Processos de fracionamento de resíduos agroindustriais para obtenção de hemiceluloses e lenhina de elevada qualidade para aproveitamento integrado no âmbito de uma biorrefinaria

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Resumo

As palhas de arroz e de milho são resíduos agroindustriais com elevada relevância mundial, para os quais não existem processos de valorização economicamente relevantes. O fracionamento seletivo dos seus componentes estruturais (hemiceluloses, celulose e lenhina) permitiria a sua valorização, mas este é ainda um problema desafiante em termos científicos e tecnológicos.

Neste trabalho, desenvolveu-se uma estratégia de fracionamento integrada, que englobou a otimização de um método hidrotérmico (auto-hidrólise) para separação das hemiceluloses e de um método organosolv (com a utilização de misturas etanol:água) para separação da lenhina. A otimização destes processos permitiu selecionar as melhores condições operatórias.

O fracionamento seletivo e sequencial, em condições suaves, da fração hemicelulósica e da lenhina permitiu a obtenção de produtos de valor acrescentado: i) xilo-oligossacáridos (rendimento máximo 53 g/100 g xilana) que foram purificados utilizando técnicas cromatográficas e cujo potencial prebiótico foi avaliado; ii) lenhina solúvel (rendimento deslenhificação máximo 42 g/100 g de sólido) passível de incorporação em materiais compósitos; iii) compostos derivados da lenhina potencialmente bioativos que foram purificados por técnicas de membrana. Obteve-se ainda uma fração celulósica com uma digestibilidade próxima dos 90% que poderá ser facilmente valorizada por fermentação.

Os resultados mostraram que a estratégia de fracionamento proposta pode ser aplicada para a valorização destes materiais como matéria-prima no âmbito de uma biorrefinaria.

Palavras-chave: auto-hidrólise, lenhina, oligossacáridos, organosolv, palhas de milho e arroz.

Abstract

Rice and corn straws are agro-industrial wastes with worldwide importance for which there are no economically relevant valorisation processes. The selective fractionation of their structural components (cellulose, hemicelluloses and lignin) would allow the valorisation of these materials, but this is still a particularly challenging scientific and technological problem. In this work, a strategy of sequential fractionation was developed, which involved a hydrothermal pretreatment (autohydrolysis) for hemicelluloses removal and an organosolv process (using ethanol:water solutions) for lignin removal which were optimized. This strategy allowed the selective and sequential fractionation of the hemicellulosic and lignin fractions, using mild conditions, and the production of a range of added-value products: i) xylo-oligosaccharides (maximum 53 g/100 g xylan) that were purified using chromatographic techniques and evaluated for their potential prebiotic effect ; ii) soluble lignin (maximum delignification yield 42 g/100 g solid) that can be incorporated in composite materials; iii) potential bioactive compounds derived from lignin that were purified using membrane techniques. A cellulosic fraction with an enzymatic digestibility close to 90% was also obtained, which can be easily upgraded by fermentation. The results supported the proposed strategy for valorisation of these residual materials as raw materials within the biorefinery concept.

Key words: autohydrolysis, lignin, oligosaccharides, organosolv, rice and corn straws

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Moniz, P., Pereira, H., Quilhó, T., Carvalheiro, F. (2013) Characterisation and hydrothermal processing optimization of corn straw towards the selective fractionation of hemicelluloses. *Industrial Crops and Products*, 50, 145-153.

Moniz, P., Pereira, H., Duarte, L.C., Carvalheiro, F. (2014) Hydrothermal production and gel filtration purification of xylo-oligosaccharides from rice straw, *Industrial Crops and Products*, 62, 460-465

Moniz, P., Ho, A.L., Duarte, L.C., A., Kolida, S., Rastall, R., Pereira, H., Carvalheiro, F. Assessment on the bifidogenic effect of corn straw xylo-oligosaccharides. Submetido

Moniz, P., Lino, J., Boeriu, C.G., Roseiro, L.B., Duarte, L.C., Pereira, H., Carvalheiro, F. Fractionation of hemicelluloses and lignin from rice straw by combining autohydrolysis and optimized mild organosolv delignification. Submetido

Moniz, P., Serralheiro C., Matos, C.T., Boeriu, C.G., Duarte, L.C., Roseiro, L.B., Pereira, H., Carvalheiro, F. Production, chemical characterization and membrane separation of lignin-derived products from rice straw using a mild ethanol organosolv process after autohydrolysis. Manuscrito

Outras publicações relevantes para esta tese:

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Vilcocq, L., Spinola, V., Moniz, P., Duarte, L.C., Carvalheiro, F., Fernandes, C., Castilho, P. Acid-modified clays as green catalysts for the hydrolysis of hemicellulosic oligosaccharides Submetido

Índice

Abreviaturas, Símbolos e Siglas

Abs	Absorvância
Ac	Ácido acético
AcOS	Oligossacáridos substituídos grupos acetilo
AOS	Arabino-oligossacáridos
Ara	Arabinose
Arn	Arabinano
ºC	Grau Celcius
CS	Fator de severidade combinado (“Combined severity factor”)
CZE	Electroforese capilar (“Capillary Zone Electrophoresis”)
Da	Dalton
DP	Grau de polimerização
EC	Comissão de enzimas
FOS	Fruto-oligossacáridos
g	Grama
Gac	Grupos acetilo
Gal	Galactose
Glc	Glucose
GlcOS	Gluco-oligossacáridos
GOS	Galacto-oligossacáridos
HAD	Hidrólise com ácido diluído
HMF	5-hidroximetil-2-furaldeído (hidroximetilfurfural)
HPLC	Cromatografia Líquida de Alta Pressão (“High Performance Liquid Chromatography”)
IR	Índice de refração
ISA	Instituto Superior de Agronomia
kcal	Quilocaloria
kg	Quilograma
L	Litro

LNEG	Laboratório Nacional de Energia e Geologia
LSR	Razão líquido-sólido (“Liquid-to-Solid Ratio”)
M	Molar
Man	Manose
mg	Miligrama
min	Minuto
mL	Mililitro
NF	Nanofiltração
nm	Nanómetro
OS	Oligossacáridos
R^2	Coeficiente de correlação
Rha	Ramnose
R_0	Ordenada de reação
rpm	Rotações por minuto
SCFA	Ácidos gordos de cadeia curta (“Short Chain Fatty Acids”)
T	Temperatura
t	Tempo
ton	Tonelada
U	Unidade de atividade enzimática
UA	Ácidos urónicos (“Uronic Acids”)
EU	União Europeia
UF	Ultrafiltração
USDA	Departamento de Agricultura dos Estados Unidos (United States Department of Agriculture)
UV	Ultravioleta
V	Volume
Xn	Xilano
XOH	Xilitol
XOS	Xilo-oligossacáridos
Xyl	Xilose

Preâmbulo

A biomassa vegetal é uma fonte renovável de matérias-primas e energia de grande importância económica dada a sua abundância e ampla distribuição geográfica, e cuja utilização também se impõe por razões de sustentabilidade económica e ambiental.

Apenas uma pequena percentagem da biomassa vegetal produzida é utilizada comercialmente e incorporada nos produtos finais, pelo que a maior parte constitui resíduos ou subprodutos dos processos de produção e transformação. Devido às grandes quantidades de biomassa processadas, esses resíduos ou subprodutos podem constituir um problema ambiental com um encargo económico significativo para o seu tratamento e remoção. A utilização de biomassa vegetal de uma forma eficiente, com a integração de várias cadeias de aproveitamento, nomeadamente energética, química e materiais, permite um retorno ambiental, ao eliminar uma fonte de contaminação e económico, ao acrescentar valor que a biomassa não tem na forma de resíduo.

O conceito de biorrefinaria tem por base a conversão da biomassa em diversos produtos, incluindo energia, e tendo subjacente uma redução de resíduos e emissões mínimas de poluentes. Este conceito pressupõe o aproveitamento integral da biomassa através da utilização integrada das suas diferentes frações químicas. Para tal pode ser vantajoso, ou necessário, fazer um fracionamento seletivo para recuperar frações diferenciadas e para a sua conversão posterior em biocombustíveis, químicos e biomateriais, preferencialmente de valor acrescentado. No entanto, este processo é complexo e depende da tecnologia existente ou do desenvolvimento de novas tecnologias, colocando assim importantes desafios para a sua utilização.

Uma biorrefinaria consiste numa unidade industrial que integra equipamentos e processos de conversão de biomassa para a produção de combustíveis, energia, materiais e produtos químicos numa ótica de utilização integral e sustentável do recurso. Entre as matérias-primas que podem ser utilizadas no âmbito de uma biorrefinaria, os subprodutos e resíduos de natureza lenhocelulósica apresentam grande interesse devido à sua abundância, composição e custo baixo.

A estrutura complexa da biomassa vegetal exige, em muitos casos, processos para o seu fracionamento, nomeadamente quando se pretende uma utilização diferenciada dos componentes químicos. Muitas vezes este fracionamento constitui uma primeira fase no processo de conversão. Entre as opções de pré-tratamento para o fracionamento seletivo dos componentes lenhocelulósicos, incluem-se a hidrólise com ácido diluído, tratamentos hidrotérmicos, tratamentos com solventes orgânicos, tratamentos alcalinos, entre outros (Carvalheiro et al. 2008; Girio et al. 2010).

Os tratamentos hidrotérmicos (auto-hidrólise) e a hidrólise com ácido diluído permitem uma separação das hemiceluloses que são recuperadas como açúcares solúveis, enquanto a celulose e a lenhina se mantem, quase na totalidade, na fase sólida (Carvalheiro et al. 2004; Garrote et al. 1999; Girio et al. 2010).

A complexidade química e estrutural das hemiceluloses tem constituído um impedimento para a sua valorização. No entanto, a possibilidade de utilizar este tipo de pré-tratamentos para a sua

remoção da matriz lenhocelulósica, permitirá a sua obtenção com elevado grau de pureza, permitindo o desenvolvimento de novos produtos químicos especializados, e assim contribuir significativamente para a economia das biorrefinarias. Por exemplo, estes processos permitem produzir oligossacáridos que, após purificação e/ou transformação, são potenciais agentes prebióticos (Carvalheiro et al. 2004; Crittenden e Playne 1996; Kabel et al. 2002) ou hidrogéis (Gabrielii et al. 2000) com aplicações na alimentação humana e animal e nas indústrias química, cosmética e farmacêutica.

A fração sólida resultante, rica em celulose, pode ser utilizada para bioconversões, nomeadamente para a produção de etanol (Chang e Holtzapple 2000; Saha et al. 2005) e ácidos orgânicos, como o ácido láctico (Rivas et al. 2004), que são produtos com um volume de mercado relativamente elevado e em crescimento.

A lenhina representa outro polímero muito importante para a economia das biorrefinarias, pois pode constituir até 35% da biomassa vegetal (Pereira et al. 2003). A sua estrutura complexa tem limitado a valorização desta fração, que é ainda maioritariamente utilizada para a produção de energia por combustão. Com a crescente importância e desenvolvimento das biorrefinarias, tem-se verificado um aumento na procura de aplicações para a lenhina a par com o desenvolvimento de metodologias de transformação adequadas que permitam a obtenção de produtos de valor acrescentado, tais como, resinas, compostos fenólicos ou fibras de carbono (Bozell et al. 2007; Bozell 2010).

