74.9% and the smallest TMR values were obtained for the higher NaOH concentrations.

The pretreatments performed at 120 ºC removed more lignin than those carried out at 100 ºC. The percentages of lignin solubilized were 38.8%, 54.7% and 63.8% for the assays with 1.0, 2.0 and 3.0% of NaOH, respectively (Table 4). The

greater percentage of lignin (63.8%) was solubilized when the pretreatment was performed with 3.0% of NaOH at 120 ºC without agitation. The higher amount of cellulose solubilized also occurred in these conditions.

Table 4. Composition of the solid fraction obtained after alkaline pretreatment (g/ 100 g of treatment solid, dry matter), total mass recovery (TMR) from the pretreated vine pruning and percentages of cellulose and lignin which were solubilized with alkaline treatment.

NaOH concentration; temperature	TMR (%)	Cellulose (%)	Solubilized Cellulose*	Lignin (%)	Solubilized Lignin*
1.0%; 120°C	62.6	65.3 ± 0.2	22.9 ± 0.2	35.7 ± 0.4	38.8 ± 0.4
1.0%; 100°C	74.9	67.1 ± 0.8	4.9 ± 0.8	32.4 ± 0.1	32.3 ± 0.1
2.0%; 120°C	54.4	69.7 ± 0.4	28.2 ± 0.4	29.8 ± 0.2	54.7 ± 0.2
2.0%; 100°C	67.5	68.1 ± 1.2	9.7 ± 1.2	31.9 ± 0.2	37.7 ± 0.2
3.0%; 120°C	52.5	75.0 ± 1.0	25.5 ± 1.0	24.7 ± 0.3	63.8 ± 0.3
3.0%, 100°C	49.9	69.3 ± 1.8	9.7 ± 1.8	30.8 ± 0.1	40.8 ± 0.1

The material which was submitted at pretreatment with NaOH contained the following composition: 54.8% of cellulose, 10.9% of hemicellulose and 35.8% of lignin. The hemicellulose that not was solubilized with the acid treatment was completely solubilized in this step. All assays were performed during 60 minutes with a substrate to alkali ratio (w/v) of 1:20. The percentages of cellulose and lignin refer to grams of the each fraction per 100 g of treated material. *Percentages of cellulose and lignin which were solubilized with alkaline treatment. Values were corrected multiplying previous column (component percentage) by TMR and divided by percentage of each component of material before delignification (material submitted at acid treatment).

In all assays performed at 100 ºC, the amount of solubilized cellulose did not exceed 10% (Table 4). However, the condition that removed most lignin was 100 ºC and 3.0% of NaOH. The choice of the delignification pretreatment should be based on the highest removal of lignin and the lowest or no solubilization of cellulose (YANG et al., 2015) along with the maximal enzymatic hydrolysis yields. Therefore, all samples obtained from the alkaline treatment were submitted to enzymatic hydrolysis.

3.4.5 Structural changes in the biomass

The main constituents of cell wall of plants are cellulose, hemicellulose and lignin distributed in one primary wall and a secondary wall with three layers. Usually, the primary wall has more lignin than the secondary wall. The plant tissues are parenchyma, collenchyma, xylem, sclerenchyma. The parenchyma is rich in cellulose, the collenchyma in hemicellulose and the xylem and sclerenchyma in lignin (FAHN, 1967; GIBSON, 2012a). To evaluate the changes promoted by the pretreaemtb, the

structure of the vine pruning before and after the pretreatment was analyzed through a SEM. The images of the untreated vine pruning (UNT) showed the complete and compact parenchyma, sclerenchyma and xylem (Figure 6). The vine pruning pretreated with diluted acid (120 ºC, 1.5% and 30 minutes) showed a slight opening of the parenchyma structure (structure composed mainly of cellulose and hemicellulose), but it was still possible to observe the original cell contours. The sclerenchyma and the xylem remained intact. In the PT1 (acid treatment followed by NaOH 1% (w/v) at 120 ºC without agitation), the parenchyma degradation started with unstruted areas and others intact. The contours of the original cell were not observed anymore. The xylem remained preserved and the sclerenchyma was slight modified. The parenchyma from the sample of pretreatment two (PT2) was similar to the previous sample (PT1) and the sclerenchyma exhibited some tiny holes at the surface.

The samples from the pretreatments 3 and 4 (both with 2.0% of NaOH, at 120 ºC and 100 ºC, respectively) (Figure 7) showed a more degraded parenchyma with tiny holes at the surface. The cell structure is also disorganized due to the chemical attack. Regarding the sample 3, the alkali started to act on the xylem structure and some cracks are seen on the residue surface. In the samples 5 and 6, the lignocellulosic structure of vine pruning has been significantly destroyed. The sclerenchyma seems to have exploded and the xylem showed a lot of cracks. The results dearly suggest that the NaOH concentration affects more the lignocellulosic structure than the temperature. These results corroborated with the observed in the quantitative analysis previously reported (Table 4).

Figure 6. SEM images of vine pruning without and with pretreatment. Subtitle: UNT – untreated vine pruning; PT0 – vine pruning pretreated with diluted sulfuric acid (1.5% H_2SO_4, 120 ºC and 30 minutes - assay 10); PT1 refer to pretreatment that used 1.0% of NaOH at 120 °C without agitation and PT2 refer to pretreatment 100 ºC with agitation. Magnifications of parenchyma and sclerenchyma in all samples was equal at 1000x and for xylem were different (UNT - 2000x; PT0 – 1000X; PT1 – 2540x; PT2 – 1000x).

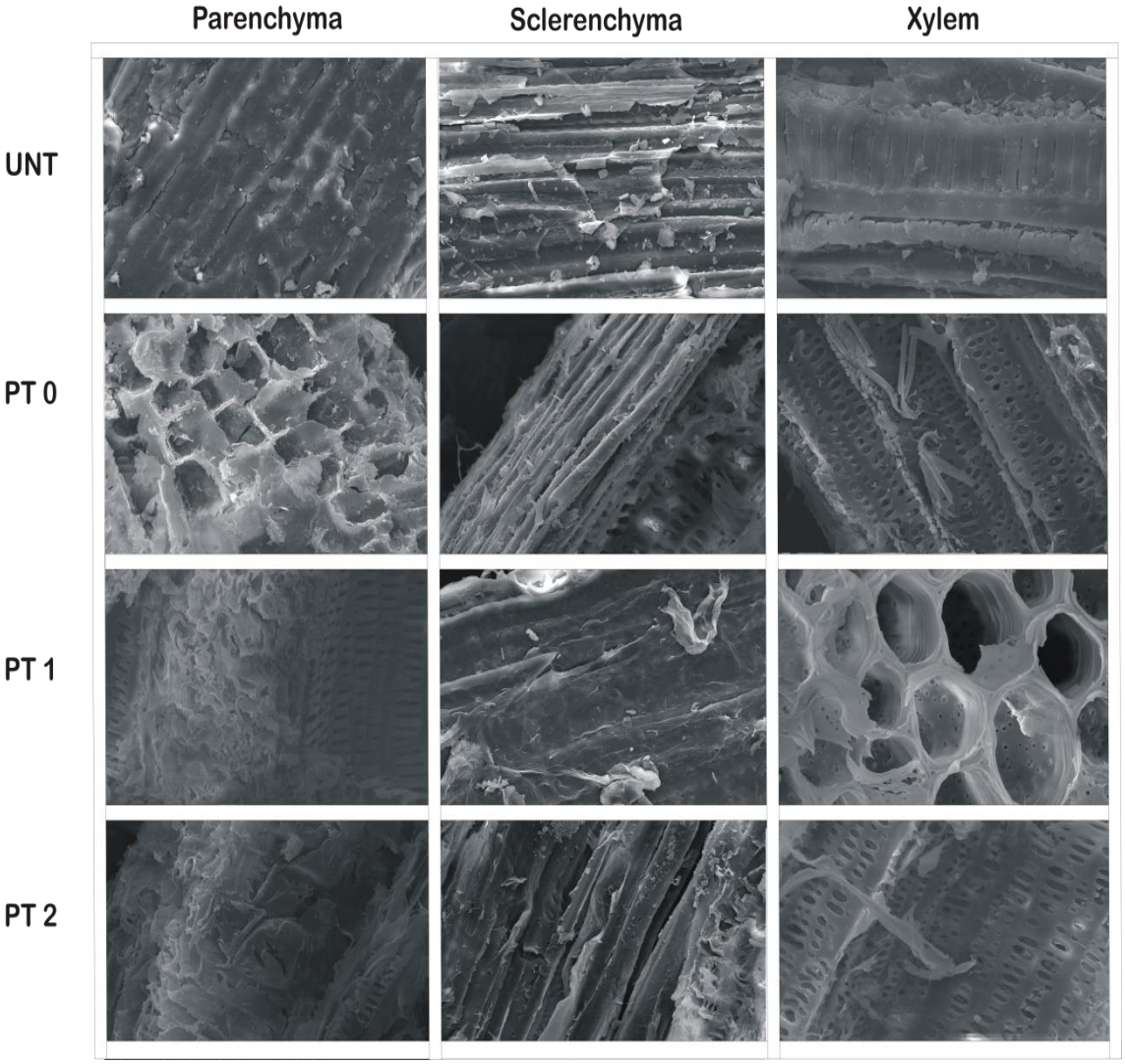
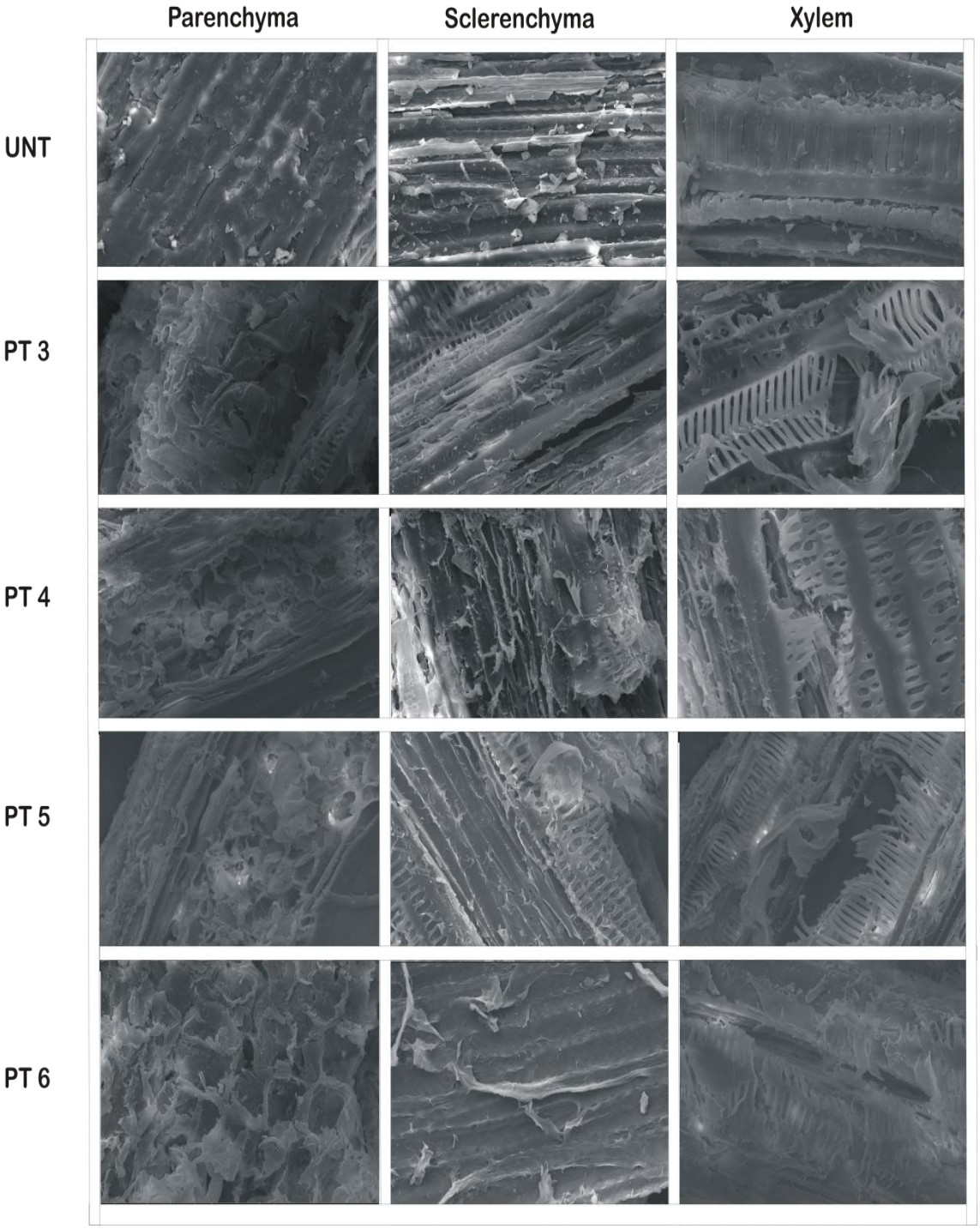


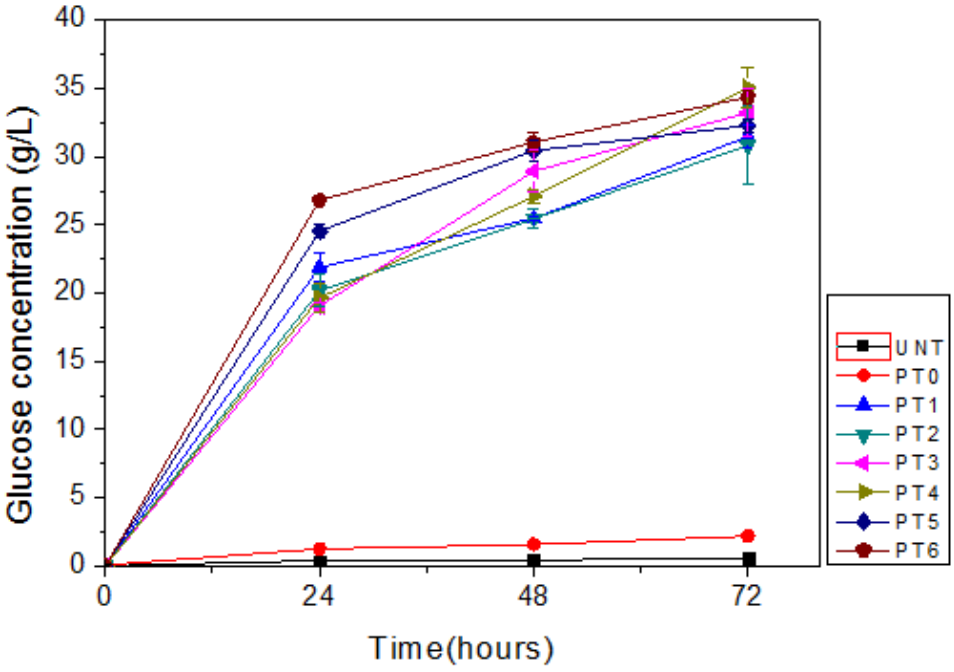
Figure 7. SEM images of vine pruning without and with pretreatment. Subtitle: UNT – untreated vine pruning; PT3 and PT 5 refer to pretreatment that used 2.0% of NaOH at 120 °C without agitation and 100 ºC with agitation, respectively. PT 4 and PT6 refer to pretreatment that used 3.0% of NaOH at without agitation and 100 ºC with agitation, respectively. Magnifications of parenchyma and sclerenchyma in all samples was equal at 1000x and for xylem were different (UNT - 2000x; PT 3 – 1000x; PT 4 – 1500x; PT5 – 692 x; PT6 – 1000x).



3.4.6 Enzymatic hydrolysis of the treated vine pruning

Kumar et al.(2010) studied the combined pretreatment of wood chips. First, they subjected the residue to steam explosion and then to the delignification. The hydrolysis of the treated material led to 95.5% of glucose. Similar results were reported for sugarcane bagasse that was submitted steam explosion and alkaline treatment (ROCHA et al., 2015). On the other hand, when steam explosion was replaced by pretreatment with dilute acid and alkaline treatment, the glucose yield was only 79% of sugar cane bagasse (ROCHA et al., 2015). For the same residue, the association of the acid treatment with ionic liquid resulted in a glucose yield of 95.5% (JIANG et al., 2013). Cara et al. (2008) studied only the acid pretreatment of olive pruning (under conditions completely different from those tested in this work) and obtained a glucose yield of 76.5%. The enzymatic hydrolysis of barley straw, submitted only acid treatment, resulted in 61.7% of glucose (YANG et al., 2015). On the other hand, Shao et al. (2017) studied the pretreatment of the corn stover by steam explosion with sulphuric acid and obtained an yield of glucose equal at 84.7% after enzymatic hydrolysis. In the present work, the enzymes tested were not able to hydrolyze the untreated vine pruning (Figure 8) and the hydrolysis of the vine pruning treated only with dilute acid sulfuric released only 2.17 g/L of glucose (yield of 8.8%) (Table 5) after 96 hours.This result showed that the pretreatment with acid was not enough to make the cellulose present in this material available for the enzymes action. This result suggests the need of the material delignification, thus, a step with this purpouse was performed after acid treatment.

Figure 8. Glucose concentration (g/L) released from of enzymatic hydrolysis of vine pruning untreated and pretreated. Subtitle: UNT – untreated vine pruning; PT0 – vine pruning pretreated with diluted sulfuric acid (1.5% H_2SO_4, 120 ºC and 30 minutes - assay 10); PT1, PT3 and PT5 refer to a pretreatment with NaOH at 120 °C without agitation (the concentrations of NaOH which were used 1.0%, 2.0% and 3.0%) and PT2, PT4 and PT6 refer to a pretreatment with NaOH at 100 ºC with agitation (the concentrations of NaOH which were used 1.0%, 2.0% and 3.0%). All these assays were performed during 96 hours and the samples were collected every 24 hours and were kept in boiling water for 5 min to inactive enzymatic activity, and centrifuged at 11975 g for 20 minutes for separated insoluble fractions.



The hydrolysis of all residues submitted to the alkaline pretreatment (PT1 to PT6) present similar profiles. In all assays, the highest rate of glucose release occurred in the first 24 hours of hydrolysis and with 72 hours of processing, the higher glucose concentrations (30 g/L and 35 g/L) (Table 5) were obtained. The glucose concentration from hydrolysis with the residue after the pretreatment 1 (1.0% of NaOH at 120 °C without agitation) was 14.5 times higher than the amount released using the material treated only with acid.

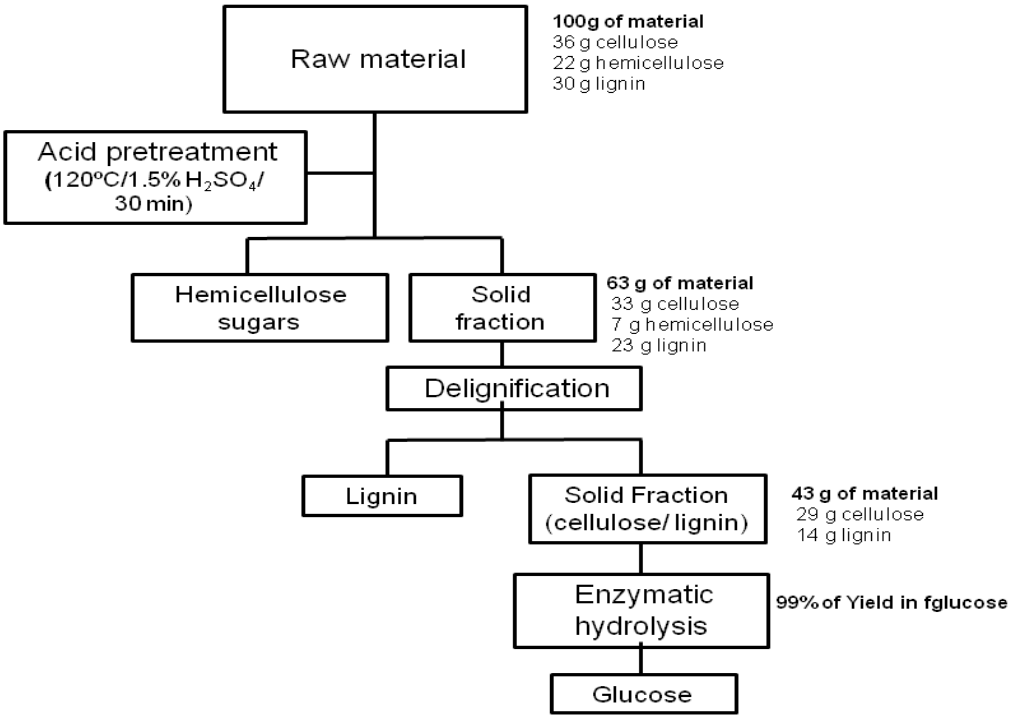
Table 5. Glucose concentration and yield after 96 hours of enzymatic hydrolysis of vine pruning. Subtitle: UNT – untreated vine pruning; PT0 – vine pruning pretreated with diluted sulfuric acid (1.5% H_2SO_4, 120 ºC and 30 minutes - assay 10); PT1, PT3 and PT5 refer to pretreatment that was realized at 120 °C without agitation and with 1.0%, 2.0% and 3.0% of NaOH, respectively and PT2, PT4 and PT6 refer to a pretreatment at 100 ºC with agitation and with 1.0%, 2.0% and 3.0% of NaOH, respectively).

Pretreatments Conditions	Glucose concentration (g/L)	Yield (%)
Untreated (UNT)	0.56 ± 0.10	---
Acid pretreatment	2.17 ± 0.1	8.8 ± 0.4
PT1	31.46 ± 0.7ab	90.78 ± 1.9ab
PT2	30.80 ± 0.0a	86.31 ± 0.0ac
PT3	33.22 ± 0.6bcd	90.96 ± 1.6ab
PT4	35.06 ± 1.2d	98.72 ± 3.4d
PT5	32.26 ± 0.4abc	80.86 ± 1.2c
PT6	34.35 ± 0.4cd	93.35 ± 1.1bd

Different letters in the same column indicate significant differences in the values of averages ($p < 0.05$) in accordance with Tukey's test.

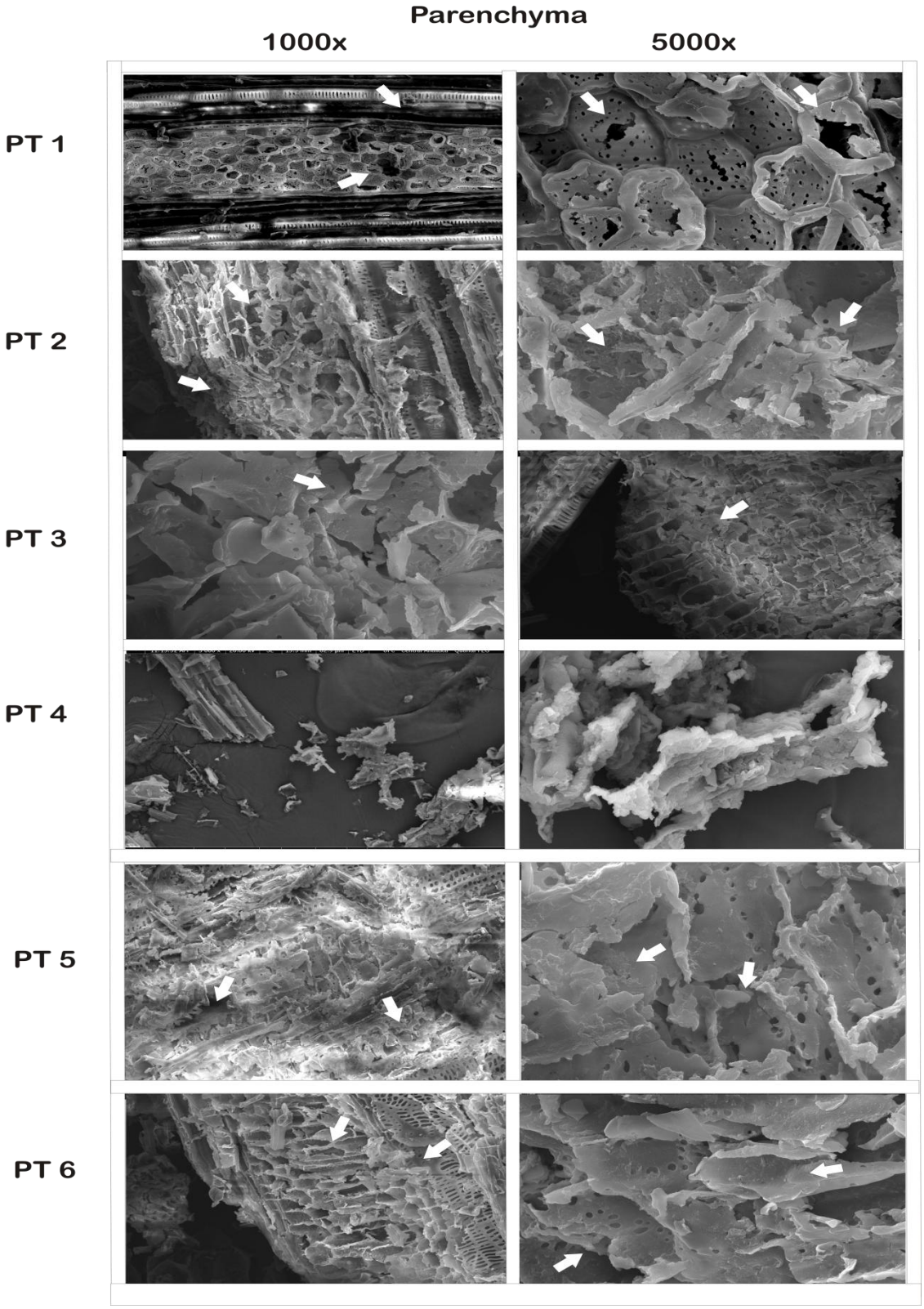
The increase in the temperature from 100 ºC to 120 ºC led to a higher level of lignin removal in all samples pretreated with alkali. In these same conditions, the content of cellulose solubilized increased (Table 4). For the same NaOH concentration, the results were not significantly different (after 96 hours of assay). These data reinforce the need to of combining the amount of lignin removed with the pretmeant, and the cellulose solubilized after the enzyme hydrolysis to choose the best treatment conditions of a given material. The complete removal of the lignin may not be required for an enzyme hydrolysis of cellulose. The greater yield in glucose was obtained from the hydrolysis of the residue obtained with the pretreatment 4 (2.0% of NaOH, at 100 ºC without agitation). As shown in Figure 9, the material treated in these conditions showed 29 g of cellulose and 14 g lignin and after enzymatic hydrolysis practically the whole cellulose was hydrolyzed in glucose (99% of yield).