Entre os métodos de deslenhificação podem distinguir-se os tradicionalmente utilizados na indústria da pasta para papel, tais como os métodos alcalinos (principalmente o processo kraft), com sulfitos e outros métodos de deslenhificação alternativos, tais como os que usam solventes orgânicos, por exemplo os métodos designados como organosolv ou acetosolv (Bozell et al. 2007). Os métodos de deslenhificação suaves, nomeadamente misturas aquosas com solventes orgânicos, permitem obter lenhinas com maior potencialidade para aplicação em produtos de valor acrescentado (Lora e Glasser 2002; Shatalov e Pereira 2005), devido à sua massa molecular baixa e reatividade elevada (Shatalov e Pereira 2002). Os solventes orgânicos, em geral, além de poderem ser eles próprios produtos das biorrefinarias, podem ainda ser facilmente recuperados (Bozell et al. 2007).

Como as hemiceluloses são, em geral, o primeiro componente polimérico a separar na cadeia de fracionamento, o método de pré-tratamento utilizado para a remover é também determinante para a qualidade da celulose e das lenhinas que se obtêm. Os métodos de hidrólise suaves, como é o caso dos tratamentos hidrotérmicos, permitem obter sólidos ricos em celulose e em lenhina de elevada qualidade, pelo facto de o único reagente utilizado ser água.

Neste trabalho, utilizar-se-ão como materiais de estudo subprodutos agroindustriais com importância nacional e internacional e atualmente ainda pouco valorizados: palha de arroz e resíduos da cultura do milho. Os subprodutos da indústria do arroz são materiais com elevada abundância mundial. Em Portugal, as cascas de arroz têm aplicações com um valor comercial baixo, sendo, por exemplo, utilizadas em aviários (Domínguez-Escribá e Porcar 2009), e não existe qualquer aplicação para as palhas que são deixadas no campo. A cultura de milho, em

particular para fins não-alimentares tais como a produção de bioetanol, tem tido um crescimento muito acentuado nos últimos anos, quer na Europa quer nos EUA e os resíduos (caules, palhas, carolo) poderão também ter um papel importante como substratos no enquadramento das biorrefinarias (Kamm e Kamm 2007).

Este trabalho tem como enquadramento geral o conceito de biorrefinaria. Este conceito pressupõe o aproveitamento integrado das diferentes frações da biomassa e a sua conversão posterior em biocombustíveis, produtos químicos e biomateriais de forma sustentável do ponto de vista económico e ambiental. Em particular, a temática deste trabalho insere-se na valorização da fração hemicelulósica e da lenhina.

O objetivo deste trabalho consiste no desenvolvimento de processos que permitam o fracionamento seletivo dos componentes químicos estruturais da biomassa vegetal para utilização posterior das diferentes frações químicas em aplicações de valor acrescentado, utilizando como materiais biomássicos subprodutos e resíduos agroindustriais com relevância no contexto nacional e internacional, palha de milho e palha de arroz.

Estudaram-se processos de pré-tratamento para a remoção seletiva e sequencial das hemiceluloses e da lenhina com uma utilização de reagentes químicos minimizada. Para a hidrólise seletiva das hemiceluloses foram otimizados processos hidrotérmicos (auto-hidrólise). Nos resíduos sólidos, ricos em celulose e lenhina, foram otimizados processos de deslenhificação com solventes orgânicos (organosolv).

Para cada tipo de pré-tratamento foram otimizadas as condições operacionais conducentes à recuperação máxima das hemiceluloses e da lenhina, sem degradação significativa da celulose.

Os produtos resultantes, principalmente oligossacáridos e lenhina, foram objeto de processos de recuperação e purificação e de uma caracterização profunda ao nível químico (composição monomérica, grau de polimerização, grupos funcionais, reatividade) e físico (densidade) tendo em vista perspetivar as suas possíveis aplicações industriais, nomeadamente como agentes prebióticos (oligossacáridos), compostos fenólicos (lenhina).

No **Capítulo I** apresenta-se uma revisão bibliográfica, focando a composição dos materiais lenhocelulósicos e os possíveis métodos para o seu aproveitamento e valorização. São descritos diversos métodos utilizados para o fracionamento seletivo das hemiceluloses e da lenhina. São também descritas as aplicações potenciais dos produtos resultantes da auto-hidrólise, nomeadamente dos oligossacáridos e é abordada a sua importância, em termos do mercado e das suas propriedades funcionais e referidos os métodos utilizados para a sua produção. Por último, abordam-se alguns exemplos de valorização da lenhina.

No **Capítulo II** é estudada a solubilização das hemiceluloses da palha de milho por auto-hidrólise. São identificadas as condições operacionais que maximizam a recuperação de xilo-oligossacáridos. São caracterizados os oligossacáridos obtidos sendo avaliada a formação de outros produtos, nomeadamente de monossacáridos e subprodutos da hidrólise. São apresentados os rendimentos para os diversos produtos, assim como a composição química e solubilização dos polímeros constituintes dos resíduos sólidos. Apresenta-se também uma

caracterização pormenorizada da fração sólida resultante do processo de auto-hidrólise.

No **Capítulo III** são apresentados os resultados da otimização das condições operacionais para a produção de xilo-oligossacáridos a partir de palha de arroz. Para os licores de XOS obtidos na condição otimizada, são apresentados os resultados da sua purificação por cromatografia de filtração gel e de caracterização das frações obtidas quanto ao seu grau de polimerização.

No **Capítulo IV** é estudada a purificação dos XOS presentes nos licores de palha de milho obtidos por auto-hidrólise. O potencial bifidogénico dos XOS purificados é estudado por fermentação *in vitro* com culturas fecais.

No **Capítulo V** é estudada a deslenhificação organosolv das palhas de arroz determinando-se as condições (tempo e concentração de etanol) com maior influência no rendimento do processo, assim como nas recuperações de xilana e de glucana após a deslenhificação. Apresenta-se uma primeira abordagem da composição das lenhinas obtidas em termos de peso molecular e determinação de compostos fenólicos.

No **Capítulo VI** estuda-se a composição das lenhinas organosolv obtidas de modo a perspetivar potenciais aplicações para as mesmas. Apresenta-se também um estudo da separação de compostos fenólicos presentes nos extratos de lenhina realizado utilizando processos com membranas (nanofiltração).

No **Capítulo VII** são apresentadas as principais conclusões deste trabalho, assim como algumas considerações sobre as possíveis linhas de trabalho futuro.

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Capítulo I. Introdução

1.1 Conceito de biorrefinaria

Ao longo dos anos, os materiais de origem fóssil foram substituindo gradualmente os recursos renováveis como principal fonte de materiais, de energia e químicos. No entanto, as reservas de combustíveis fósseis são cada vez mais escassas, o que torna imperativa a procura de alternativas sustentáveis e economicamente viáveis. As biorrefinarias incluem-se nesta procura. Os avanços da investigação na área química e biológica, assim como nos processos que lhes estão associados, terão um grande impacto nas biorrefinarias do século XXI, que serão instalações que convertem recursos renováveis, como por exemplo biomassa, em produtos químicos úteis obtidos a partir de tecnologias pouco poluentes e inovadoras. A variedade de produtos obtidos a partir da biomassa começa já a constituir uma alternativa aos produtos de base petroquímica (Uihlein e Schebek 2009).

Uma biorrefinaria consiste numa unidade industrial que integra equipamentos e processos de conversão de biomassa para a produção de combustíveis, energia, materiais e produtos químicos, preferencialmente de valor acrescentado (Figura 1) numa perspetiva de sustentabilidade global.

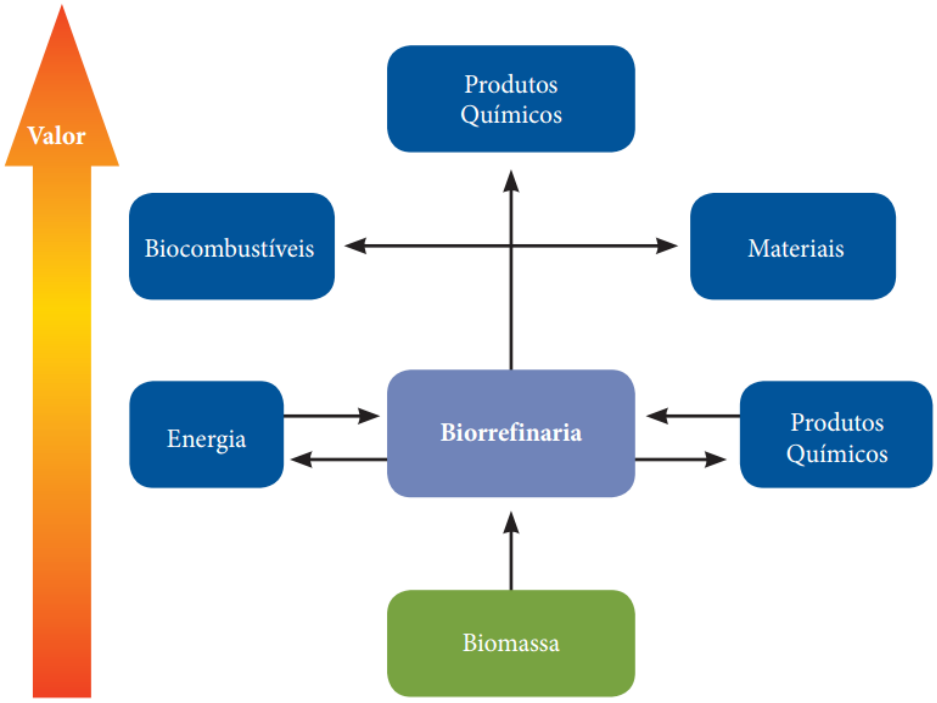


Figura 1- Ilustração do conceito de biorefinaria (adaptado de *siadeb.org*)

O conceito de biorrefinaria surgiu por analogia ao das refinarias de petróleo, que produzem combustíveis e derivados de petróleo. As biorrefinarias industriais têm sido vistas como o início de um percurso promissor para uma nova indústria de base biológica. No entanto, utilizar biomassa para produzir múltiplos produtos tendo como base tecnologias complexas não é um processo fácil (Kamm e Kamm 2004; Kamm e Kamm 2007). O objetivo principal de uma biorrefinaria será tirar

partido da complexidade da biomassa de forma a produzir produtos de elevado valor e de baixo volume e produtos de baixo valor e elevado volume, de forma a otimizar a produção de valor acrescentado (Fernando et al. 2006). Embora o conceito seja similar ao das refinarias petroquímicas, a natureza química e física da biomassa requer uma atenção especial aos processos de separação e transformação quer sejam físicos, químicos ou biológicos (Ragauskas et al. 2006).

As biorrefinarias podem ser classificadas de acordo com: i) o processo tecnológico, ii) a matéria-prima utilizada e iii) o desenvolvimento tecnológico do processo (Kamm e Kamm 2007).

Nas biorrefinarias podem estar envolvidos diferentes processos tecnológicos, que incluem desde os processos de extração e separação até às conversões termoquímicas, químicas e bioquímicas da biomassa, de forma a obter um maior número de produtos, preferencialmente de valor acrescentado. A classificação mais usual das biorrefinarias baseia-se no tipo de processos tecnológicos envolvidos, definindo-se em diferentes plataformas: termoquímica e bioquímica, entre outras (Kamm et al. 2006; Kamm e Kamm 2007). A plataforma termoquímica envolve a decomposição de biomassa com recurso a calor e a catalisadores, através de gaseificação ou pirólise. A plataforma bioquímica envolve a utilização de pré-tratamentos (físicos, físico-químicos, químicos) tendo em vista a conversão de biomassa em açúcares simples na presença de biocatalisadores e posterior fermentação de forma a produzir combustíveis líquidos, materiais e produtos químicos de modo sustentável. Esta permite operar a uma escala inferior à plataforma termoquímica e de modo mais descentralizado. Apesar de enfrentar barreiras tecnológicas e económicas provenientes da complexidade e resistência da parede celular da biomassa vegetal, nesta plataforma ocorre a desconstrução da biomassa utilizando uma abordagem baseada em fracionamento seletivo (Naik et al. 2010).