Figure 9. Mass Balance of the selected pretreatment (1.5% H_2SO_4, 120 ºC and 30 minutes and delignification with 2.0% NaOH, 100 ºC without agitation) and the enzymatic hydrolysis of vine pruning.



The structure of the vine pruning after enzymatic hydrolysis was also analyzed through a SEM (Figure 10). The enzymatic hydrolysis was performed at the same conditions for all assays because the aim was to evaluate the effect of the pretreatment on the enzymes action. The material obtained from the enzymatic hydrolysis became porous breaking up by manual maceration. In figure 10, it was possible to observe which structure of vine pruning was damaged. The parenchyma was partially destroyed with small fissures (or cracks) at the surface as shown by the arrows. However, it was still possible to observe the original cell contours. The images of the samples 2, 3, 5 and 6 showed a parenchyma destroyed and the contours of the original cell were not observed anymore. The sample 4 structure (pretreatment with 2.0% of NaOH at 100 ºC with agitation) exhibited the most destroyed kneaded aspect. This result was fully aligned with the quantitative results obtained for this sample (glucose yield equal to 98.7%).

Figure 10. SEM images of vine pruning after enzymatic hydrolysis with the commercial enzyme Cellic® CTec2 and Novozyme 188. Subtitle: PT1, PT3 and PT5 refer to pretreatment that used 1.0%, 2.0% and 3.0% of NaOH, respectively and it was realized at 120 °C without agitation) and PT2, PT4 and PT6 refer to a pretreatment 100 ºC with agitation and in the same concentrations of NaOH (1.0%, 2.0% and 3.0% of NaOH, respectively).



3.5 Conclusions

The combination of the acid and alkali pretreatments that resulted in the best hydrolysis of the cellulose of vine pruning was 1.5% of H_2SO_4 at 120 ºC during 30 minutes (in autoclave) followed by delignification with 2.0% of NaOH at 100 ºC during 60 minutes. The material pretreated at these conditions was then submitted to an enzymatic hydrolysis and released 98.7% of glucose. It was also possible to obtain visual information on the structural modification caused by the pretreatments on the vine pruning. The SEM results were fully aligned with the quantitative ones, showing that the pretreatment really removed the loginifn and opened the fiber structure allowing the enzyme attack and the material sacchrification. The low levels of acetic acid, furfural and HMF obtained in the liquid fraction of the acid preteament allows its use after neutralization in processing where xylose is fermented.

3.6 Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit, COMPETE 2020 (POCI-01-0145-FEDER-006684) and the project Multibiorefinery (POCI-01-0145-FEDER-016403) funded by the European Regional Development fund under the scope of Norte 2020. In Brazil the study was supported by CAPES, FUNCAP and CNPQ. The authors would like to thank at Central analytical (Physical Department) of Federal University of Ceará by the microscopic analysis carried out. E. Gudina acknowledgments FCT for the Post-Doctoral Grant (CEB-BPD/01/2015/07).

3.7 Referências

ALVIRA, P.; TOMÁS-PEJÓ, E.; BALLESTEROS, M.; NEGRO, M. J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. **Bioresource Technology**, v. 101, n. 13, p. 4851–4861, 2010.

BANERJEE, D.; MUKHERJEE, S.; PAL, S.; KHOWALA, S. Enhanced saccharification efficiency of lignocellulosic biomass of mustard stalk and straw by salt pretreatment. **Industrial Crops and Products**, v. 80, p 42–49, 2016.

BIAGINI, E.; BARONTINI, F.; TOGNOTTI, L. Gasification of agricultural residues in

a demonstrative plant: Vine pruning and rice husks. **Bioresource Technology**, v. 194, p. 36–42, 2015.

BRITO, P. S. D.; OLIVEIRA, A. S.; RODRIGUES, L. F. Energy valorization of solid vines pruning by thermal gasification in a pilot plant. **Waste and Biomass Valorization**, v. 5, n. 2, p. 181–187, 2014.

BUSTOS, G.; MOLDES, A. B.; CRUZ, J. M.; DOMINGUEZ, J. M. Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by Lactobacillus pentosus. **Journal of the Science of Food and Agriculture**, v. 84, n. 15, p. 2105–2112, 2004.

BUSTOS, G.; MOLDES, A. B.; CRUZ, J. M.; DOMINGUEZ, J. M. Production of lactic acid from vine-trimming wastes and viticulture lees using a simultaneous saccharification fermentation. **Journal of the Science of Food and Agriculture,** v. 85, p. 466–472, 2005.

CARA, C.; RUIZ, E.; OLIVE, J. M.; SAEZ, F; CASTRO, E. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. **Bioresource Technology**, v. 99, n. 6, p. 1869–1876, 2008.

CUEVAS, M.; GARCÍA, J. F.; SÁNCHEZ, S. Enhanced enzymatic hydrolysis of pretreated almond-tree prunings for sugar production. **Carbohydrate Polymers**, v. 99, p. 791–799, 2014.

CARVALHO, D. M.; SEVASTYANOVA, O.; PENNA, L. S.; SILVA, B. P.; LINDSTROM, M. E.; COLODETTE, J. L. Assessment of chemical transformations in eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid, and alkaline pretreatments. **Industrial Crops and Products**, v. 73, p. 118–126, 2015.

DOWE, N.; NREL, J. M. SSF Experimental Protocols — Lignocellulosic Biomass Hydrolysis and Fermentation Laboratory Analytical Procedure (LAP) Issue Date : 10 / 30 / 2001 SSF Experimental Protocols — Lignocellulosic Biomass Hydrolysis and Fermentation Laboratory Analytical Pro. **National Renewable Energy Laboratory**, v. NREL/TP-51, n. 10/20/2001, 2008.

FAHN, A. **Plant anatomy**. [s.l: s.n.].

FERREIRA, S.; DUARTE, A. P.; RIBEIRO, M. H. L.; QUEIROZ, J. A.; DOMINGUES, F. C. Response surface optimization of enzymatic hydrolysis of Cistus ladanifer and Cytisus striatus for bioethanol production. **Biochemical Engineering Journal**, v. 45, n. 3, p. 192–200, 2009.

GIBSON, L. J. The hierarchical structure and mechanics of plant materials. **Journal of The Royal Society Interface**, v. 9, p. 2749-2766, 2012.

HAFID, H. S.; RAHMAN, N. A.; MOKHTAR, M. N.; TALIB, A. T. Over production of fermentable sugar for bioethanol production from carbohydrate-rich Malaysian food waste via sequential acid-enzymatic hydrolysis pretreatment. **Waste Management**, v. 67, p. 95–105, set. 2017.

JIANG, L. Q.; FANG, Z.; LI, X. K.; LUO, J.; FAN, S. P. Combination of dilute acid and ionic liquid pretreatments of sugarcane bagasse for glucose by enzymatic hydrolysis. **Process Biochemistry**, v. 48, n. 12, p. 1942–1946, 2013.

KUMAR, L.; CHANDRA, R.; CHUNG, P. A.; SADDLER, J. Can the same steam pretreatment conditions be used for most softwoods to achieve good, enzymatic hydrolysis and sugar yields? **Bioresource Technology**, v. 101, n. 20, p. 7827–7833, 2010.

LIM, J. S.; MANAN, Z. A.; ALWI, S. R. W.; HASHIM, H. A review on utilisation of biomass from rice industry as a source of renewable energy. **Renewable and Sustainable Energy Reviews**, v. 16, n. 5, p. 3084–3094, 2012.

MAEDA, R. N.; BARCELOS, C. A.; ANNA, L. M. M. S.; JUNIOR, N. P. Cellulase production by Penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. **Journal of biotechnology**, v. 163, n. 1, p. 38–44, 10 jan. 2013.

MATEO, S.; PUENTES, J. G.; ROBERTO, I. C.; SANCHEZ, S.; MOYA, A. J. Optimization of acid hydrolysis of olive tree pruning residue . Fermentation with Candida guilliermondii. **Biomass and Bioenergy**, v. 69, p. 39-46, 2014.

MILLER, S. A.; BROOM, F. D.;THORP, T. G.; BARNETT, A. M. Effects of leader pruning on vine architecture , productivity and fruit quality in kiwifruit (Actinidia deliciosa cv. Hayward). **Scientia Horticulturae**, v. 91, p. 189–199, 2001.

MOLDES, A. B.; BUSTOS, G.; TORRADO, A.; DOMINGUEZ, J. M. Comparison between different hydrolysis processes of vine-trimming waste to obtain hemicellulosic sugars for further lactic acid conversion. **Applied Biochemistry and Biotechnology**, v. 143, n. 3, p. 244–256, 2007.

NARRA, M.; JAMES, J. P.; BALASUBRAMANIAN, V. Simultaneous saccharification and fermentation of delignified lignocellulosic biomass at high solid loadings by a newly isolated thermotolerant Kluyveromyces sp. for ethanol production. **Bioresource Technology**, v. 179, p. 331–338, 2015.

NEVES, P. V.; PITARELO, A. P.; RAMOS, L. P. Production of cellulosic ethanol from sugarcane bagasse by steam explosion: Effect of extractives content, acid catalysis and different fermentation technologies. **Bioresource Technology**, v. 208, p. 184–194, 2016.

NTALOS, G. A.; GRIGORIOU, A. H. Characterization and utilisation of vine prunings as a wood substitute for particleboard production. **Industrial Crops and Products**, v. 16, n. 1, p. 59–68, 2002.

ROCHA, G. J. M.; GONÇALVES, A. R.; NAKANISHI, S. C.; NASCIMENTO, V. M.; SILVA, V. F. N. Pilot scale steam explosion and diluted sulfuric acid pretreatments: Comparative study aiming the sugarcane bagasse saccharification. **Industrial Crops and Products**, v. 74, p. 810–816, 2015.

SARATALE, G. D.; SARATELE, R. G.; GHODAKE, G. S.; JIANG, Y. Y.; CHANG,J. S.; SHIN, H. S.; KUMAR, G. Characterization and its application in the saccharification of rice waste biomass for ethanol production : An integrated biotechnological approach. **Journal of the Taiwan Institute of Chemical Engineers**, v. 0, p. 1–8, 2017.

SARATALE, G. D.; JUNG, M. Y.; OH, M. K. Reutilization of green liquor chemicals for pretreatment of whole rice waste biomass and its application to 2,3-butanediol production. **Bioresource Technology**, v. 205, p. 90–96, 2016.

SARKAR, N.; KUMAR, G.; BANNERJEE, S.; AIKAT, K. Bioethanol production from agricultural wastes: An overview. **Renewable Energy**, v. 37, n. 1, p. 19–27, 2012.

SHAO, S.; ZHANG, J.; HOU, W.; QURESHI, A. S. Q.; BAO, J. Lower pressure heating steam is practical for the distributed dry dilute sulfuric acid pretreatment. **Bioresource Technology**, v. 238, p. 744–748, 2017.

SLUITER, A.; HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D. Determination of Extractives in Biomass. **Biomass Analysis Technology Team Laboratory Analytical Procedure**, n. January, p. 1–6, 2008a.

SLUITER, A. HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D. Determination of Ash in Biomass. **Biomass Analysis Technology Team Laboratory Analytical Procedure**, n. January, p. 1–6, 2008b.

SLUITER, A.; HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D.; CROCKER, D. Determination of Structural Carbohydrates and Lignin in Biomass. **Biomass Analysis Technology Team Laboratory Analytical Procedure,** v. 2011, n. July, 2012.

SU, C. H.; CHUNG, M. H.; HSIEH, H. J.; CHANG, Y. K.; DING, J. C.; WU, H. M. Enzymatic hydrolysis of lignocellulosic biomass in ionic liquid media for fermentable sugar production. **Journal of the Taiwan Institute of Chemical Engineers**, v. 43, n. 4, p. 573–577, 2012.

SUN, S.; SUN, S.; CAO, X.; SUN, R. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. **Bioresource Technology**, v. 199, p. 49–58, 2015.

VAZ DE ARRUDA, P.; SANTOS, J. C.; RODRIGUES, R. C. L. B.; SILVA, D. D. V.; YAMAKAWA, C. K.; ROCHA, G. J. M.;JUNIOR, J. N.; PRADELLA, J. G. C.; ROSSELL, C. E. V.; FELIPE, M. G. A. Scale up of xylitol production from sugarcane bagasse hemicellulosic hydrolysate by Candida guilliermondii FTI 20037. **Journal of Industrial and Engineering Chemistry**, v. 47, p. 297–302, 2017.

YAN, Z.; LI, J.; CHANG, S.; CUI, T.; JIANG, Y.; YU, M.; ZHANG, L.; ZHAO, G.; QI, P.; LI, S. Lignin relocation contributed to the alkaline pretreatment efficiency of sweet sorghum bagasse. **Fuel**, v. 158, p. 152–158, 2015.

YANG, M.; ZHANG, J.; KUITTINEN, S.; VEPSALAINEN, J.; SOININEN, P.; KEINANEN, M.; PAPPINEN, A. Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone-butanol-ethanol fermentation. **Bioresource Technology**, v. 189, p. 131–137, 2015.

YOO, H. Y.; LEE, J. H.; KIM, D. S.; LEE, J. H.; LEE, S. K.; LEE, S. J.; PARK, C.; KIM, S. W. Enhancement of glucose yield from canola agricultural residue by alkali pretreatment based on multi-regression models. **Journal of Industrial and Engineering Chemistry**, v. 51, p. 303–311, jul. 2017.

4 VALORIZATION OF CHESTNUT SHELL: ALKALINE PRETREATMENT AND ENZYMATIC HYDROLYSIS OF CELLULOSE

T. C. MACIEL[1], E. GUDIÑA[2], E. C. MIGUEL[2], L. R. RODRIGUES[3], S. RODRIGUES[4]

[1] Federal University of Ceará, Centre of Technology, Department of Chemical Engineering, Campus do Pici, Bloco 709, CEP 60440-900, Fortaleza-CE-Brazil.
[2] Federal University of Ceará, Physics Department/Analytical Center, Campus do Pici Bloco 928, CEP 60440-900, Fortaleza-CE-Brazil
[3] CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[4] Federal University of Ceará, Centre of Agricultural Sciences, Department of Food Engineering, Campus do Pici, Bloco 858, CEP 60440-900, Fortaleza-CE-Brazil

4.1 Abstract

An increased interest in the production of renewable energy from lignocellulosic biomass has been registered in the recent years. These materials are mainly composed of lignin, hemicellulose and cellulose. In the present study, the chestnut shell, a residue obtained from the chestnut fruits processing (*Castanea sativa* Mill.), was evaluated as a potential material to be used in a biorefinery context. The chesnut shell herein used is composed of 27.3% of cellulose, 12.6% of hemicellulose and 43.6% of lignin. The chemical composition of the untreated and pretreated Chestnut shell residue was determined according to the analytical protocols described by National Renewable Energy Laboratory (NREL) (U.S Department of Energy). Thus, for the saccharification of this residue, it is necessary first to include a pretreatment able to remove the lignin. The parameters evaluated in this pretreatment were the temperature and NaOH concentration. The treated residues were then submitted to an enzymatic hydrolysis with commercial cellulases (Cellic® CTec2 and Novozyme 188). The condition that led to the greatest reduction of the lignin content was 2.0% of NaOH and 100 ºC. However, a good lignin removal was also obtained with 1.0% of NaOH at 60 ºC for 1 hour. Under this condition it was possible to obtain a material containing 49.1% (w/w) of cellulose, 20.6% (w/w) of hemicellulose and 27.8% (w/w) of lignin. The highest saccharification yield obtained was 86% and the glucose concentration was 22.8 g/L in the treatment at 60 ºC with 1.0% of NaOH. The results obtained by the classical analysis recommended by NREL were corroborated by eletronic microscopy in which it is clearly shown the lignin removal and the exposure of the internal vegetal cell structure in the biomass.

Keywords: Alkaline pretreatment; Cellulase; Enzymatic hydrolysis; Chestnut shell.

4.2 Introduction

Chesnut fruits (*Castanea sativa* Mill.) are important food products not only due to their nutritional value (LI et al., 2016) but also because they contain carbohydrates with low glycemic index, high amounts of fiber and are gluten free (SERVILLO et al., 2016). Worldwide there are about 12 different species of chestnut. In Europe, the *Castanea sativa Miller* is the only species found. About 108.000 tons of chestnuts are produced per year in Europe (LI et al., 2016) and in the Galicia (Spain), around 8000 tons per year are used in the food industry (VÁZQUEZ et al., 2012). The chestnut shell is a lignocellulosic material that represents about 10% of the total weight of the fruit (LEE et al., 2016). Different biotechnological applications of the chestnut residues have been reported, such as its use for the absorption of heavy metals (COBAS et al., 2016) and as a source of antioxidants and natural colorants (ZHAO; FENG; WANG, 2014).

The chestnut shell is a lignocellulosic material formed basically by cellulose, hemicellulose and lignin. Its composition varies according to the cultivation area. In the chestnut shell (Chestnut fruits from Trás-os-Montes, Portugal) used in the present study were quantified 27.3% (w/w) of cellulose, 12.6% (w/w) of hemicellulose and 43.6% (w/w) of lignin. A similar composition (28.4% of cellulose, 7.9% of hemicellulose and 41.7% of lignin) was reported by Maurelli et al. (2013) for the chestnut shell originating from Liuan city (China).

In the last years, the concept of biorrefinery of the lignocellulosic biomass has received great attention due to the various products that can be manufactured in this process (VECINO et al., 2017; YOO et al., 2017). The lignocellulosic materials are mainly composed of lignin, hemicellulose and cellulose. The lignin can be used in the production of aromat(ic polymers of value-added, as phenols, vanillin, syringaldehyde that are interesting raw materials for the industries of flavor and fragance (MOTA et al., 2016). Moreover, Lactic lactic acid can be produced from hemicellulose sugars and biofuels can be produced from cellulose (YAN et al., 2015). In this context, the chestnut shell being an abundant low-cost lignocellulosic material exhibits a great potential to be used as a renewable sugars

source.

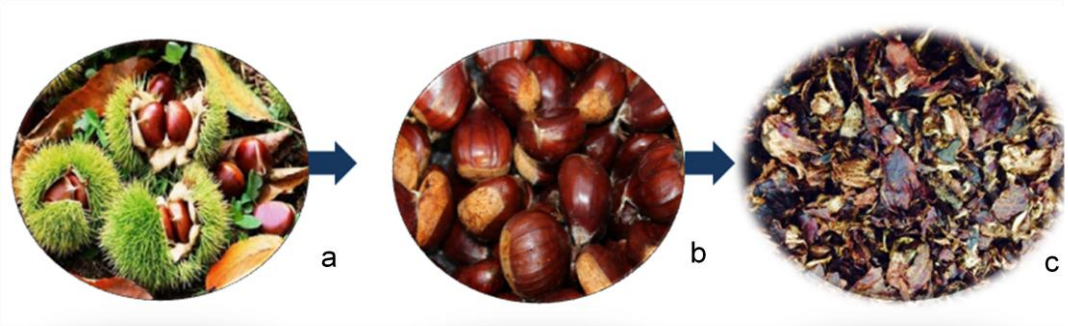
The lignocellulosic materials are highly resistant to enzymatic and microbial degradation due to their structure that presents cross-linkages between the cellulose and hemicellulose, ester bonds between ester and lignin and crystalline cellulose(LIU et al., 2016). Therefore, for its reuse an appropriate pretreatment that can guarantee the release of its fermentable sugar constituents its necessary (YOO et al., 2017). Some examples of pretreatments applied to lignocellulosic biomass are: steam explosion, dilute acid and alkaline (HE et al., 2016). The alkaline pretreatment breaking the ester bonds between lignin, hemicellulose and cellulose, promoting the saponification of ester bonds. The delignification occurs due to the disruption of the crosslinks between hemicellulose and lignin (YOO et al., 2017). In this way, the access to the internal cellulose increases and its crystallinity degree decreases (CANILHA et al., 2012). When compared with other pretreatments, this process uses low temperatures, solubilizes less hemicellulose and generates less carbohydrate degradation products (JÖNSSON; MARTÍN, 2016). This pretreatment is appropriate for agricultural residues and its efficiency greatly depends on the lignin content of the biomass (TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010), besides factors as alkali concentration, time and temperature of process (YOO et al., 2017). Therefore, the choice of an adequate pretreatment method is important for the bioprocess efficiency and competitiveness. In summary, the aim of this work was to study the pretreatment of the chestnut shell and to evaluate its enzymatic hydrolysis towards the production of fermentable sugars.

4.3 Materials and methods

4.3.1 Raw material

The chestnut shell (*Castanea sativa Miller*) used in this work was kindly provided by a local farm in the Trás-os-Montes (Portugal). The residue (outer brown peel with the inner pellicle) was used as it came from the industry (Figure 11), being only washed and dried in an oven at 60 ºC for 24 hours.

Figure 11. Chestnut fruits hell from a local farm in Trás-os-Montes (Portugal). Subtitle: chestnut fruits inside of the urchin, chestnut fruits, chestnut shell after removal of fruit.



4.3.2 Alkaline pretreatment

A pretreatment with sodium hydroxide in two concentrations (1.0% and 2.0% w/v) was carried out at 60 ºC, 80 ºC and 100 ºC under agitation in a stirred-heating plate. All experiments were done with the biomass to alkali ratio (w/v) of 1:20 during 60 minutes. After each treatment, the solid and the liquid fractions were separated by vacuum filtration (in a Buchner funnel with a soft cloth), and the solid fraction was washed until neutral pH. The recovered solid fraction was dried in an oven at 60 ºC for 24 hours, weighted and represented as total mass recovery (TMR) according to the Equation (1). The chemical composition was determined as described in section 3.2.5

$$TMR\ (\%) = \frac{mass\ of\ insoluble\ fraction\ in\ pretreatment\ (g)}{initial\ mass\ \ (g)} * 100 \qquad (1)$$

The mass of insoluble fraction in the pretreatment is the mass recovered after the filtration and successive washings of the treated material; and the *initial mass* is the mass of the residue before treatment.

The lignin solubilized with the treatment was calculated as the percentage of delignification (D) according to the Equation (2).

$$D\ (\%) = 100 - \frac{\mathrm{TMR\%-LBT\%}}{LBU\%} \qquad (2)$$

LBT is the percentage of lignin in the pretreated biomass and LBU is the percentage of lignin in the untreated biomass.

The percentage of cellulose and hemicellulose, which was not solubilized due tothe treatment, was calculated based the ratio between the fraction present in the treated biomass and the onre presented in the untreated biomass according to the Equations (3) and (4).

$$CNS\ (\%) = \frac{\mathrm{TMR\%-CBT\%}}{\mathrm{CBU\%}} \quad (3)$$

CNS is the percentages of cellulose which not solubilized with treatment, CBT is the percentage of cellulose in the treated biomass and CBU is the percentage of cellulose in the untreated biomass.

$$HNS\ (\%) = \frac{\mathrm{TMR\%-HBT\%}}{\mathrm{HBU\%}} \quad (4)$$

HNS is the percentage of hemicellulose which not solubilized with treatment, HBT is the percentage of hemicellulose in the treated biomass and HBU is the percentage of hemicellulose in the untreated biomass.

4.3.3 Enzymatic hydrolysis

The enzymatic hydrolysis of the treated chestnut shell was performed with a blend of commercial enzymes: Cellic® CTec2 (30 FPU/ g of biomass) and Novozyme 188 (15 CBU/ g of biomass). The enzymatic activities of filter paper cellulase (FPcellulase), carboxymethyl cellulase (CMCase), cellobiase (cellobiose) and xylanase (which acts on the xylan from the hemicellulose) were determined for the enzymes used.

The total cellulase (FPcellulase) was assayed by measuring the release of reducing sugars from Whatman Nº. 1 filter paper (1.0 x 6.0 cm) hydrolysis in 50 mM sodium citrate buffer (pH 5.0) at 50 ºC during 60 minutes [18]. Carboxymethylcellulase CMCase) was assayed by measuring the reducing sugars released from the reaction mixture containing 0.5 mL of enzyme and 0.5 mL of 1.0% (w/v) of CMC solution in 50 mM sodium citrate buffer (pH 5.0) at 50 ºC during 60 minutes [18]. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method. One unit of enzymatic activity

was defined as the amount of enzyme that released 1 µmol of the hydrolysis product from the respective substrate, per minute per mL under the assay conditions [18]. Cellobiase activity was assayed by measuring the glucose released from the reaction mixture containing 0.5 mL of enzyme and 0.5 mL of a cellobiose solution (1% w/v) in 50 mM sodium citrate buffer (pH 5.0) at 50 ºC during 60 min. The mixture was boiled for 15 minutes to stop the reaction by the enzyme denaturation [18]. For the cellobiase assay, the glucose concentration was quantified by High Performance Liquid Chromatography (HPLC) as described in the section 2.4. The DNS method is not suitable to quantify cellobiase activity because celobiose is a reducing sugar.

The assays of enzymatic hydrolysis were performed with a blend of commercial enzymes: Cellic® CTec2 (30 FPU/ g of biomass) and Novozyme 188 (15 CBU/ g of biomass). The assays were performed in 100 mL Erlenmeyer's flasks with caps. The reaction medium (40 mL) was composed of sodium citrate buffer (50 mM, pH 5.0 containg sodium azide 0.1% w/v)), the enzymes and 5% (w/v) of biomass. The assays were done in triplicate and carried out in a rotatory shaker (Model SL-223/ SOLAB®) at 200 rpm, 50 °C for 96 hours [19]. The samples (1.0 mL of each Erlenmeyer) were collected every 24 hours and were kept in boiling water for 5 min to inactivate the enzymes. Then, the samples were centrifuged at 12000 *g* for 20 minutes to separate the insoluble fractions. The soluble fraction was filtered through a 0.45 µm filter (cellulose acetate ester) and the concentrations of cellobiose, glucose, xylose, arabinose present in the samples were measured by HPLC as described in section 3.2.4 The enzymatic hydrolysis yield (%) was calculated according to the Equation. (5):

$$Enzymatic\ hydrolysis\ Yield\ (\%) = \left(\frac{[glucose]+1.05\,[cellobiose]}{1.11\,f\,[biomass]}\right) * 100 \qquad (5)$$

where [glucose] is the glucose concentration (g/L), [cellobiose] is the cellobiose concentration (g/L), [biomass] is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L), f is the cellulose fraction in dry biomass (g/g), 1.05 is the factor that converts cellobiose to equivalent glucose and 1.11 is the factor that converts cellulose to equivalent glucose. An assay of enzymatic hydrolysis with the untreated chestnut shell was also conducted.

4.3.4 Analytical methods

The chemical composition of the untreated and pretreated chestnut shell residue was determined according to the analytical protocols described by National Renewable Energy Laboratory (NREL) (U.S Department of Energy).