O uso de qualquer uma das plataformas vai depender do tipo de biorrefinaria e desenvolvimento tecnológico que esta envolva. O desenvolvimento das biorrefinarias passa pela otimização dos processos envolvidos nas plataformas termoquímica e bioquímica, pelo estabelecimento de sinergias entre ambas de modo a reduzir desperdícios, alargar a origem da matéria-prima e fazer face à sua heterogeneidade e complexidade estrutural. Em simultâneo, há que conceber processos e equipamentos de modo a minimizar o desperdício e a maximizar o rendimento não só em combustíveis líquidos como também em produtos de valor acrescentado (Bozell et al. 2007; Werpy et al. 2004).

1.2 Os materiais lenhocelulósicos

A crescente escassez das tradicionais fontes de matérias-primas na indústria química, ou seja, os recursos fósseis, tem impulsionado o aproveitamento mais racional de recursos e fontes alternativas e renováveis. Como matérias-primas, os materiais lenhocelulósicos (ML) apresentam a vantagem de serem atrativos para uma grande variedade de processos químicos, serem abundantes, renováveis e estarem disponíveis a baixo custo.

Os ML incluem diferentes tipos de biomassa de origem vegetal que têm como característica comum serem constituídos por polissacáridos (celulose e hemiceluloses) e lenhina (Fengel e

Wegener 1984). Os ML representam cerca de 50% da biomassa vegetal global e têm uma produção anual estimada de 10-50 mil milhões de toneladas (Duarte et al. 2007).

De acordo com a sua origem e composição química e com as características físicas e mecânicas, estes materiais podem ser classificados em dois tipos principais: materiais lenhosos de espécies resinosas ou folhosas e materiais não-lenhosos (materiais herbáceos como é o caso de algumas culturas agrícolas).

Os ML são normalmente considerados abundantes, de baixo custo e potencialmente valorizáveis. Devido às elevadas quantidades processadas, podem constituir um problema ambiental em alguns casos, tornando-se assim um encargo significativo. Os subprodutos e os resíduos lenhocelulósicos podem também ser classificados como: florestais (provenientes das florestas e das indústrias da pasta de papel e da transformação da madeira, incluindo aparas e serradura), agrícolas (palhas, cascas, caules e espigas de cereais, podas de árvores de fruto e de videiras), agroindustriais (bagaço de azeitona extratado, dreche cervejeira, polpa, casca e sementes de frutos e vegetais) e urbanos (papéis, cartões e lixo doméstico constituídos por celulose) (Duarte et al. 2007).

Tem havido um interesse crescente pelos resíduos agroindustriais devido à elevada quantidade produzida anualmente, à possibilidade de valorização industrial, à sua disponibilidade e baixo custo e ao elevado potencial de bioconversão (Carvalheiro et al. 2008). No entanto, estes materiais, assim como todos os ML na sua generalidade, possuem uma estrutura complexa, requerendo pré-tratamentos e processos de fracionamento para serem eficientemente convertidos em produtos.

Do ponto de vista químico, os componentes dos ML podem ser classificados em componentes de massa molecular elevada e de massa molecular baixa. Os de massa molecular elevada são os principais constituintes da parede celular: a celulose (35-50%), as hemiceluloses (20-35%) e a lenhina (10-25%). Nos materiais de massa molecular baixa estão incluídos os extrativos (compostos orgânicos) e a cinza (compostos inorgânicos) (Quadro 1).

Quadro 1 Composição química (componentes estruturais) de diversos materiais lenhocelulósicos[a].

Material	Celulose	Hemiceluloses	Lenhina	Referências
Materiais agrícolas e agroindustriais				
Bagaço de azeitona	36,4	26,8	26	(Brás et al. 2014)
Bagaço de cana-de-açúcar	35-42,8	26,2-35,8	16,1-25,2	(Geddes et al. 2011;Imman et al. 2013;Mesa et al. 2011b)
Cana-de-açúcar	36	17	17	(Fonseca et al. 2011)
Cana de bambu	38,8	24,9	23,9	(González et al. 2011)
Carolo de milho	31,7-39,4	31,9-34,7	15,9-22,3	(Cheng et al. 2010;Oliveira et al. 2010)
Casca de amêndoa	28	34,6	28,3	(Montané *et al.* 1993; Nabarlatz et al. 2007)
Cascas de arroz	36,7-37,7	16,7-17,3	21,3-22,1	(Vila *et al.* 2002, Mansilla *et al.*1998)
Casca de noz	25-30	25-30	30-40	(Sun e Cheng 2002);
Farelo de cevada	23	32,7	21,4	(Cruz *et al.* 2001)
Fibra de milho	30	33	8	(Allen *et al.* 2001)
Folhas de milho	37,6	34,5	12,6	(Cruz *et al.* 2001)
Palha de cevada	13,8	16,7	33,75	(Magee e Kosaric 1985)
Palha de trigo	38.9	23.5	18.0	(Carvalheiro et al. 2009a)
Palha de sorgo	35,1	24	25,4	(Téllez-Luis *et al.* 2002)-
Polpa de beterraba	39,1	22,3	22,6	(Saska e Ozer 1995)
Sorgo	36	18	16	(Mok e Antal 1992)
Serradura de choupo	39,6-45,2	15,8-18,7	18,4-27,1	(Kim *et al.* 2001a; Xiang *et al.* 2004).
Madeiras resinosas				
Abeto	43-51,1	15,2-26	27,3-29	(Fengel e Wegener 1984;Olsson e Hahn-Hägerdal 1996;Rydholm 1965)
Pinheiro	42,9-52,2	13,5-26	26,3-30,2	(Fengel e Wegener 1984;Olsson e Hahn-Hägerdal 1996;Rivas et al. 2012)
Madeiras folhosas				
Amieiro	40,5	18,4	20,8	(Taherzadeh et al. 1997)
Carvalho	38,9-44	18,7-23,8	21,5-24,7	(Kim et al. 2000)
Choupo	39-51,3	21-28,4	20,3-26	(Mok e Antal 1992 ; Capek-Ménard et al. 1987)
Eucalipto	38-54,0	15-30	23,1-37	(Carrasco et al. 1986; Miranda e Pereira 2002);
Faia	43,2-49,8	18-28,2	16-24	(Taherzadeh et al. 1997)

[a] valores expressos em g/100 g de material seco (adaptado de *Carvalheiro, 2005*).

A composição química dos ML depende da origem do material e dos fatores genéticos e, assim como das condições de crescimento e da sua origem geográfica.

A Figura 2 representa esquematicamente a localização dos polímeros estruturais na parede das células vegetais. O esqueleto microfibrilhar de celulose encontra-se envolto em hemiceluloses, estando a lenhina a ocupar os espaços vazios deixados entre as hemiceluloses. No seu conjunto formam um material compósito resistente a ataques microbiológicos e enzimáticos e, consequentemente ao seu fracionamento (Fengel e Wegener 1984; Pereira et al. 2003).

Os extrativos encontram-se no lúmen celular e nas células parenquimatosas (Fengel e Wegener 1984; Pereira et al. 2003).

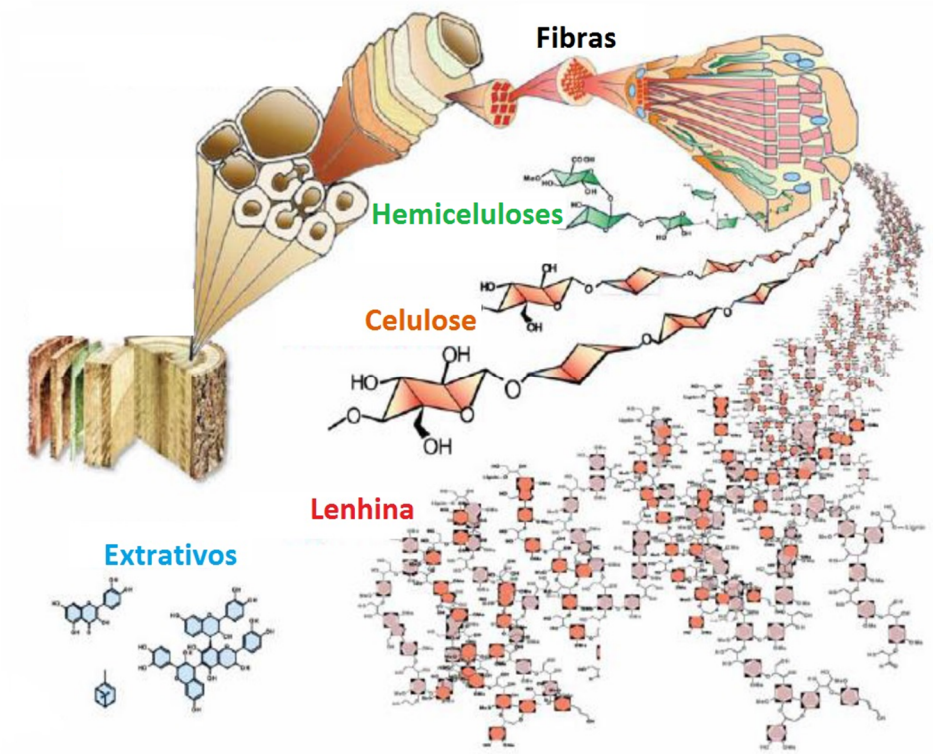


Figura 2 - Organização estrutural dos componentes macromoleculares de materiais lenhocelulósicos (adaptado de *Hoffmann; Faix; Lehnen. in Wild, 2014*)

1.2.1 Celulose

A celulose é o principal componente na maioria dos ML, podendo representar 35-50% da sua massa, e está localizada predominantemente na parede secundária. É um polímero constituído por moléculas de β-D-glucopiranose unidas por ligações glucosídicas β-(1,4). Cada molécula de glucose apresenta uma rotação 180º em relação às moléculas vizinhas, pelo que a unidade básica de repetição é, na realidade, uma molécula de celobiose (Nag, 2008). É esta organização que permite o estabelecimento de ligações entre as várias cadeias de celulose, associada ao arranjo estrutural desta molécula. A orientação das ligações e pontes de hidrogénio entre cadeias poliméricas fazem da celulose um polímero altamente estável e cristalino, apresentando um elevado grau de polimerização (Khanal et al. 2010, Pereira et al. 2003).

As cadeias de celulose dispõem-se ordenadamente agregando-se em microfibrilhas, ocorrendo ao

longo das cadeias zonas de maior ordenação (cristalinas) e que constituem a maior parte da celulose, e com zonas de menor ordenação (amorfas).

Como resultado da sua estrutura cristalina e das ligações de hidrogénio, a celulose apresenta uma elevada resistência química sendo insolúvel na maioria dos solventes (Agbor et al. 2011; Fengel e Wegener 1984; Saka, 1991; Sjöström, 1981). Porém é solubilizada após despolimerização em ácidos, nomeadamente ácido sulfúrico, ou em outras soluções iónicas (Carvalheiro et al. 2005).