The content of lignin and sugars was determined quantifying the acid hydrolysis products carried out in a two-step biomass fractionation. The first step consisted of the sulfuric acid (72% v/v) hydrolysis at 30 ºC during 1 hour. In the second step, the mixture was diluted with water to an acid concentration of 4.0% (v/v), and it was autoclaved at 120 ºC for 60 minutes. Afterwards, the solid fraction containing the insoluble acid lignin (Klason lignin) was separated from the liquid fraction containing sugars and the soluble acid lignin through vacuum filtration (using a Buchner funnel with a sintered glass porous plate nº 2). The Klason lignin was determined gravimetrically, and the soluble acid lignin was determined using a UV-Visible spectrophotometer at 215 nm and 280 nm. The concentrations of cellobiose, glucose, xylose, arabinose were measured by HPLC (SLUITER et al., 2011) using an Agilent 1260 infinity system (Wilmington, Delaware, EUA) equipped with a Refractive Index (IR) at 35ºC and a UV-visible detector (285 nm). Separation was achieved using an Aminex HPX-87H (Bio-Rad Laboratories Inc., America) column at 60 ºC. The mobile phase was 5 mmol/L sulfuric acid, and the flow rate was 0.7 mL/min.

The extractives, which represents the compounds that can be extracted from the treated solid, were determined through a two-step extraction. The first extraction was done with distilled water and the second with ethanol 95% (v/v), both during 6 hours at 80 ºC without agitation using an automated Soxhlet extractor (Soxtec™ 8000). The extractives mass was quantified by gravimetry. The extractives include the inorganic materials (e.g. soil or fertilizers associated with the biomass), nonstructural sugars, nitrogenous material, chlorophyll and waxes (SLUITER et al., 2008a). The ash content was determined through dry oxidation of the biomass in a muffle at 575 ºC for 4 hours. The ash mass was quantified by gravimetry (SLUITER et al., 2008b).

The concentration of cellobiose, glucose, xylose, arabinose released from the enzymatic hydrolysis was measured by HPLC (Agilent 1260 infinity, Wilmington, Delaware, EUA) using the conditions previously described.

4.3.5 The morphology of biomass

The morphology of the untreated and treated chestnut shell were visualized using a Scanning Electron Microscope - SEM (Quanta 450 FEG – FEI equipment). Prior to analysis, the samples were freeze-dried and fixed in a stub. Then, the samples were coated with a thin layer of gold by vacuum metallization (Quorum QT150ES).

4.3.6 Statistics analysis

All experiments were carried out in triplicate. Results were expressed as Mean ± SD. The Tukey's test was applied using the software Statistica 13.0 (StatSoft) at 95% of confidence level.

4.4 Results and Discussion

4.4.1 Raw material composition

The composition of chestnut shell determined by acid hydrolysis (NREL methodology) was 27.3% (w/w) of cellulose, 43.6% (w/w) of lignin, 12.6% (w/w) of hemicellulose, 16.3% (w/w) of extractives and 0.9% (w/w) of ash. The lignocellulosic materials can be used as raw material in various bioprocesses (WANG et al., 2017), the lignin, for example, is the most abundant fraction in these materials and can be used for the production of chemicals such as aromatic polymers (YOO et al., 2017). On the other hand, the cellulose, after acid or enzymatic hydrolysis can be used in the production of biofuels or to produce several chemicals (LEE et al., 2016). Aside from the high content of lignin, chestnut shell showed a higher amount of water soluble pigments compared to other residues such as sugarcane with 1.5% (w/w) of extractives (SLUITER et al., 2008a). Thus, the extractives can be used for instance as natural colorants in the textile industry (FANG; ZHAO; SONG, 2010). Zhao; Feng; Wang, (2014) developed a flax fabric colored with a colorant extracted from the chestnut shell.

4.4.2 Alkaline pretreatment of the chestnut shell

The composition of untreated and pretreated chestnut shell is summarized in Table 6. The total mass recovery (TMR) after the alkaline pretreatment (different NaOH concentrations and temperature) ranged from 15.3% to 34.3% (Table 6). The smallest TMR values were observed when at temperature and alkali concentration were larger, because, in these conditions more lignin was removed from the material. At 60 º, the effect of NaOH concentration about the removal of lignin was more evident than the effect of temperature. This because, the alkali acts mainly on the esters bonds between the polysaccharides and lignin (YOUNG et al., 2017).

According to the results presented in Table 6, all samples after the treatments showed an increased content of cellulose and hemicellulose. This occurred due to the removal of lignin and extractives during the treatment. About 80% of the composition of the treated material was found to be cellulose and hemicellulose because of the dissolution of a high amount of lignin in all assays and the high content of natural extractives in the raw material. The treatment that results in the best cellulose and hemicellulose recovery is the desired one. The lignin solubilized in the treatment remained in the liquid fraction (liquor) and can be separated from the solution (hydrolysate) by several methods as acidification (pH adjustment with acid), filtration with membranes and electrocoagulation (WANG et al., 2017) and used for other purposes. Gogoi and Hazarika (GOGOI; HAZARIKA, 2017) studied the pretreatment of rice straw with ionic liquids. To recover the lignin solubilized in the ionic liquids, the authors used 0.1 N H_2SO_4 to reduce the solubility of lignin in the medium. Wang et al. (WANG et al., 2017) studied the fermentation of alkaline hydrolysate of sugarcane bagasse, and acetic acid (the amount required quantity reach pH 4.8) was used for the lignin recovery.

Table 6. Composition of the solid fraction of the pretreated chestnut shell (g/100 g of pretreated solid, dry matter) and total mass recovery (TMR, %). The results correspond to the average of triplicate experiments ± S.D.

NaOH (%, w/v)/	Cellulose (%)		Hemicellulose (%)		Lignin (%)	
Temp. (ºC)/TMR (%)	Content	Recovery	Content	Recovery	Content	Solubilization
Untreated	27.3 ± 0.1a	100	12.6 ± 0.7a	100	43.6 ± 3.9a	0
1 (1.0, 60, 38.3)	49.1 ± 0.9b	68.9	20.6 ± 0.8bc	62.6	27.8 ± 1.0b	75.6
2 (2.0, 60, 34.7)	54.5 ± 0.1c	69.3	24.2 ± 0.2d	66.6	18.8 ± 0.1cd	85.0
3 (1.0, 80, 32.8)	57.2 ± 0.7c	68.7	21.1 ± 0.5b	54.9	21.4± 0.2c	83.9
4 (2.0, 80, 29.5)	55.5 ± 0.9c	60.0	22.2 ± 0.1bd	52.0	19.3 ± 0.1cd	87.0
5 (1.0, 100, 24.3)	62.7 ± 1.6f	55.8	21.4 ± 0.9b	41.3	15.5 ± 0.0d	91.4
6 (2.0, 100, 15.3)	63.6 ± 2.4f	42.9	18.4 ± 1.2c	22.3	16.2 ± 0.1d	94.3

All the pretreatments were conducted for 60 minutes with agitation in a stirred-heating plate. The percentages of cellulose, hemicellulose, and lignin in the treated material were expressed as g of each per 100 g of treated material. The percentages of cellulose and hemicellulose recovered after the treatment and the percentage of solubilized lignin in each treatment were expressed as a percentage of the untreated biomass, as described in section 2.5. Different letters in the same column indicate significant differences in the values of averages ($p<0.05$) by Tukey's test.

Table 6 also shows the percentages of cellulose and hemicellulose recovered in the solid fraction after the treatments, as well as the solubilized lignin (all based on the raw material weight). At 80 ºC and 100 ºC, the increase in NaOH concentration resulted in a slight increase on the solubilized lignin (3.1% and 1.9%, respectively). At 60 ºC, this effect was more pronounced with an increase of 10% on the solubilized lignin. Thus, the temperature imparted a higher effect on the lignin solubilization than the NaOH concentration, as can be observed in the assays carried out with 1.0% (w/v) of NaOH.

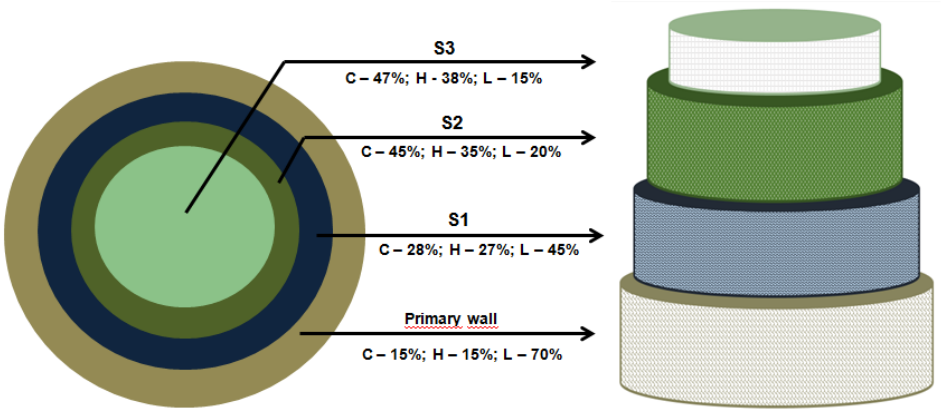
The most aggressive treatment condition among the tested ones (2.0% of NaOH and 100 ºC) was able to provide a material with 63.6% of cellulose (w/w, grams of cellulose by 100 grams of pretreated biomass), 18.4% (w/w) of hemicellulose and 16.2% (w/w) of lignin. Nonetheless, in this condition, a great loss of cellulose (58.8%) in relation of cellulose content in the untreated biomass was also observed. On the other hand, for the treatment carried out at 100 ºC and 1.0% of NaOH, the cellulose loss was 36.2% with 91.4 % of lignin solubilization. An ideal delignification treatment should be able to remove the largest content of lignin preserving the cellulose in the solid fraction. Thus, the best conditions are the treatments 1 to 3, where high amounts of lignin were solubilized with a good preservation of the cellulose and hemicellulose. Those conditions are also the ones exhibiting the higher biomass recovery.

Maurelli et al. (2013) studied the pretreatment of the chestnut shell from the Avelino region in Italy (untreated chestnut shell with 28.4% of cellulose, 7.9% of hemicellulose and 41.7% of lignin). In the treatment with 10% of NaOH at 70 ºC for 22 hours, 75.5% of lignin was removed from the raw material. The chestnut shell used in the present work and the reported by Maurelli et al. (2013), present a similar composition regarding the cellulose and lignin content. In the present work, when the delignification was performed at 100 ºC, the lignin removal was similar to the one reported by those authors (MAURELLI et al., 2013), but using a NaOH concentration ten times lower (1 % w/v) and with only one hour of processing. The use of less alkali in shorter processing time clearly affects the total processing costs. He et al. (2016) studied the pretreatment of the chestnut shell from Liuan (China) (Untreated chestnut shell with 28.1% of cellulose, 16.7% of hemicellulose and 23.2% of) with ethylene glycol-$HCIO_4$ followed by delignification with NaOH (1.0 % of NaOH at 90 ºC during 1 hour). After the combined treatment, the authors achieved a delignification of 77.5% of the raw material, a similar value to the one obtained in our work using a single step

treatment. Carvalho et al., 2015) studied the pretreatment of eucalyptus at 175 ºC with 15% of NaOH. Under these conditions, 51% of lignin was removed. Several authors studied the delignification of sugarcane bagasse with solubilization of 63.9%, 86.7% and 90.0% of lignin at the following pretreatment conditions: 0.5% of NaOH, 121 ºC and 60 minutes (NARRA; JAMES; BALASUBRAMANIAN, 2015); 1.5% of NaOH, 100 ºC and 60 minutes (OLIVEIRA et al., 2013); and 15% of NaOH, 175 ºC and 90 minutes (CARVALHO et al., 2015). Liu et al. (LIU et al., 2016) studied the pretreatment of corn stover and reported that 78.7% of lignin was solubilized with 12% of NaOH at 140 ºC during 30 minutes. The same amount of lignin was removed with only 0.5% NaOH at 120ºC in rice straw (NARRA; JAMES; BALASUBRAMANIAN, 2015). Based on the abovementioned results, it is possible to conclude that there is not a single pretreatment condition that is equally interesting for all residues. The combination of temperature, time and alkali concentration greatly depends on the type and the composition of the lignocellulosic material under study. It is well known, for example, that syringyl lignin is easier to break than guaiacyl lignin (SANTOS et al., 2011). Comparing the delignification of chestnut shell with others residues, the lignin of this material could be easily removed at mild temperature, and using a low alkali concentration enabled to remove up to 91% of the lignin of the original material.

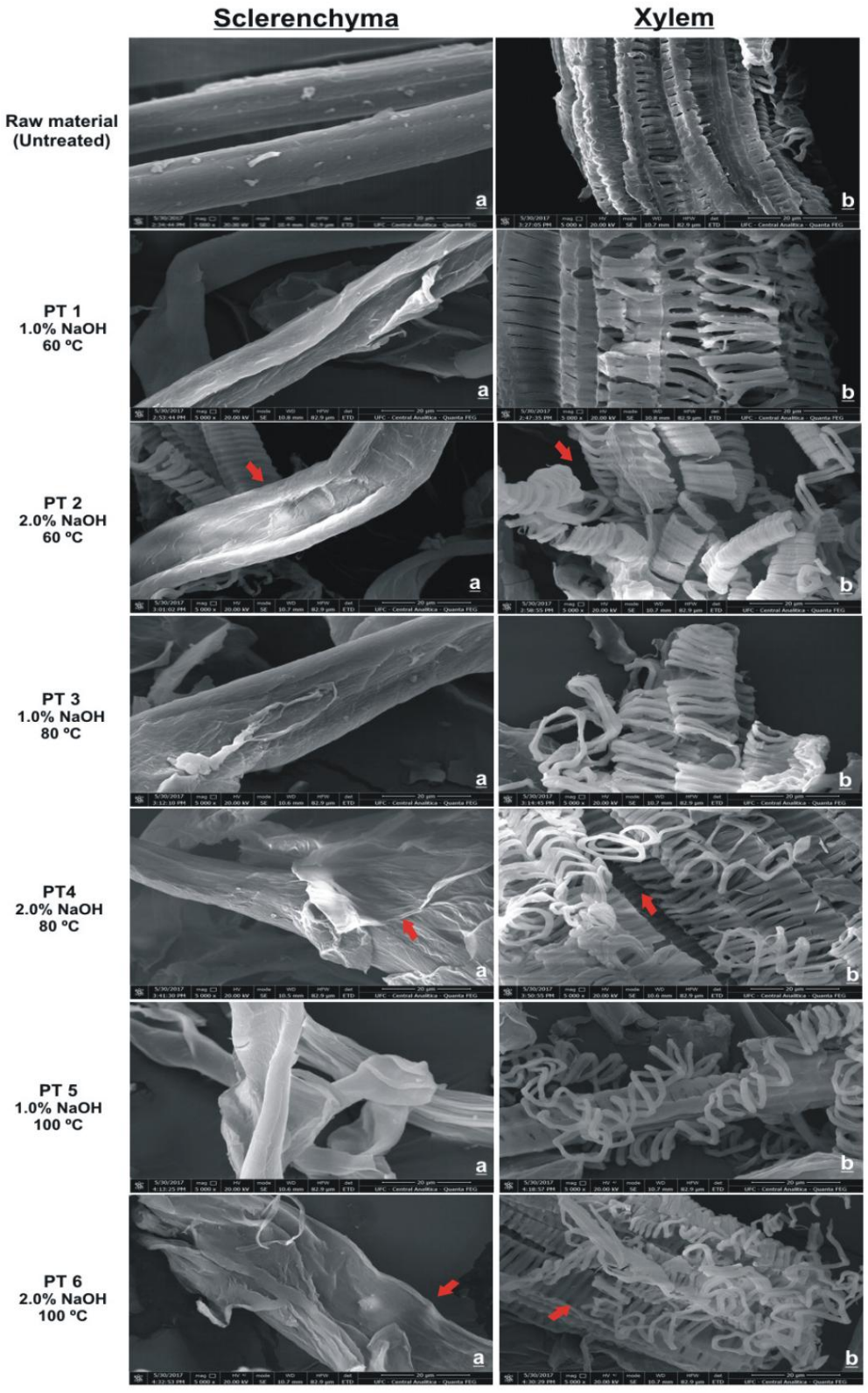
The cell wall of plants is composed of cellulose, hemicellulose and lignin in various layers that overlap one another. It contains a primary wall and three layers of the secondary cell wall (outer layer - S1, middle layer - S2, inner layer - S3). The content of each one of the components in cell wall of wood was showed in Figure 12. The greatest content of lignin is present in the primary layer, and the secondary layers show more cellulose and hemicellulose (GIBSON, 2012b). Normaly, the plants are divided in several tissues as parenchyma, collenchyma, xylem, sclerenchyma. The parenchyma shows higher content of cellulose and collenchyma, higher content of pectin and hemicellulose. However, the proportions are variables in accordance with specie (FAHN, 1967). The sclerenchyma and xylem are tissues with the primary and secondary wall almost always lignified (GIBSON, 2012b).

Figure 12. The cell wall of plants with the primary and the three secondary layers (outer layer - S1, middle layer - S2, inner layer - S3). The average content of Cellulose (C), Hemicellulose and Lignin (L) in each layer was described in figure



The structure of the chestnut shell prior and after the alkali pretreatment was visualized by electronic microscopy SEM (Figure 13). Since most of the lignin is found in sclerenchyma and xylem, these tissues were observed through MEV in order to identify possible changes occurred in its structure due to pretreatment. The sclerenchyma of the untreated chestnut shell exhibited long smooth and cylindrical filaments (Figure 13- UNTa). After the treatment with NaOH, the bundles of Sclerenchyma lost their cylindrical structure highly compact (Figure 13-1a, 2a, 3a, 4a, 5a, and 6a). It is possible to see the occurrence of significant damages in their structure that seems to have exploded in some areas. The damages in the structure increase with the increase of the treatment temperature as indicated by the arrows (Figure 13- PT 2a, PT 4a and PT 6a). The xylem in the untreated biomass showed walls with intact helical thickening. After the pretreatment with NaOH it was possible to observe the occurrence of damages in the xylem structure, which intensified as the temperature treatment increased (arrows in Figure 13- PT 2b, PT 4b and PT 6b). These results suggest that the temperature of the treatment had more impact than the alkali concentration on the biomass structure. These results corroborate the quantitative chemical analysis previously discussed (Table 1).

Figure 13. SEM images of chestnut shell before and after the pretreatments. Magnification of Sclerenchyma and xylem 5000X. Subtitle: UNT – untreated chestnut shell; 1, 3 and 5 – pretreatments with 1.0% of NaOH and 60 ºC, 80ºC and 100 ºC, respectively; 2, 4 and 6 – pretreatments with 2.0% of NaOH and 60 ºC, 80 ºC and 100 ºC, respectively.



4.4.3 Characterization of enzymes

The enzyme activiy (FPase, Carboxymethylcellulase, cellobiase and xylanase) quantified for the enzymes Cellic® CTec2 and Novozyme 188 are presented in Table 7. The FPase and xylanase activities were found in Cellic® CTec2. On the other hand, the Novozyme 188 practically did not present FPase activity but it was found to be rich in cellobiase, the enzyme responsible for the cellobiose breakdown in two glucose molecules. Therefore, the enzymes complemented each other and have to be used together.

Table 7. Characterization of the enzymes Cellic® CTec2 and Novozyme 188 regarding their activity when exposed to different substrates. The results correspond to the average of triplicate experiments ± S.D.

Enzymes	FPase (FPU/ mL)	Cellobiase (UI/ mL)	CMCase (UI/ mL)	Xylanase (UI/ mL)
Cellic® CTec2	362.1 ± 9.1a	1.4 ± 0.4a	38.9 ± 0.9a	137.9 ± 5.6
Novozyme 188	3.8 ± 0.2b	28.2 ± 0.9b	2.6 ± 0.0b	Not activity

Different letters in the same column indicate significant differences in the values of averages ($p<0.05$) by Tukey's test.

4.4.4 Enzymatic hydrolysis

The commercial enzymes used in this work were not able to hydrolyze the cellulose from the untreated chestnut shell, as the demonstrated by the small amount of glucose quantified after 72 hours of enzymatic hydrolysis (2.3 g/L) (Table 8). This result was expected given the high amount of lignin present in this material (43.6%). The lignin acts as a physical barrier in the biomass limiting the enzymes access to the cellulose. The decrease of lignin content, in addition to the improvement of cellulose accessibility, also reduces the occurrence of nonspecific enzyme bindings with the lignin (TANG et al., 2017), thus favoring the hydrolysis as seen in the results obtained with the treated material. The hydrolysis of the untreated biomass released only 1.3 g/L of xylose, while 11.4 g/L of this sugar was obtained from the hydrolysis of biomass treated with 2.0% of NaOH at 60 ºC. Cellic® CTec2 showed the highest activity of xylanase (Table 7). Thus, xylose was also found in all hydrolysates.

The low values of cellobiose after 72 hours of hydrolysis (Table 8) are due to the presence of the enzyme Novozyme 188, which has a high cellobiase activity. Because, the accumulation of cellobiose would cause action inhibition of the Cellic®

CTec2. The hydrolysis results emphasize the importance of taking into account the saccharification yield along with the pretreatment as a criterion to choose the best processing conditions. The enzymatic hydrolysis of the chestnut shell samples pretreated with alkali released between 22.8 to 27.6g/L of glucose after 72 hours (Table 8). No significant differences were observed on the glucose yield obtained within the saccharification of treated samples (Tukey test at 95% of confidence). The results showed that the conditions tested in PT1 (smoothest condition) and PT6 (more aggressive treatment condition) led to similar hydrolysis outcomes. The glucose content and the saccharification yield in the hydroslysates showed that the enzymatic hydrolysis is not only influenced by the lignin content of the biomass. The acetyl groups of the hemicellulose can inhibit the hydrogen bridges between the cellulose and the catalytic domain of cellulases, consequently affecting negatively the action of the enzymes (YAN et al., 2015). As presented in Table 3, the treatment that removes more lignin (PT6) does not necessarily leads to the best enzymatic biomass saccharification.

Although, the conditions of the treatments PT5 and PT6 (both carried out at 100 ºC) led to higher glucose concentrations, according to the Tukey's test the glucose yields of these treatments are not statistically ($p<0.05$) different from the one obtained in the treatment PT1. The temperature in PT1 was 60 ºC, therefore the energy requirements is lower when compared to the assays conducted at 100 ºC. Hence, based on the saccharification yield and in the energy requirements, the condition selected to treat chestnut shell was 1.0% of NaOH at 60 ºC for 1 hour (pretreatment PT1), which leads to the highest saccharification yield.

Table 8. Glucose concentration and saccharification yield after 72 hours of enzymatic hydrolysis of chestnut shell. The results correspond to the average of triplicate experiments ± S.D.

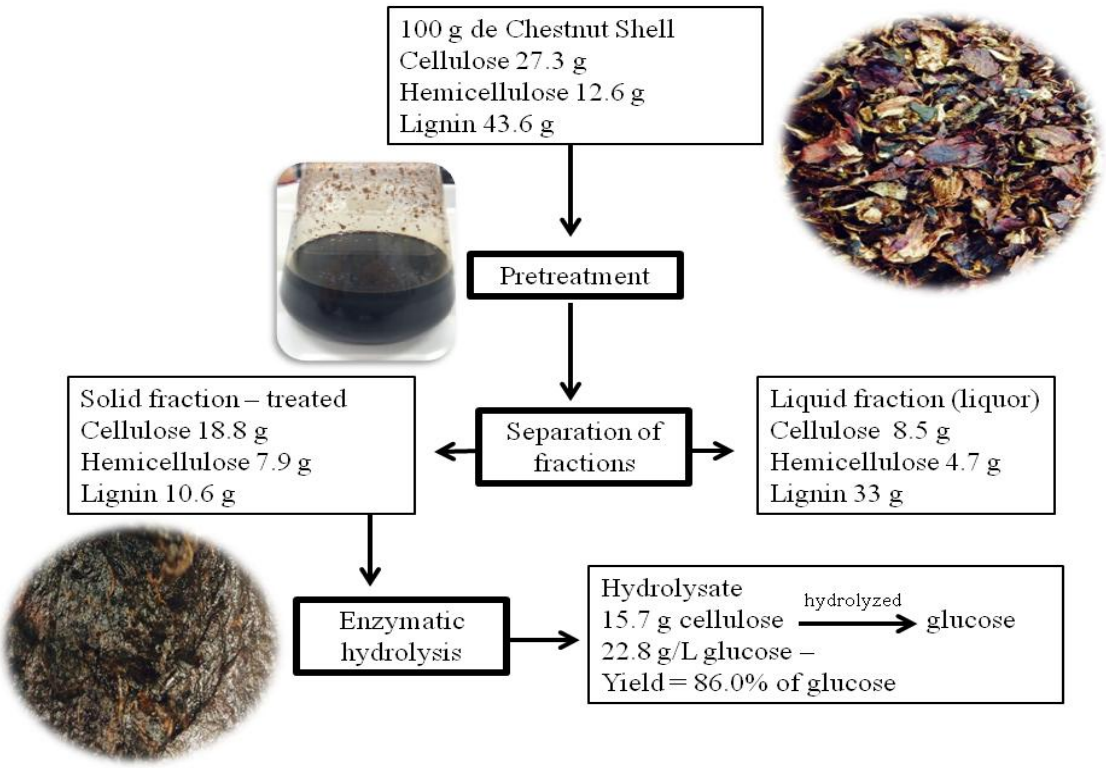
Pretreatments Conditions (Temp./ Alkali conc.)	Concentration (g/L)			Glucose yield (%)
	Xylose	Cellobiose	Glucose	
Untreated (UNT)	1.3 ± 0.4a	0.4 ± 0.1ac	2.3 ± 0.8a	17.9 ± 0.3a
1 (60ºC/ 1.0%)	8.3 ± 0.1b	0.6 ± 0.1ab	22.8 ± 0.2b	86.0 ± 0.7b
2 (60ºC/ 2.0%)	11.4 ± 1.1c	0.6 ± 0.1ab	23.7 ± 0.9b	80.3 ± 4.1b
3 (80ºC/ 1.0%)	8.0 ± 0.4b	0.1 ± 0.0c	26.7 ± 0.6c	84.0 ± 2.6b
4 (80ºC/ 2.0%)	8.7 ± 0.9b	0.4 ± 0.2abc	24.6 ± 0.5b	81.1 ± 2.3b
5 (100ºC/ 1.0%)	6.6 ± 0.3b	0.7 ± 0.3ab	27.6 ± 0.7c	81.5 ± 1.6b
6 (100ºC/ 2.0%)	6.8 ± 0.6b	0.8 ± 0.1b	27.6 ± 1.0c	80.6 ± 3.8b

Different letters in the same column indicate significant differences in the values of averages ($p<0.05$) by Tukey's test.