1.2.2 Hemiceluloses

As hemiceluloses são o segundo polímero mais abundante da biomassa lenhocelulósica (20-50%). São heteropolímeros constituídos a partir de vários açúcares, compreendendo dois grandes grupos: pentoses (β-D-xilose, α-L-arabinose) e hexoses (β-D-manose, β-D-glucose, α-D-galactose). Também apresenta pequenas quantidades de L-ramnose, L-fucose e de ácidos urónicos (ácidos α-D-glucurónico, α-D-4-*O*-metilglucurónico, α-D-galacturónico). Os grupos hidroxilo dos açúcares podem ser parcialmente substituídos por grupos acetilo (Kumar et al. 2008; Pereira et al. 2003).

Entre os componentes estruturais da parede celular, as hemiceluloses são mais suscetíveis a tratamentos químicos e termoquímicos, uma característica atribuída em grande parte ao seu carácter amorfo e ao baixo grau de polimerização (comparativamente à celulose), permitindo que seja mais facilmente hidrolisável em meios ácidos e solúvel em meios alcalinos, à temperatura ambiente. A sua solubilização ocorre a partir dos 180ºC.

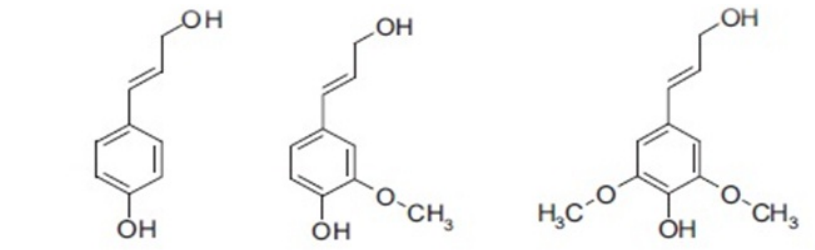
As hemiceluloses apresentam diferenças quanto à sua estrutura e composição em função da sua origem biológica. Nas madeiras folhosas, as hemiceluloses predominantes são as xilanas (*O*-acetil-metilglucuronoxilana) e as glucomananas (em menor quantidade). Por outro lado, no grupo das resinosas, as hemiceluloses são maioritariamente do tipo galactoglucomananas (*O*-acetil-galactoglucomanana), contendo também algumas xilanas (arabino-4-*O*-metilglucuronoxilana) (Fengel e Wegener 1984). No entanto, as resinosas possuem uma proporção superior de unidades de manose e glucose comparativamente às hemiceluloses das folhosas e resíduos agrícolas. O conteúdo de manose pode chegar aos 10% nas folhosas e até 5% nos resíduos agrícolas. Nestes dois últimos grupos de materiais, em geral, cerca de 80% dos açúcares hemicelulósicos correspondem a xilose, pelo que é frequente associar o conteúdo em hemiceluloses ao conteúdo em xilanas (Moure et al. 2006). As xilanas mais comuns são formadas por uma cadeia principal de xilose ligada por ligações β-1,4, onde as unidades estruturais são substituídas por arabinose, ácido glucurónico, metil-glucorónico e acético (Moure et al. 2006).

As hemiceluloses são solúveis em soluções alcalinas e facilmente hidrolisáveis por ácidos nos seus componentes monoméricos, apresentando uma estabilidade química e térmica inferior à celulose, provavelmente devido à falta de cristalinidade e ao grau de polimerização mais baixo (Pereira et al. 2003). Da hidrólise das hemiceluloses são obtidos hidrolisados que podem conter hexoses (glucose, manose e galactose), pentoses (xilose e arabinose), pequenas quantidades de outras hexoses (fucose e ramnose) e ainda ácidos urónicos e acético que se encontram ligados a alguns açúcares.

1.2.3 Lenhina

A lenhina é um heteropolímero complexo de massa molecular elevada e de natureza polifenólica, constituído por unidades básicas de fenilpropano, unidas por ligações éter e carbono-carbono com diversos tipos de ligação. Apresenta uma estrutura tridimensional complexa e de difícil degradação microbiana (Fengel e Wegener 1984; Pereira et al. 2003). A sua função nos ML é reforçar e impermeabilizar a parede das células, tendo um papel fundamental no suporte mecânico, na condução de solutos e na proteção contra agentes exteriores nas plantas superiores (Fengel e Wegener 1984; Sakakibara 1991; Sjöström 1981).

A lenhina é um polímero amorfo, construído a partir de três unidades de fenil-propano, álcool *p*-cumarílico, álcool coniferílico e álcool sinapílico, unidos por ligações C-O-C (éter) e C-C (Figura 3). Estas estruturas monoméricas compreendem o mesmo esqueleto de fenil-propano, diferindo apenas no grau de substituição do átomo de carbono no anel aromático que é designado, de acordo com os substituintes presentes como guaiacilo (G), siringilo (S) e hidroxifenilo (H) derivados do álcool coniferílico, álcool sinapílico e álcool *p*-cumarílico, respetivamente (Fengel e Wegener 1984; Pereira et al. 2003; Sarkanen e Hergert 1971). Este último não é metoxilado, enquanto os anteriores têm um e dois grupos metoxilo, A percentagem dos três percursores varia de acordo com a origem dos ML. As lenhinas provenientes de resinosas são usualmente referidas como lenhinas do tipo G porque os seus elementos estruturais resultam principalmente do álcool coniferílico, enquanto as provenientes de folhosas são designadas por lenhinas GS e são essencialmente constituídas por álcoois do tipo coniferílico e sinapílico. As lenhinas de materiais não-lenhosos apresentam quantidades variáveis das três unidades (HGS).



Álcool *p*-cumarílico (H) | Álcool coniferilico (G) | Álcool sinapílico (S)

Figura 3 - Precursores da lenhina

A massa molecular de lenhinas isoladas varia, sendo difícil a quantificação do grau de polimerização tal como existe na natureza, devido às fragmentações inevitáveis que ocorrem durante a sua extração. Apesar de ser evidente a existência de interações físicas e químicas entre a lenhina e os polissacáridos (ligações de hidrogénio, forças de van der Waals e ligação covalente), não se conhece com exatidão a natureza e o número dessas ligações. Considera-se que a lenhina se encontra ligada a parte das hemiceluloses, sendo as ligações covalentes do tipo éter ou glucosídicas as mais frequentemente sugeridas (Alén 2000; Gübitz et al. 1998; Kosiková e

Ebringerová 1994).

A estrutura da lenhina é bastante complexa e a sua composição depende da origem da matéria-prima e varia de acordo com a técnica de extração usada (Figura 4).

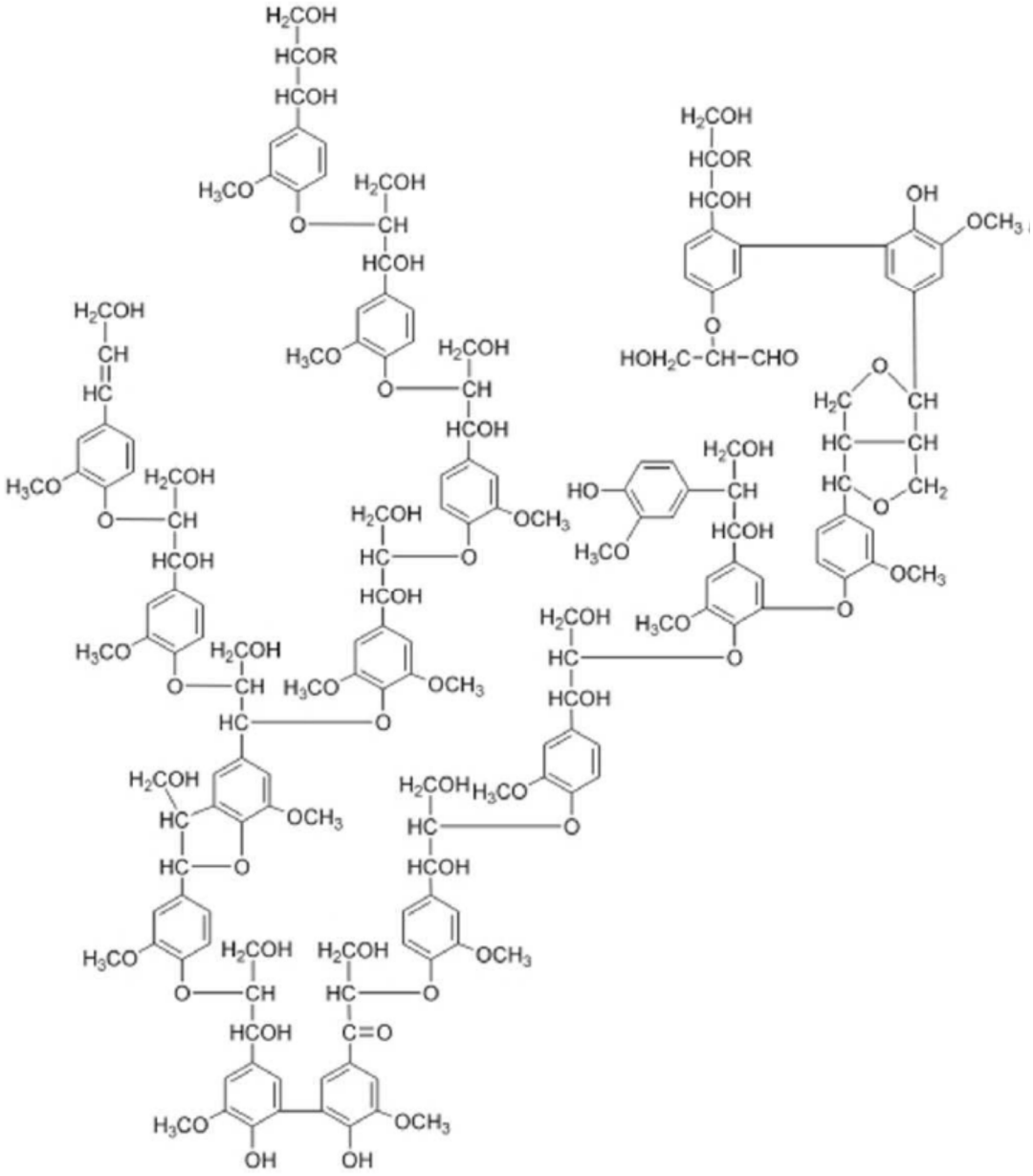


Figura 4 - Modelo da estrutura da lenhina de abeto proposta por *Adler 1977*.

Devido à sua natureza química, a lenhina é um dos polímeros naturais mais resistentes a reações de hidrólise alcalina, ácida e enzimática, tornando-se mais suscetível do que os polissacáridos a reações de oxidação ou a ação de solventes orgânicos (Kneževic et al. 2013).

1.2.4 Compostos de massa molecular pequena

Para além dos componentes macromoleculares, todas as espécies vegetais contêm outras substâncias químicas nas suas células. Essas substâncias químicas representam, na sua maioria, uma pequena fração dos materiais lenhocelulósicos (em geral inferior a 10%), mas no entanto podem influenciar as suas propriedades e afetar o seu processamento (Fengel e Wegener 1984). Estes compostos podem ser divididos em orgânicos (extrativos) e inorgânicos (cinza). A composição e quantidade relativa desses compostos dependem de fatores como a espécie, a proveniência, a idade e a localização na planta, à semelhança do que acontece com os compostos macromoleculares (Fengel e Wegener, 1984; Miranda e Pereira, 2002).