Liu et al. (2016) studied the delignification of corn stover with 12% of NaOH (140 ºC, 20 min) and submitted the treated material to an enzymatic hydrolysis with a mixture of Celluclast 1.5 L and Novozyme 188 reporting a glucose yield of 71.9%. Oliveira et al. (2013) assessed the combination of the sugarcane straw pretreatment by steam explosion followed by delignification with NaOH (1.5%, 100 °C, 60 minutes). The treated material was submitted to enzymatic hydrolysis with the same enzymes previous mentioned and the glucose yield obtained was 85.1%. YOO et al. (2017) studied the pretreatment of canola straw with NaOH through the experimental design. The higher glucose yield (93.9%) from enzymatic hydrolysis with Celluclast e cellobiase occured with treated canola straw with 15% NaOH, 80 ºC during 24 hours. In this work, with NaOH concentration 14 times less than that and only 1 hour of process was possible get 86% of glucose in the enzymatic hydrolysis. Maurelli et al. (2013) studied several pretreatments of the chestnut shell and the treated samples were also submitted to enzymatic hydrolysis. The hydrolysis yields of the chestnut shell treated with NaOH (10% of NaOH at 70 ºC and 22 hours) were 67.8% and 53.6% for the cocktail 1 (Accellerase®1500, 6.7; Accellerase®XY, 196; Accellerase®BG, 358) and cocktail 2 (Cellic®CTec, 6.7; Cellic®HTec, 196; Accellerase BG, 358), respectively. These yields were lower than those found in our work, in which the treatment was faster (60 minutes), it was carried out at lower temperature (60 ^{o}C) and it is less aggressive regarding the alkali concentration (1%). Although the enzymes used in the previous work may differ from the ones herein used, it is important to note that the glucose yields obtained in our work were higher. The enzymatic hydrolysis of the material pretreated with an alkali concentration ten times lower (1.0%) at 60 ºC led to a glucose yield of 86.0%.

As shown in Figure 14, 18.8 g of cellulose, 7.9 g of hemicellulose and 10.6 g of lignin were obtained after the chestnut shell treatment (1.0% of NaOH, 60 ºC, 1 hour). After enzymatic hydrolysis, 15.7 g of cellulose was hydrolyzed in glucose, which represents a glucose yield of 86.0%.

Figure 14. Mass Balance of the selected pretreatment (condition 1 – 1.0% NaOH and 60 ºC) and the enzymatic hydrolysis of chestnut shell.



4.5 Conclusions

The alkaline pretreatment of the chestnut shell was efficient in the all conditions tested, even for the mildest condition (60 ºC and 1.0% NaOH) it was possible to remove 75.0% of lignin. The treatments carried out at 100 ºC with 1.0% and 2.0% of NaOH were the ones that removed the higher amounts of lignin 91.4% and 94.3%. Under these conditions, it was possible to obtain a material with 62.7% and 63.6% of cellulose, respectively. The increase of temperature was more effective on the lignin removal compared to the increase of NaOH concentration. The results obtained by the traditional methodology recommended by the NREL were corroborated by the microscopy analysis, i.e. the changes on the biomass structure due to the pretreatments were clearly visualized by SEM. The results of enzymatic hydrolysis showed that not necessarily the condition that promotes the highest lignin removal results in the best hydrolysis yield. Regarding the energy requirements and the saccharification yield, the pretreatment carried out with 1.0% of NaOH at 60 ºC for 1 hour was found to be the best one exhibiting the highest yield (86.0%). In the present work, a

mild pretreatment carried out with ten time less alkali at lower temperature for only one hour, was found to be effective for the chesnut shell saccharification.

4.6. Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit, COMPETE 2020 (POCI-01-0145-FEDER-006684) and the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462). The authors also acknowledge financial support from BioTecNorte operation (NORTE-01-0145-FEDER-000004) and the project MultiBiorefinery (POCI-01-0145-FEDER-016403) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. E. J. Gudiña was supported by the Post-Doctoral grant CEB-BPD/01/2015/07 from the project UID/BIO/04469/2013, funded by FCT. Additionally, the authors acknowledge CAPES for financial support. The authors would like to thank the Analytical Central (Physical Department) of the Federal University of Ceará that conducted the microscopic analysis.

4.6 Rererências

ALVIRA, P.; TOMÁS-PEJÓ, E.; BALLESTEROS, M.; NEGRO, M. J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. **Bioresource Technology**, v. 101, n. 13, p. 4851–4861, 2010.

BALI, G.; MENG, X.; DENEFF, J. I.; SUN, Q.; RAGAUSKAS, A. J. The effect of alkaline pretreatment methods on cellulose structure and accessibility. **ChemSusChem**, v. 8, n. 2, p. 275–279, 2015.

BANERJEE, D.; MUKHERJEE, S.; PAL, S.; KHOWALA, S. Enhanced saccharification efficiency of lignocellulosic biomass of mustard stalk and straw by salt pretreatment. **Industrial Crops and Products**, v. 80, p 42–49, 2016.

BANSAL, N.; TEWARI, R.; SONI, R.; SONI, S. K. Production of cellulases from Aspergillus niger NS-2 in solid state fermentation on agricultural and kitchen waste residues. **Waste Management**, v. 32, n. 7, p. 1341–1346, 2012.

BEHERA, S. S.; RAY, R. C. Solid State Fermentation for Production of Microbial Cellulases: Recent Advances and Improvement Strategies. **International Journal of Biological Macromolecules**, v. 86, p. 656–669, 2016.

BIAGINI, E.; BARONTINI, F.; TOGNOTTI, L. Gasification of agricultural residues in a demonstrative plant: Vine pruning and rice husks. **Bioresource Technology**, v. 194, p. 36–42, 2015.

BILAL, M.; ASGHER, M.; IQBAL, H. M. N.; HU, H,; ZHANG, X. Biotransformation of lignocellulosic materials into value-added products − A review. **International Journal of Biological Macromolecules**, v. 98, p. 447–458, 2017.

BRITO, P. S. D.; OLIVEIRA, A. S.; RODRIGUES, L. F. Energy valorization of solid vines pruning by thermal gasification in a pilot plant. **Waste and Biomass Valorization**, v. 5, n. 2, p. 181–187, 2014.

BUSTOS, G.; MOLDES, A. B.; CRUZ, J. M.; DOMINGUEZ, J. M. Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by Lactobacillus pentosus. **Journal of the Science of Food and Agriculture**, v. 84, n. 15, p. 2105–2112, 2004.

CANILHA, L.;CHANDEL, A. K.; MILESSI, T. S. S.; ANTUNES, F. A. F.; FREITAS, W. L. C.; FELIPE, M. G. A.; SILVA, S. S. Bioconversion of sugarcane biomass into ethanol: An overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. **Journal of Biomedicine and Biotechnology**, v. 2012, p. 1-15; 2012.

CARA, C.; RUIZ, E.; OLIVE, J. M.; SAEZ, F; CASTRO, E. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. **Bioresource Technology**, v. 99, n. 6, p. 1869–1876, 2008.

CUEVAS, M.; GARCÍA, J. F.; SÁNCHEZ, S. Enhanced enzymatic hydrolysis of pretreated almond-tree prunings for sugar production. **Carbohydrate Polymers**, v. 99, p. 791–799, 2014.

CERDA, A.; GEA, T.; VERGAS-GARCIA, M. C.; SANCHEZ, A. Towards a competitive solid state fermentation: Cellulases production from coffee husk by sequential batch operation and role of microbial diversity. **Science of The Total Environment**, v. 589, p. 56–65, 2017.

CHEN, W. H.; TU, Y. J.; SHEEN, H. K. Disruption of sugarcane bagasse lignocellulosic structure by means of dilute sulfuric acid pretreatment with microwave-assisted heating. **Applied Energy**, v. 88, n. 8, p. 2726–2734, 2011.

CHEN, Z.; WAN, C. Biological valorization strategies for converting lignin into fuels and chemicals. **Renewable and Sustainable Energy Reviews**, v. 73, n. March 2016, p. 610–621, 2017.

COBAS, M.; MEIJIDE, J.; SANROMAN, M. A.; PAZOS, M. Chestnut shells to mitigate pesticide contamination. **Journal of the Taiwan Institute of Chemical Engineers**, v. 61, p. 166–173, 2016.

CARVALHO, D. M.; SEVASTYANOVA, O.; PENNA, L. S.; SILVA, B. P.; LINDSTROM, M. E.; COLODETTE, J. L. Assessment of chemical transformations in

eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid, and alkaline pretreatments. **Industrial Crops and Products**, v. 73, p. 118–126, 2015.

CASTRO, A. M.; PEREIRA, N. Produção, propriedades e aplicação de celulases na hidrólise de resíduos agroindustriais. **Quimica Nova**, v. 33, n. 1, p. 181–188, 2010.

SANTOS, T. C.; GOMES, D. P. P.; BONOMO, R. C. F.; FRANCO, M. Optimisation of solid state fermentation of potato peel for the production of cellulolytic enzymes. **Food Chemistry**, v. 133, n. 4, p. 1299–1304, 2012.

DOWE, N.; NREL, J. M. SSF Experimental Protocols — Lignocellulosic Biomass Hydrolysis and Fermentation Laboratory Analytical Procedure (LAP) Issue Date : 10 / 30 / 2001 SSF Experimental Protocols — Lignocellulosic Biomass Hydrolysis and Fermentation Laboratory Analytical Pro. **National Renewable Energy Laboratory**, v. NREL/TP-51, n. 10/20/2001, 2008.

FAHN, A. **Plant Anatomy**. [s.l: s.n.].

FANG, H.; ZHAO, C.; SONG, X.-Y. Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: response surface methodology or using cellulase from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger NL02. **Bioresource Technology**, v. 101, n. 11, p. 4111–9, jun. 2010.

FENG, Q.; LIN, Y. Integrated processes of anaerobic digestion and pyrolysis for higher bioenergy recovery from lignocellulosic biomass: A brief review. **Renewable and Sustainable Energy Reviews**, n. November 2016, p. 1–16, 2017.

FERREIRA, S.; DUARTE, A. P.; RIBEIRO, M. H. L.; QUEIROZ, J. A.; DOMINGUES, F. C. Response surface optimization of enzymatic hydrolysis of Cistus ladanifer and Cytisus striatus for bioethanol production. **Biochemical Engineering Journal**, v. 45, n. 3, p. 192–200, 2009.

GIBSON, L. J. The hierarchical structure and mechanics of plant materials. **Journal of The Royal Society Interface**, 2012.

GOGOI, G.; HAZARIKA, S. Coupling of ionic liquid treatment and membrane filtration for recovery of lignin from lignocellulosic biomass. **Separation and Purification Technology**, 2017.

HAFID, H. S.; RAHMAN, N. A.; MOKHTAR, M. N.; TALIB, A. T. Over production of fermentable sugar for bioethanol production from carbohydrate-rich Malaysian food waste via sequential acid-enzymatic hydrolysis pretreatment. **Waste Management**, v. 67, p. 95–105, set. 2017.

HE, Y-C.; LIU, F.; DI, J-H.; DING, Y.; ZHU, Z-Z.; WU, Y-Q.; CHEN, L.; WANG, C.; XUE, Y-F.; CHONG, G-G.; MA, C-L. Effective enzymatic saccharification of dilute NaOH extraction of chestnut shell pretreated by acidified aqueous ethylene glycol media. **Industrial Crops and Products**, v. 81, p. 129–138, 2016.

IDRIS, A. S. O.; PANDEY, A.; RAO, S. S.; SUKUMARAN, R. K. Cellulase

production through solid-state tray fermentation, and its use for bioethanol from sorghum stover. **Bioresource Technology**, 2017.

JIANG, L-Q.; FANG, Z.; LI, X-K.; LUO, J.; FAN, S-P. Combination of dilute acid and ionic liquid pretreatments of sugarcane bagasse for glucose by enzymatic hydrolysis. **Process Biochemistry**, v. 48, n. 12, p. 1942–1946, 2013.

JÖNSSON, L. J.; MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource Technology**, v. 199, p. 103–112, 2016.

KOYAMA, M.; WATANABE, K.; KUROSAWA, N.; ISHIKAWA, K.; BAN, S.; TODA, T. Effect of alkaline pretreatment on mesophilic and thermophilic anaerobic digestion of a submerged macrophyte: inhibition and recovery against dissolved lignin during semi-continuous operation. **Bioresource Technology**, v. 238, p. 666–674, 2017.

KUMAR, L.; CHANDRA, R.; SADDLER, J. Can the same steam pretreatment conditions be used for most softwoods to achieve good, enzymatic hydrolysis and sugar yields. **Bioresource Technology**, v. 101, n. 20, p. 7827–7833, 2010.

LE FLOCH, A.; JOURDES, M.; TEISSEDRE, P. L. Polysaccharides and lignin from oak wood used in cooperage: Composition, interest, assays: A review. **Carbohydrate Research**, v. 417, p. 94–102, 2015.

LEE, N. K.; JUNG, B. S.; NA, D. S.; YU, H. H.; KIM, J-S.; PAIK, H-D. The impact of antimicrobial effect of chestnut inner shell extracts against Campylobacter jejuni in chicken meat. **LWT - Food Science and Technology**, v. 65, p. 746–750, 2016.

LIM, J. S.; MANAN, Z. A.; ALWI, S. R. W.; HASHIN, H. A review on utilisation of biomass from rice industry as a source of renewable energy. **Renewable and Sustainable Energy Reviews**, v. 16, n. 5, p. 3084–3094, 2012.

LI, Q.; SHI, X.; ZHAO, Q.; CUI, Y.; OUYANG, J.; XU, F. Effect of cooking methods on nutritional quality and volatile compounds of Chinese chestnut (Castanea mollissima Blume). **Food Chemistry**, v. 201, p. 80–86, 2016.

LI, Y.; ZHANG, X.; XIONG, L.; MEHMOOD, M. A.; ZHAO, X.; BAI, F. On-site cellulase production and efficient saccharification of corn stover em- ploying cbh2 overexpressing Trichoderma reesei with novel induction system. **Bioresource Technology**, v. 238, p. 643–649, 2017.

LIU, H., PANG, B.; ZHOU, J.; HAN, Y.; LU, J.; LI, H.; WANG, H.; Comparative study of pretreated corn stover for sugar production using cotton pulping black liquor (CPBL) instead of sodium hydroxide. **Industrial Crops and Products**, v. 84, p. 97–103, 2016.

MAEDA, R. N.; BARCELOS, C. A.; ANNA, L. M. M. S.; JUNIOR, N. P. Cellulase production by Penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. **Journal of Biotechnology**, v. 163, n. 1, p. 38–44, 10 jan. 2013.

MATEO, S.; PUENTES, J. G.; ROBERTO, I. C.; SANCHEZ, S.; MOYA, A. J. Optimization of acid hydrolysis of olive tree pruning residue . Fermentation with Candida guilliermondii. **Biomass and Bioenergy**, v. 69, p. 39-46, 2014.

MARTINS, G. M.; BOCCHINI-MARTINS, D. A.; BEZZERRA-BUSSOLI, C.; PAGNOCCA, F. C.; BOSCOLO, M.; MONTEIRO, D. A.; SILVA, R.; GOMES, E. The isolation of pentose-assimilating yeasts and their xylose fermentation potential. **Brazilian Journal of Microbiology**, p. 1–7, 2017.

MAURELLI, L.;IONATA, E.; CARA, F. L.; MORANA, A. Chestnut shell as unexploited source of fermentable sugars: Effect of different pretreatment methods on enzymatic saccharification. **Applied Biochemistry and Biotechnology**, v. 170, n. 5, p. 1104–1118, 2013.

MISHRA, V.; JANA, A. K.; JANA, M. J.; GUPTA, A. Bioresource Technology Enhancement in multiple lignolytic enzymes production for optimized lignin degradation and selectivity in fungal pretreatment of sweet sorghum bagasse. **Bioresource Technology**, v. 236, p. 49–59, 2017.

MILLER, S. A.; BROOM, F. D.;THORP, T. G.; BARNETT, A. M. Effects of leader pruning on vine architecture , productivity and fruit quality in kiwifruit (Actinidia deliciosa cv . Hayward). **Scientia Horticulturae** , v. 91, p. 189–199, 2001.

MOLDES, A. B.; BUSTOS, G.; TORRADO, A.; DOMINGUEZ, J. M. Comparison between different hydrolysis processes of vine-trimming waste to obtain hemicellulosic sugars for further lactic acid conversion. **Applied Biochemistry and Biotechnology**, v. 143, n. 3, p. 244–256, 2007.

MOOD, H. S; GOLFESHAN, A. H.; TABATATABAEI, M.; JOUZANI, G. S.; NAJAFI, G. H.; GHOLAMI, M.; ARDJMAND, M. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. **Renewable and Sustainable Energy Reviews**, v. 27, p. 77–93, 2013.

MOTA, M. I. F.; PINTO, P. C. R.; LOUREIRO, J. M.; RODRIGUES, A. E. Successful recovery and concentration of vanillin and syringaldehyde onto a polymeric adsorbent with ethanol/water solution. **Chemical Engineering Journal**, v. 294, p. 73–82, 2016.

NARRA, M.; JAMES, J. P.; BALASUBRAMANIAN, V. Simultaneous saccharification and fermentation of delignified lignocellulosic biomass at high solid loadings by a newly isolated thermotolerant Kluyveromyces sp. for ethanol production. **Bioresource Technology**, v. 179, p. 331–338, 2015.

NEVES, P. V.; PITARELO, A. P.; RAMOS, L. P. Production of cellulosic ethanol from sugarcane bagasse by steam explosion: Effect of extractives content, acid catalysis and different fermentation technologies. **Bioresource Technology**, v. 208, p. 184–194, 2016.

NTALOS, G. A.; GRIGORIOU, A. H. Characterization and utilisation of vine prunings as a wood substitute for particleboard production. **Industrial Crops and Products**, v.

16, n. 1, p. 59–68, 2002.

NYGAARD, I.; DEMBELE, F.; DAOU, I.; MARIKO, A.; KAMISSOKO, F.; COULIBALY, N.; BORGSTROM, R.; BRUUN, T. B. Lignocellulosic residues for production of electricity, biogas or second generation biofuel: A case study of technical and sustainable potential of rice straw in Mali. **Renewable and Sustainable Energy Reviews**, v. 61, p. 202–212, 2016

OLIVEIRA, F. M. V.; PINHEIRO, I. O.; SOUTO-MAIOR, A. M.; MARTIN, C.; GONÇALVES, A. R.; ROCHA, G. J. M. Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products. **Bioresource Technology**, v. 130, p. 168–173, 2013.

PATERAKI, C.; ALMQVIST, H.; LADAKIS, D.; LIDEN, G.; KOUTINAS, A. A.; VLYSIDIS, A. Modelling succinic acid fermentation using a xylose based substrate. **Biochemical Engineering Journal**, v. 114, p. 26–41, 2016.

PERCIVAL ZHANG, Y. H.; HIMMEL, M. E.; MIELENZ, J. R. Outlook for cellulase improvement: Screening and selection strategies. **Biotechnology Advances**, v. 24, n. 5, p. 452–481, 2006.

RABEMANOLONTSOA, H.; SAKA, S. Various pretreatments of lignocellulosics. **Bioresource Technology**, v. 199, p. 83–91, 2015.

ROCHA, G. J. M.; GONÇALVES, A. R.; NAKANISHI, S. C.; NASCIMENTO, V. M.; SILVA, V. F. N. Pilot scale steam explosion and diluted sulfuric acid pretreatments: Comparative study aiming the sugarcane bagasse saccharification. **Industrial Crops and Products**, v. 74, p. 810–816, 2015.

SANTOS, R. B.; CAPANEMA, E. A.; BALAKSHIN, M. Y.; CHANG, H-M.; JANEEL, H. Effect of hardwoods characteristics on kraft pulping process: Emphasis on lignin structure. **BioResources**, v. 6, n. 4, p. 3623–3637, 2011.

SARATALE, G. D.; JUNG, M.-Y.; OH, M.-K. Reutilization of green liquor chemicals for pretreatment of whole rice waste biomass and its application to 2,3-butanediol production. **Bioresource Technology**, v. 205, p. 90–96, 2016.

SARATALE, G. D.; SARATELE, R. G.; GHODAKE, G. S.; JIANG, Y-Y.; CHANG,J-S.; SHIN, H-S.; KUMAR, G. Characterization and its application in the saccharification of rice waste biomass for ethanol production : An integrated biotechnological approach. **Journal of the Taiwan Institute of Chemical Engineers**, v. 0, p. 1–8, 2017.

SARKAR, N.; GHOSH, S. K.; BANNERJEE, S.; AIKAT, K. Bioethanol production from agricultural wastes: An overview. **Renewable Energy**, v. 37, n. 1, p. 19–27, 2012.

SERVILLO, L.; GIOVANE, A.; CASALE, R.; BALESTRIERI, M. L.; CAUTELA, D.; PAOLUCCI, M.; SIANO, F.; VOLPE, M. G.; CASTALDO, D. Betaines and related ammonium compounds in chestnut (Castanea sativa Mill.). **Food Chemistry**, v. 196, p. 1301–1309, 2016.

SHAO, S.; ZHANG, J.; HOU, W.; QURESHI, A. S.; BAO, J. Lower pressure heating steam is practical for the distributed dry dilute sulfuric acid pretreatment. **Bioresource Technology**, v. 238, p. 744–748, 2017.

SLUITER, A.; HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D. Determination of Extractives in Biomass. **Biomass Analysis Technology Team Laboratory Analytical Procedure**, n. January, p. 1–6, 2008a.

SLUITER, A. HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D. Determination of Ash in Biomass. **Biomass Analysis Technology Team Laboratory Analytical Procedure**, n. January, p. 1–6, 2008b.

SLUITER, A.; HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D.; CROCKER, D. Determination of Structural Carbohydrates and Lignin in Biomass. **Biomass Analysis Technology Team Laboratory Analytical Procedure,** v. 2011, n. July, 2012.

SU, C-H.; CHUNG, M-H.; HSIEH, H-J.; CHANG, Y-K.; DING, J-C.; WU, H-M. Enzymatic hydrolysis of lignocellulosic biomass in ionic liquid media for fermentable sugar production. **Journal of the Taiwan Institute of Chemical Engineers**, v. 43, n. 4, p. 573–577, 2012.

SUN, S.; SUN, S.; CAO, X.; SUN, R. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. **Bioresource Technology**, v. 199, p. 49–58, 2015.

TALEBNIA, F.; KARAKASHEV, D.; ANGELIDAKI, I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. **Bioresource Technology**, v. 101, n. 13, p. 4744–4753, 2010.

TANG, C.; SHAN, J.; CHEN, Y.; ZHONG, L.; SHEN, T.; ZHU, C.; YING, H. Organic amine catalytic organosolv pretreatment of corn stover for enzymatic saccharification and high-quality lignin. **Bioresource Technology**, v. 232, p. 222–228, 2017.

T. K. GHOSE. Measurement of cellulase activities. **Pure and Applied Chemistry**, v. 59, n. 2, p. 257–268, 1987.

VAZ DE ARRUDA, P.; SANTOS, J.C.; RODRIGUES, R. C. L. B.; SILVA, D. D. V.; YAMAKAWA, C. K.; ROCHA, G. J. M.;JUNIOR, J. N.; PRADELLA, J. G. C.; ROSSELL, C. E. V.; FELIPE, M. G. A. Scale up of xylitol production from sugarcane bagasse hemicellulosic hydrolysate by Candida guilliermondii FTI 20037. **Journal of Industrial and Engineering Chemistry**, v. 47, p. 297–302, 2017.

VÁZQUEZ, G.; MOSQUERA, O.; FREIRE, M. S.; ANTORRENA, G.; GONZALEZ-ALVAREZ, J. Alkaline pre-treatment of waste chestnut shell from a food industry to enhance cadmium, copper, lead and zinc ions removal. **Chemical Engineering Journal**, v. 184, p. 147–155, 2012.

VECINO, X.; RODRIGUEZ-LOPEZ,L.; GUDINA, E. J.; CRUZ, J. M.; MOLDES, A. B.; RODRIGUES, L. R. Vineyard pruning waste as an alternative carbon source to produce novel biosurfactants by Lactobacillus paracasei. **Journal of Industrial and Engineering Chemistry**, v. 55, p. 40–49, 2017.

WANG, W.; CHEN, X.; TAN, X.; WANG, Q.; LIU, Y.; HE, M.; YU, Q.; QI, W.; LUO, Y.; ZHUANG, X.; YUAN, Z. Feasibility of reusing the black liquor for enzymatic hydrolysis and ethanol fermentation. **Bioresource Technology**, 2017.

XIN, D.; SUN, Z.; VIIKARI, L.; ZHANG, J. Role of hemicellulases in production of fermentable sugars from corn stover. **Industrial Crops and Products**, v. 74, p. 209–217, 2015.

YAN, Z.; LI, J.; CHANG, S.; CUI, T.; JIANG, Y.; YU, M.; ZHANG, L.; ZHAO, G.; QI, P.; LI, S. Lignin relocation contributed to the alkaline pretreatment efficiency of sweet sorghum bagasse. **Fuel**, v. 158, p. 152–158, 2015.

YANG, M.; ZHANG, J.; KUITTINEN, S.; VEPSALAINEN, J.; SOININEN, P.; KEINANEN, M.; PAPPINEN, A. Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone-butanol-ethanol fermentation. **Bioresource Technology**, v. 189, p. 131–137, 2015.

YOO, H. Y.; LEE, J. H.; KIM, D. S.; LEE, J. H.; LEE, S. K.; LEE, S. J.; PARK, C.; KIM, S. W. Enhancement of glucose yield from canola agricultural residue by alkali pretreatment based on multi-regression models. **Journal of Industrial and Engineering Chemistry**, v. 51, p. 303–311, jul. 2017.

ZHAO, Q.; FENG, H.; WANG, L. Dyeing properties and color fastness of cellulase-treated flax fabric with extractives from chestnut shell. **Journal of Cleaner Production**, v. 80, p. 197–203, 2014.

ZHENG, W.; ZHENG, Q.; XUE, Y.; HU, J.; GAO, M-T. Influence of rice straw polyphenols on cellulase production by Trichoderma reesei. **Journal of Bioscience and Bioengineering**, v. 123, n. 6, p. 731–738, 2017.

ZHUANG, X.; WANG, W.; YU, Q.; QI, W.; WANG, Q.; TAN, X.; ZHOU, G.; YUAN, Z. Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products. **Bioresource Technology**, v. 199, p. 68–75, 2016.

5 CONCLUSÕES FINAIS

Neste trabalho foi otimizar os pré-tratamentos de dois resíduos abundantes em Portugal, a poda da vide e a casca da castanha, de forma a obter rendimentos elevados em glicose após a hidrólise enzimática da celulose dos materiais. O que coloca esses resíduos como promissoras matérias-primas para diversos bioprocessos de produção de produtos de alto valor agragado.