Os compostos orgânicos, do tipo lipofílico e hidrofílico, compreendem uma grande variedade de terpenóides, esteróis, ácidos gordos esterificados com glicerol ou com álcoois de elevado peso molecular (ceras), ácidos gordos livres, compostos fenólicos (como os taninos), aminoácidos, pectinas, amidos e alcaloides (Pereira et al. 2003). São normalmente quantificados tendo em conta a sua solubilidade (total ou parcial) em solventes orgânicos neutros e/ou água, designando-se, por essa razão, extrativos. A maior parte deles são metabolitos secundários, importantes para o desenvolvimento e crescimento celular e como agentes de defesa contra os ataques microbianos (Pereira et al. 2003).

Os compostos inorgânicos incluem elementos como o potássio, magnésio e silício, no geral em quantidades inferiores a 1% (Fengel e Wegener, 1984). Designam-se normalmente como cinzas pois correspondem a substâncias inorgânicas que são geralmente determinadas pela incineração do material a temperaturas entre 575-850˚C. Os seus componentes principais são os carbonatos, fosfatos, oxalatos e silicatos de cálcio, potássio e magnésio, bem como de sílica (Angles et al. 1997). O teor de cinzas varia substancialmente com o tipo de ML considerado. Na madeira é inferior a 1%, enquanto nas herbáceas pode atingir valores elevados. É o caso de certas palhas de cereais, como o trigo e o milho que apresentam percentagens de cinza até 3% e cascas ou palhas de arroz podem mesmo variar entre 8-14% (Kim et al. 2012; Vegas et al. 2004).

1.2.5 Outros materiais poliméricos

Os ML podem também conter outros polímeros, geralmente presentes em menores quantidades, e em quantidades muito variáveis tais como as pectinas, proteínas e amido.

As pectinas são compostas por cadeias lineares e em conjunto com as hemiceluloses e com a lenhina, interagem com as fibrilhas de celulose criando uma estrutura rígida que reforça a parede celular (de Vries e Visser, 2001).

Na parede celular, as proteínas podem estar ligadas covalentemente, através de ligações cruzadas com a lenhina e polissacáridos (Lamport, 1965). Comparativamente às madeiras, que são praticamente desprovidas de proteína, os materiais não-lenhosos podem apresentar teores de proteína muito mais elevados (Alén, 2000).

O amido localiza-se no endosperma das espécies vegetais, podendo ainda estar presente em quantidade apreciável nos resíduos vegetais, nomeadamente nas cascas.

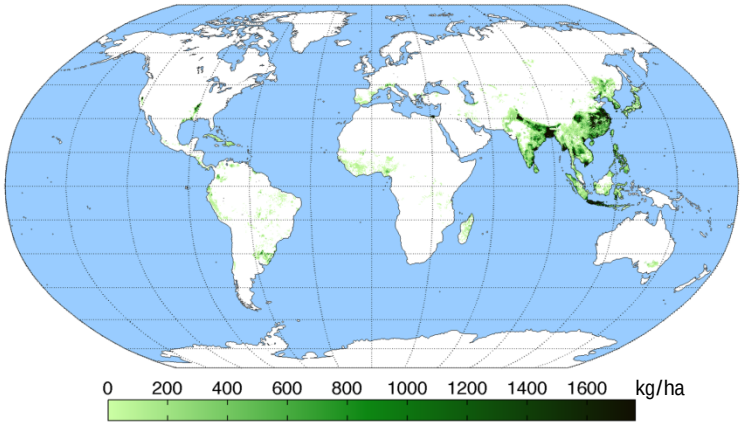
1.3 Aproveitamento de resíduos agroindustriais

1.3.1 A cultura do milho e do arroz

O milho é uma planta da família das gramíneas e corresponde à espécie *Zea mays*. Trata-se de um cereal com um valor nutricional elevado, sendo fundamentalmente utilizado na alimentação humana e em rações para animais. Atualmente é cultivado e consumido em todos os continentes e só rivaliza com a cultura do trigo e do arroz (Figura 5).

A cultura do arroz é uma das culturas com maior relevância a nível mundial e os seus resíduos (palhas e cascas) são consequentemente produzidos em grandes quantidades. Também o arroz é uma planta da família das gramíneas (constituído por sete espécies, *Oryza barthii, Oryza glaberrima, Oryza latifolia, Oryza longistaminata, Oryza punctata, Oryza rufipogon e Oryza sativa*, sendo esta última a mais comum em Portugal.

A



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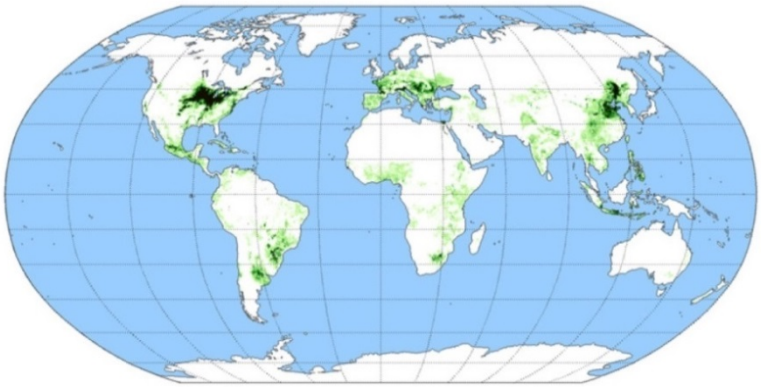


Figura 5 - Principais zonas geográficas dedicadas ao cultivo de arroz (A) e milho (B) (adaptado de *http://pt.wikipedia.org*)

Sendo duas culturas amplamente produzidas e distribuídas a nível mundial, tanto a cultura do milho como a cultura do arroz geram resíduos agroindustriais em quantidades muito elevadas. Estima-se que a cultura de milho gere cerca de 206 milhões de toneladas/ano de resíduos (Kadam e McMillan, 2003) e a cultura de arroz entre 650–975 milhões de toneladas/ano (Binod et al. 2010).

1.3.2 Disponibilidade e localização geográfica

A produção mundial de milho, de acordo com os dados divulgados pelo Departamento de Agricultura dos Estados Unidos (USDA) (apps.fas.usda.gov, 2014), foi no ano de 2012/2013 de 868,61 milhões de toneladas, sendo que os resultados preliminares apontam para 988,57 milhões de toneladas na colheita de 2013/2014 e as projeções sugerem um valor de 990,69 milhões de toneladas para 2014/2015. Este aumento que se tem vindo a verificar na produção dever-se-á sobretudo ao aumento da produtividade (toneladas de milho produzidas por hectare cultivado) visto que a área cultivada (milhões de hectares) se mantém aproximadamente constante nos mesmos anos (Figura 6).

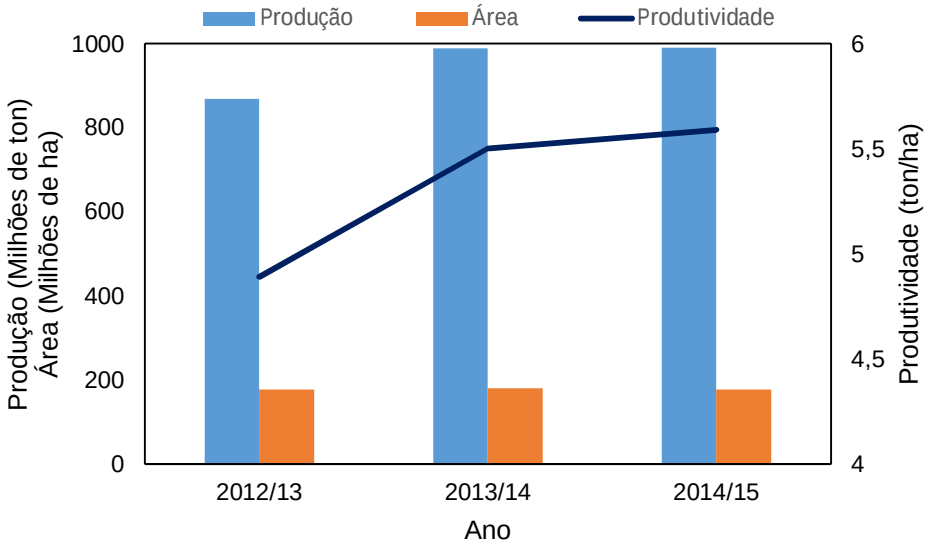


Figura 6 - Produção mundial de milho

O milho lidera a área total em Portugal, chegando aos 40% da área de cereais cultivados; em 1999, a cultura do milho chegava aos 163 497 hectares e a sua produção atingia aproximadamente um milhão de toneladas (Anpromis, 2012). A região agrária da Beira Litoral e a zona agrária do Baixo Mondego eram as que mais contribuíam para a produção do milho. Apesar das condições climáticas e económicas, em 2012 semearam-se cerca de 140 000 hectares de milho, mais 7 500 hectares do que em 2010 (Quadro 2) (Anpromis, 2012).

Quadro 2 - Plantação mundial (Milhões de ha) dos principais cereais (Anpromis, 2012).

	Arroz	**Cevada**	**Milho**	**Trigo**
2010	28 985	20 322	132 491	51 026
2011	31 213	16 213	137 413	39 546
2012	30 965	17 516	140 723	50 693

Também as potencialidades que surgiram devido ao regadio na zona de Alqueva e o interesse crescente dos produtores de milho têm levado a elevadas produções deste cereal. O peso da cultura de milho que já se posiciona como a segunda maior na região do Alqueva, ocupando uma área de regadio de 18% (Anpromis, 2012), superior a outras culturas arvenses e suscetível a

crescimento nos próximos anos. De acordo com os dados disponibilizados pelo Instituto Nacional de Estatística (www.ine.pt), a produção de milho aumentou cerca de 33%, quando comparada com 2011.

A produção mundial de arroz tem aumentado nos últimos anos e prevê-se que atinja o valor de 481 milhões de toneladas em 2014/15 (apps.fas.usda.gov, 2014), que atribui esse aumento de produção ao aumento de área para 162 milhões de hectares (Figura 7). A produtividade não tem grandes alterações e está calculada em 4,44 toneladas por hectare. O maior crescimento de produção ocorre nos países asiáticos. Num clima mediterrâneo com influência atlântica, o arroz é maioritariamente cultivado em condições de regadio. Em Portugal, a produção de arroz é feita apenas nas envolventes de cinco rios (Mira, Sado, Sorraia, Tejo e Mondego); mais a norte a cultura é impedida pelo frio. A produção de arroz no ano de 2013 rondou as 160 mil toneladas (www.ine.pt).

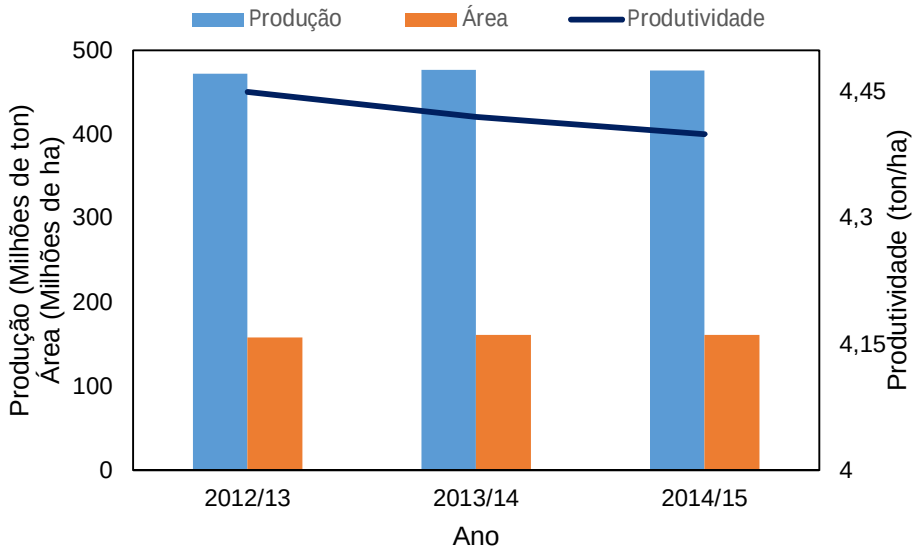


Figura 7 - Produção mundial de arroz

1.3.3 Os resíduos das culturas de milho e arroz

Após a colheita do milho, os principais resíduos são o caule e as folhas, que no seu conjunto constituem a palha, e o carolo. No caso do arroz são os principais resíduos são a palha (caule e folhas) e cascas.

A estimativa das quantidades de resíduos disponíveis a partir das culturas agrícolas nem sempre é uma tarefa fácil. Ao contrário do que acontece para os produtos agrícolas, para os quais se encontram estatísticas oficiais em diversos organismos por exemplo, FAO (www.faostat.fao.org) e Instituto Nacional de Estatística (www.ine.pt), os quantitativos dos resíduos e subprodutos não estão facilmente disponíveis. Assim, o critério mais utilizado passa pela estimativa de uma razão entre as quantidades de resíduos e a quantidade de produto, mas mesmo esta estimativa pode ser afetada pelas espécies/variedades cultivadas, técnicas de recolha utilizadas e principalmente pelas diferentes práticas agrícolas utilizadas que definem diferentes quantidades de material a

deixar no campo para proteção e fertilização do solo. O Quadro 3 apresenta os valores típicos das razões grão/resíduo reportados na literatura para o arroz e para o milho. Como se pode observar, existe alguma disparidade de valores.

Quadro 3 - Razão entre resíduo e grão para as culturas de arroz e de milho. Valores em ton/ton, base seca

Cultura	**Razão**	**Referência**
Arroz (casca)	0,29	(Matsumura et al. 2005)
Arroz (palha)	1,15	(Matsumura et al. 2005)
Arroz (palha)	1,35	(Matsumura et al. 2005)
Milho (*Corn stover*)	1,0	(Ramachandra et al. 2004)
Milho (*Corn stover*)	1,1	(Matsumura et al. 2005)
Milho (*Corn stover*)	0,9 -1,1	(Kadam e McMillan 2003)

Corn stover – resíduos da cultura do milho incluindo palha e carolo

A estimativa do valor de casca e palha de arroz produzida em Portugal, por cada 10 ton de grão, é de 2 ton de casca e 7,5 ton de palha. Esta estimativa foi obtida através de dados fornecidos pela Orivárzea (www.orivarzea.pt), empresa distribuidora de arroz. Quanto à cultura do milho, foi obtida uma caracterização das duas principais variedades de milho cultivadas em Portugal. Os dados encontrados, obtido no âmbito do desenvolvimento de um Projeto Proder (SecMILHO 2014) estão apresentados nos Quadros 4, 5 e 6 e foram obtidos para plantas cortadas manualmente, à altura de corte da máquina (10-15 cm), pelo que os valores representam o máximo possível de resíduos que poderiam ser retirados dos campos de cultura.

Quadro 4 - Altura e partição da planta de milho para as duas principais variedades cultivadas em Portugal. Os valores apresentados são a média ± desvio padrão para um mínimo de 17 plantas para cada variedade. Os valores de peso apresentados são dados em base húmida.

	Variedades de milho	
	PY1574	PO725
Altura média (cm)	287 ± 26	286 ± 17
Barba (g)	0,9 ± 0.2	1,1 ± 0.2
Folhelho (g)	22 ± 5	19 ± 5
Carolo (g)	50 ± 6	44 ± 4
Caule (g)	194 ± 48	114 ± 39
Folhas (g)	58 ± 11	54 ± 14
Grão (g)	279 ± 32	262 ± 26
Planta completa (g)	597 ± 81	495 ± 67

Quadro 5 - Valores de humidade estimados para as principais frações da planta de milho (%)

	Variedades de milho	
	PY1574	PO725
Carolo	43,2	34,1
Folhas	12,2	9,3
Grão	28,4	23,4
Caule	65,4	62,0
Folhelho	31,8	14,2

Como se pode observar, há diferenças significativas de humidade entre as principais frações e entre as variedades. Estas diferenças são apenas atribuíveis à variabilidade biológica, pois a amostragem foi feita no mesmo dia e em parcelas próximas (menos de 200 m entre si).

No Quadro 6 são apresentadas as razões resíduo/grão para as duas variedades mais cultivadas em Portugal.

Quadro 6 - Razão entre resíduo e grão para a cultura de milho. Valores em ton/ton de grão, base seca

	Variedades de milho	
	PY1574	PO725
Carolo	0,14	0,15
Folhelho	0,08	0,08
Caule	0,34	0,22
Folhas	0,25	0,24
Total	0,81	0,69

Os valores encontrados são inferiores aos reportados na literatura, mas dentro do esperado, pois estes valores variam também com a produtividade agrícola. As parcelas com maior produtividade apresentam tipicamente razões de resíduo/grão mais baixas (Kadam e McMillan 2003). Este efeito é especialmente significativo para produtividades acima das 9 ton/ha, que apresentam valores próximos dos 0,9. A produtividade destas variedades nas parcelas estudadas superior a 12 ton/ha, o que explica os valores encontrados.

Estes resíduos têm pouco ou nenhum aproveitamento. No caso particular da palha, esta pode ser deixada no solo como cobertura após a colheita, o que até uma certa percentagem, é favorável como adubação. Estes valores deverão ser da ordem dos 30% da palha produzida (www.anpromis.pt). Quando em excesso, pode causar problemas ambientais (Agronegócio, 2001). Será ainda de referir que as quantidades de palha produzidas são muito significativas, representando quantitativamente 80% em relação ao grão produzido (Pordesimo et al. 2004).

Atualmente, também a palha de arroz tem uma baixa valorização, sendo usada apenas para a produção de energia por combustão e em camas para animais. Atualmente, as palhas têm um valor de 10 cêntimos/kg e a casca cerca de 4 cêntimos/kg (dados fornecidos pela empresa Orivárzea). Este material, como todas as palhas, apresenta uma baixa densidade mas caracteriza-

se em particular por apresentar quantidades significativas de compostos alcalinos e um teor elevado de cinza (10-17%) que tem dificultado a sua valorização e das quais se destaca uma elevada percentagem em sílica, o que dificulta também a sua utilização para alimentação animal. A sílica pode ainda provocar alguns problemas durante a sua combustão.

1.3.4 Aproveitamento fracionado dos resíduos das culturas de milho e arroz

Devido á composição em polissacáridos das palhas de milho e de arroz (Quadro 7), estes materiais podem ser fracionados e pré-tratados para um aproveitamento integral de todas as suas frações.

Quadro 7 - Composição da palha de milho (% base seca) segundo vários autores

	Componentes	(Egues et al. 2012b)	(Zhong et al. 2013)	(Santos et al. 2013)
Palha de milho	Celulose	49,2	44.8	40,9
	Hemiceluloses	25,6	28.1	31,5
	Lenhina	17,9	7.5	23,1
	Outros			9,5
	Componentes	(Zhong et al. 2009)	(Yu et al. 2010)	(Sun e Sun 2002)
Palha de arroz	Celulose	44,0	36,5	34,7
	Hemiceluloses	20,1	33,8	15,1
	Lenhina	19,0	12,3	19,1
	Cinza	9,8	13,3	16

Para além da atual utilização alimentar, o milho pode ser utilizado para a produção de biocombustíveis ou biopolímeros, o que confere a esta cultura um grande potencial para uma possível aplicação em diversas indústrias (Quadro 8).

Quadro 8 - Utilizações atuais e em investigação para a palha e carolo de milho

Palha de Milho	Produção de biogás	(Zhong et al. 2013)
	Produção de xilitol	(Kadam et al. 2008;Wang et al. 2011)
	Produção de energia (combustão)	(Kumar e Patel 2011)
	Produção de etanol	(Binod et al. 2010;Varga et al. 2004)
Carolo de Milho	Produção absorventes*	(Agrisent, 2014b, 2014c; BestCobLLC, 2014; Morris, 2012
	Produção de xilitol	(Rivas et al. 2006;Wang et al. 2011)
	Produção de materiais de construção*	(Pinto et al., 2012)
	Produção compósitos*	(BestCobLLC, 2014)
	Produção de xilo-oligossacáridos (XOS)*	(Carvalheiro et al. 2009c;Suntory 2001)
	Produção de etanol	(Garrote et al. 2008)

* Produtos industriais comercializados

Os resíduos desta cultura também se assumem como uma fonte de biomassa alternativa para a

produção de energia, por exemplo, biocombustíveis líquidos, como o etanol (Silva et al. 2011) com a vantagem de não competirem com as matérias-primas para utilizações alimentares. Para além disso, como qualquer outro ML pode ainda ser utilizada para a obtenção de vários químicos entre eles, produtos de valor acrescentado, tais como oligossacáridos (Gullón et al. 2012; Kaparaju e Felby 2010). Os resíduos de milho, palha, casca e carolo (*corn stover)*, apresentam também inúmeras aplicações, nomeadamente produção de etanol, compostos fenólicos, xilitol, etc. (Egues et al. 2012a;Gonzalez et al. 2012; Mancilha e Karim 2003).

Tal como os resíduos do milho, também os resíduos da cultura do arroz apresentam potencialidades para a obtenção de produtos de valor acrescentado. A possibilidade de realização de algum tipo de pré-tratamento desta biomassa que permita a remoção parcial das cinzas, nomeadamente da sílica, pode torná-lo num resíduo muito promissor para diferentes utilizações como por exemplo a produção de energia (biocombustíveis líquidos ou gasosos) e a obtenção de produtos químicos de alto valor quer a partir das hemiceluloses quer da lenhina. A partir da lenhina parece bastante promissor a possibilidade de obtenção de compostos fenólicos biologicamente ativos (Quadro 9).

Quadro 9 - Utilizações atuais e em investigação para a palha e da casca de arroz

Palha de Arroz	Produção de xilitol	(Baek e Kwon 2007)
	Produção de energia (combustão)	(Kumar e Patel 2011)
	Camas para animais (aviários)	(Domínguez-Escribá e Porcar 2009)
	Produção de etanol	(Binod et al. 2010)
Casca de Arroz	Produção de xilo-oligossacáridos (XOS)	(Gullon et al. 2011)
	Produção de energia (combustão)	(Park e Jang 2011)
	Produção de carvão ativado (pirólise)	(Park e Jang 2012)
	Produção de etanol	(Yañez et al. 2006)

1.4 Métodos de fracionamento dos materiais lenhocelulósicos

No âmbito de uma biorrefinaria, um pré-requisito para a conversão biológica da biomassa é que esta seja sujeita a processos de pré-tratamento/fracionamento. No Quadro 10 estão representados alguns exemplos.

Para tal, poderão ser utilizados processos físicos, químicos, e/ou biológicos. A principal limitação dos métodos de fracionamento existentes reside na dificuldade de separação de um tipo de componente sem que ocorra alguma degradação na estrutura química dos restantes, pelo que a seleção do método e das condições operacionais do pré-tratamento é fundamental. Existem diversos pré-tratamentos que permitem a desconstrução da biomassa, com o objetivo de remover e despolimerizar parcialmente as hemiceluloses, reduzir a cristalinidade da celulose e remover a lenhina (Taherzadeh e Karimi, 2004).

Quadro 10 – Comparação de alguns processos para o fracionamento de materiais lenhocelulósicos (adaptado de *Carvalheiro et al. 2013*)

	Ácidos			Alcalinos		Hidrotérmicos		Organosolv	Liquidos iónicos	Fluidos supercríticos
	Diluído	Concent.	Sólidos	Hidróxidos	AFEX	Auto-hidrólise	Explosão com vapor			
Temperatura	↑	↓/0	0	↓/0	0	↑	↑	↓/0/↑	↓/0	0/↑
Remoção das hemiceluloses	↑	↑	EE	0	↓	↑	↑	↓/0	↑	↑
Recuperação das hemiceluloses	↑	0	EE	0	↓	↑	0	↓	↑	EE
Remoção da celulose	↓	↑	EE	↓	↓	↓	↓	↓	↓/0/↑	↓
Digestibilidade enzimática	↑	N.A.	EE	↑	↑↑	↑	↑↑	0	↑	EE
Remoção da lenhina	↓	↓	↓↓	↑	↓	↓	↓	↑↑	↑	↑
Formação de inibidores	↓/0	↓/↑	↓	↓	N.A.	↓	↓/0	↓	↓	↓/0
Corrosão de equipamentos	0	↑	↓	↑	↑	↓	↓	0	↓	↓
Energia necessária	↑	↓	↓/0	↓	↑	↑	↑	↓/0/↑	0	↑
Recuperação dos catalisadores	Difícil	Necessário	Fácil	Fácil	Necessário	N.A.	N.A.	Necessário	Necessário	N.A./ Necessário
Formação de resíduos	↑	↓	N.A.	↓	N.A.	↓	↓	↓	N.A.	N.A.
Implementado à escala piloto	Sim	Sim	Não	Sim/Não	Não	Sim	Sim	Sim	Não	Sim

↑, elevado; ↓, baixo; 0, moderado; EE, em estudo; N.A., não aplicável

O principal objetivo é conseguir uma separação eficiente das hemiceluloses, da celulose e da lenhina, evitando a formação de subprodutos indesejáveis como ácidos alifáticos e furanos (resultantes da degradação de açúcares) e de compostos fenólicos, minimizando os gastos energéticos e o recurso a produtos químicos.

1.4.1 Tratamentos seletivos para a hidrólise das hemiceluloses

1.4.1.1 Métodos ácidos

Os processos catalisados por ácidos podem ser divididos em processos que utilizam ácidos concentrados, com recurso a temperaturas moderadas, e processos que utilizam ácidos diluídos, a temperaturas elevadas. Nestes processos, o catalisador mais utilizado é o ácido sulfúrico (H_2SO_4), embora outros ácidos inorgânicos, como o ácido clorídrico (HCl), nítrico (HNO_3) e trifluoroacético (TFA), também possam ser utilizados (Carvalheiro et al. 2008;Girio et al. 2010).

O mecanismo de hidrólise das hemiceluloses em meio ácido envolve três passos principais (Figura 8): no primeiro dá-se a protonação do oxigénio da ligação glucosídica seguida da rutura da ligação glucosídica com a formação de um carbocatião e no fim dá-se a regeneração do ião H_3O^+, por reação do carbocatião com a molécula de água, formando uma molécula final estável.

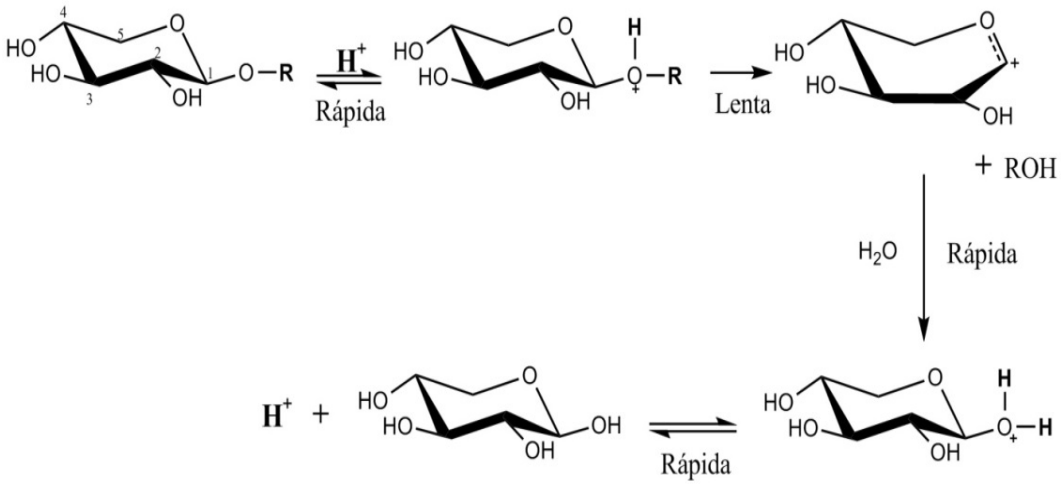


Figura - 8 - Mecanismo proposto para a cisão das ligações glucosídicas na despolimerização das hemiceluloses em meio ácido (adaptado de *Belkacemi et al. 1991*)

No caso da celulose, a formação do carbocatião está dificultada pelas ligações de hidrogénio intramoleculares das cadeias de celulose. Este facto conduz a que a hidrólise das zonas amorfas da celulose ocorra muito mais depressa do que nas zonas cristalinas (Alvira et al. 2010). Uma vez que as hemiceluloses não possuem uma estrutura cristalina, é de esperar que sejam solubilizadas muito mais facilmente do que a celulose. Assim, devido a estas diferenças entre celulose, hemiceluloses e lenhina, é possível selecionar as condições de operação de forma que a hidrólise seja mais seletiva. Deste modo, podem hidrolisar-se os materiais lenhocelulósicos com ácido diluído a temperaturas elevadas que solubilizam a quase totalidade das hemiceluloses ou utilizar-

se ácido concentrado a temperaturas moderadas e provocar a hidrólise total da celulose e das hemiceluloses, permanecendo a lenhina como resíduo insolúvel (Sun e Cheng 2002). As condições de operação vão depender assim dos objetivos que se pretendam.

Hidrólise com ácidos concentrados

Os ácidos concentrados permitem a solubilização da celulose e das hemiceluloses, obtendo-se um resíduo sólido constituído essencialmente por lenhina. Os ácidos concentrados podem atacar as pontes de hidrogénio existentes entre as cadeias de celulose, destruindo a sua cristalinidade. Este tipo de processo ocorre geralmente a temperaturas moderadas, 20-50ºC e em tempos relativamente curtos, 20-60 minutos. A solubilização dos polissacáridos é possível utilizando concentrações variadas, por exemplo, 72% de H_2SO_4, 41% de HCl ou 100% de TFA (Girio et al. 2010). Os dois últimos ácidos têm a vantagem de serem facilmente recuperáveis.

Apesar da formação de quantidades reduzidas de produtos de degradação e a possibilidade de operar a temperaturas e pressões baixas, os custos envolvidos na neutralização dos hidrolisados, recuperação dos ácidos (fundamental para a viabilidade económica do processo) e os problemas associados à corrosão de equipamentos, tornam este processo desvantajoso em relação à hidrólise com ácidos diluídos e métodos hidrotérmicos, por exemplo (Gírio et al. 2010;Taherzadeh e Karimi 2008).

Hidrólise com ácidos diluídos

A hidrólise com ácido diluído é, provavelmente, o método de pré-tratamento químico mais utilizado. Este pode ser usado, por exemplo, como método de pré-tratamento dos ML antes da hidrólise enzimática da celulose. No entanto, este não é suficientemente eficiente na dissolução da lenhina, podendo afetar a sua estrutura e aumentar a suscetibilidade da celulose à hidrólise enzimática. Para além dessa fraca eficiência na dissolução da lenhina, este método é também eficaz na hidrólise seletiva da fração hemicelulósica para a obtenção de monossacáridos, apresentando como vantagens a possibilidade de, em condições controladas, evitar a formação de produtos indesejáveis e corrosão dos equipamentos (Carvalheiro et al. 2008; Taherzadeh e Karimi 2007).

No entanto, para que a fermentação dos hidrolisados seja possível, é necessário proceder à neutralização e/ou destoxificação dos hidrolisados.

Na literatura é possível observar uma grande variedade nas condições de hidrólise com ácido diluído para uma grande diversidade de ML. As diferenças encontram-se fundamentalmente no tipo de ácido utilizado, bem como na sua concentração, temperatura e duração da reação da hidrólise (Quadro 11).

Quadro 11 - Condições operacionais utilizadas na hidrólise com ácido diluído de diferentes materiais lenhocelulósicos.

Material	Ácido	Concent. de ácido (%)	Temperatura (ºC)	Duração (min)	Referências
Resíduos agroindustriais					
Cana-de-açúcar	H_2SO_4	4	120-140	20	(Watson et al. 1984)
Carolo de milho	HCl	2	100	120	(Dominguez et al. 1997)
Palha de arroz	H_2SO_4	3	140-145	20	(Roberto et al. 2003)
Sorgo	H_3PO_4	6	134	300	(Vázquez et al. 2007)
Madeiras Folhosas					
Carvalho	H_2SO_4	2,5	190	30	(Wilson et al. 1989)
Eucalipto	H_2SO_4	3	100-130	60	(Parajó et al. 1997)
Faia	HCl	2,5	165-240	20-40	(Schwiderski et al. 2014)
Madeiras Resinosas					
Abeto	H_2SO_4	2,4	200	30	(Larsson et al. 1999)

1.4.1.2 Métodos hidrotérmicos

Os métodos hidrotérmicos baseiam-se na utilização de água, vapor, ou ambos e calor para o tratamento da biomassa. Nestas condições, ocorre hidrólise dos grupos acetilo das hemiceluloses, com a solubilização das hemiceluloses, deixando a celulose mais acessível, por exemplo, para uma posterior hidrólise enzimática (Agbor et al. 2011;Alvira et al. 2010). A principal diferença destes processos em relação à hidrólise com ácido diluído é que as hemiceluloses são maioritariamente recuperadas na forma oligomérica, enquanto nos processos que utilizam ácidos se obtém fundamentalmente monossacáridos.

Entre os métodos hidrotérmicos distinguem-se dois principais: a auto-hidrólise (*liquid hot water*, LHW) e a explosão com vapor.

Auto-hidrólise

Bobleter e Pape (1968) descreveram pela primeira vez a hidrotermólise como método de pré-tratamento para a posterior hidrólise enzimática. Não existe um termo único para designar este tipo de processos hidrotérmicos, sendo comuns as seguintes designações: auto-hidrólise (Carrasco 1989; Garrote et al. 1999c; Lora e Wayman 1978; Rubio et al. 1998; Schultz e McGinnis 1984; Tortosa et al. 1995), liquefação aquosa (Heitz et al. 1986), extração aquosa (Saska e Ozer 1995), pré-tratamento aquoso (Allen et al. 2001; Overend e Chornet 1987), hidrotermólise (Bonn et al. 1983;Hörmeyer et al. 1988;Walch et al. 1992), aquasolv (Allen et al. 1996), pré-tratamento hidrotérmico (Bobleter et al. 1989), pré-hidrólise aquosa (Conner 1984) e cozimento em água sob pressão (Weil et al. 1997). A auto-hidrólise é um processo cuja eficiência pode ser afetada por

vários fatores, tais como: o tamanho das partículas, a razão líquido/sólido, a temperatura e tempo de reação (Taherzadeh et al. 1997; Vidal et al. 2011), assim como a configuração e grau de polimerização e as suas interações com proteínas e elemento minerais (Magee e Kosaric 1985).

O método de auto-hidrólise baseia-se no uso de água e de calor, com temperaturas entre os 130 e os 240ºC (Quadro 12). Estes tratamentos levam à obtenção de hidrolisados, compostos essencialmente por derivados de hemiceluloses e um resíduo sólido composto maioritariamente por celulose e lenhina (Boussarsar et al. 2009).

Os catalisadores da hidrólise são primeiramente os iões H_3O^+ provenientes da auto-ionização da água e numa fase seguinte os iões H_3O^+ provenientes dos grupos acetilo das hemiceluloses também atuam como catalisadores (Garrote et al. 1999a). Os ácidos urónicos podem também contribuir para a libertação de iões hidrónio mas os seus efeitos não estão bem estabelecidos (Conner 1984). As uniões éter heterocíclicas das hemiceluloses são as mais suscetíveis a este tipo de reação e a sua rutura conduz à formação de oligossacáridos com diferentes graus de polimerização. Uma vez que os açúcares são principalmente obtidos na forma oligomérica, este poderá ser um processo preferencial para a obtenção de oligossacáridos que são compostos potencialmente prebióticos (Carvalho et al. 2013;Gullon et al. 2011; Rivas et al. 2012) (Quadro 12)

Quadro 12 – Condições operacionais utilizadas na auto-hidrólise de diferentes materiais lenhocelulósicos

Material	Temperatura (ºC)	Duração (min)	Referências
Resíduos agroindustriais			
Cana-de-açúcar	200	5-20	(Imman et al. 2013)
Carolo de milho	150-240	0	(Carvalheiro et al. 2009b)
Fibras de milho	210-220	2	(Allen et al. 2001)
Palha de trigo	150-240	0	(Carvalheiro et al. 2009a)
Madeiras Folhosas			
Aparas de oliveira	190-230	0	(Cara et al. 2012)
Eucalipto	145-190	0-7,5	(Garrote et al. 1999c)
Madeiras Resinosas			
Pinheiro	175	26	(Rivas et al. 2012)

Pelo facto de a auto-hidrólise não utilizar outro reagente químico para além da água, apresenta algumas vantagens, nomeadamente problemas de corrosão reduzidos (Carvalheiro et al. 2008; Gullón et al. 2012), a reciclagem de ácidos assim como a remoção de precipitados podem não ser necessários, simplificando o processo. Tudo isto possibilita a redução de custos operacionais de capital, o que proporciona vantagens económicas e impacto ambiental reduzido (Carvalheiro et al. 2008; Garrote et al. 1999c; Girio et al. 2010; Gullón et al. 2012) dos tratamentos aquosos

relativamente a outras tecnologias.

Fator de Severidade

Para determinar as condições operacionais ótimas do tratamento de hidrólise para um determinado material lenhocelulósico, é necessário estabelecer a relação entre as variáveis do processo e as modificações químicas que ocorrem no substrato.

Um modo de analisar os pré-tratamentos é através de parâmetros de severidade, que procuram combinar num único parâmetro empírico o efeito das diferentes variáveis operacionais (Carvalheiro et al., 2009). Estes parâmetros, em geral, incluem a temperatura e o tempo como principais variáveis do processo para monitorizar a extensão da conversa ou rendimento em produtos. O parâmetro R_0, denominado ordenada de reação, proposto por (Overend e Chornet 1989) é traduzido pela seguinte expressão:

$$R_0 = \int_0^t \exp\left(\frac{T-100}{14{,}75}\right).dt$$

Tendo T a temperatura da reação (ºC), t o tempo de residência (min), 100 a temperatura de referência (ºC), temperatura abaixo do qual não ocorre hidrólise e 14,75 um parâmetro empírico relacionado com a energia de ativação.

Explosão com vapor

O processo de explosão com vapor utiliza vapor de água saturado para o aquecimento, o que permitirá um aumento de velocidade de transferência de calor. Este pré-tratamento é caracterizado como um método hidrotérmico, que submete o material lenhocelulósico a altas pressões (0,69-4,83 MPa) e temperaturas (160-240ºC) por um curto período de tempo (Sun e Cheng 2002). Após a conclusão da operação, o material é sujeito a uma descompressão súbita, o que provoca a vaporização da água contida nas fibras, resultando numa explosão (Sun e Cheng 2002). As forças resultantes da descompressão provocam uma desagregação da matriz lenhocelulósica, rompendo ligações inter e intramoleculares. Devido à descompressão, ocorrem ainda modificações estruturais, nomeadamente despolimerização e degradação considerável das fibras do material.

Este método é fundamentalmente utilizado como um pré-tratamento antes da hidrólise enzimática da celulose. A sua utilização como método de obtenção de hidrolisados hemicelulósicos tem a desvantagem de conduzir a uma degradação dos açúcares relativamente elevada (Alvira et al. 2010; Carvalheiro et al. 2008).

1.4.1.3 Métodos enzimáticos

Os pré-tratamentos referidos anteriormente, dão origem a soluções ricas em hemiceluloses, na

forma oligomérica e/ou monomérica. No caso de se encontrarem na forma oligomérica será necessária a sua conversão a monossacáridos de modo a possibilitar a sua utilização pelos microrganismos (Palmqvist e Hahn-Hagerdal 2000). Uma das possibilidades consiste do uso de enzimas, nomeadamente de xilanases (Girio et al., 2010),que ajudam a hidrólise completa das moléculas, por despolimerização da cadeia principal e remoção dos grupos laterais terminais do polímero ou oligómero. São diversas as enzimas envolvidas na hidrólise, sendo as xilanas as principais (Quadro 13).

Quadro 13 - Principais enzimas envolvidas na hidrólise de xilanos (adaptado de(Carvalheiro 2005))

Enzimas	**Modo de ação**
Endo-xilanases	Hidrolisam principalmente as ligações internas de xilose β-1,4 da cadeia principal de xilana
Exo-xilanases	Hidrolisam as ligações de xilose β-1,4, produzindo xilobiose
β-Xilosidades	Produzem xilose a partir da xilobiose e de oligossacáridos de cadeia curta
α-L-arabinofuranosidades	Hidrolisam as extremidades não redutoras de arabinofuranase
α-glucuronidases	Hidrolisam os resíduos de ácido glucurónico e os seus ésteres 4-*O*-metilo

No âmbito da bioconversão dos ML, a hidrólise enzimática com celulases também é muito utilizada para a hidrólise da celulose, após pré-tratamento hidrotérmico ou com ácido diluído.
Uma das vantagens deste processo reside na utilização de temperaturas moderadas, em meios não corrosivos, o que conduz a vantagens económicas, em termos de custos de equipamento e energia. Por outro lado, o preço da enzima bem como a sua recuperação no final do processo acarretam custos e além disso este é um processo demorado, em comparação por exemplo com a hidrólise ácida (Duarte et al. 2004).

1.4.2 Tratamentos seletivos para a solubilização da lenhina

A extração da lenhina é feita em condições em que esta é progressivamente despolimerizada resultando em alterações nas suas propriedades físico-químicas. No entanto, para se conseguirem lenhinas de elevada qualidade para potenciais utilizações de valor acrescentado é necessário o seu fracionamento seletivo. A matéria-prima de onde é extraída assim como o método de extração utilizado tem uma influência significativa sobre a composição e as propriedades desta. Para a valorização da lenhina é preferível a utilização de métodos que promovam a máxima remoção desta fração e que a sua degradação seja a menor possível.

1.4.2.1 Métodos alcalinos

Os métodos alcalinos podem ser divididos em dois grupos principais, dependendo do tipo de catalisador utilizado: tratamentos com soluções de hidróxidos (sódio, cálcio ou potássio) e tratamentos com amónia: AFEX (*ammonia fibre explosion*), ARP (*ammonia recycle percolation*) e

SAA (*soaking in aqueous ammonia*) (Carvalheiro et al., 2008). Estes processos promovem em geral uma maior dissolução da lenhina do que a fração hemicelulósica (Harmsen et al. 2011) ainda que sejam de um modo geral utilizados com o objetivo de solubilizar as hemiceluloses.

Nos processos alcalinos ocorre uma redução da cristalinidade da celulose, aumentando assim a superfície de contacto e a sua porosidade. Nestes processos a recuperação das bases é fundamental para garantir a sua viabilidade económica.

Os processos de deslenhificação propriamente ditos envolvem o uso de bases como os hidróxidos de sódio, de potássio, de cálcio ou de amónio onde a aplicação do composto alcalino provoca a degradação dos ésteres das cadeias laterais resultando em alterações na lenhina, descristalinização parcial da celulose e da hemicelulose. Estes tratamentos alcalinos são, em geral menos severos comparativamente a outros tratamentos (Ruiz et al. 2013b) e têm como objetivo principal a dissolução da lenhina. Um exemplo destes métodos, muito utilizado na indústria da pasta e do papel é o processo Kraft.

1.4.2.2 Processo kraft

Os dois principais processos alcalinos de deslenhificação são o processo soda e o processo kraft. Os dois processos são similares no facto de utilizarem o hidróxido de sódio como principal reagente de deslenhificação, diferindo o processo kraft na aplicação de um componente adicional, o sulfureto de sódio. O processo kraft é um método tradicional no processo de produção de pastas para papel por ser um processo eficaz que facilmente se adapta a vários tipos de madeiras e permite produzir pastas de boa qualidade. Através do uso de hidróxido de sódio e sulfureto de sódio sob condições alcalinas fortes dá-se a clivagem das ligações C-O-C da lenhina resultando na sua solubilização uma vez que as ligações C-C são muito estáveis (Smook, 1988). Este processo de deslenhificação é usualmente efetuado a temperaturas entre 150ºC e 170°C, podendo ser utilizadas temperaturas de cozimento superiores. Após a extração alcalina, a lenhina é recuperada da fração líquida, por diminuição do pH (Rydholm, 1976). Durante este tratamento ocorrem várias reações e para além da lenhina ocorre também a dissolução de parte das hemiceluloses. A lenhina é fragmentada essencialmente pela ação do ião hidroxilo (OH^-) e hidrosulfureto (SH^-) produzindo-se lenhina com grupos tiol alifáticos chamada lenhina Kraft (Figura 9a).

1.4.2.3 Processo sulfito

O processo sulfito é também um processo tradicional utilizado na indústria da pasta para papel. Este método envolve a utilização de sais de amónio ou cálcio e dióxido de enxofre em solução aquosa a diferentes valores de pH (Sixta 1998). Os grupos sulfonato são introduzidos na estrutura da lenhina na posição α da cadeia lateral do propano e assim chamados lenhosulfonatos (Figura 9b). Devido a este grupo a maioria das lenhinas resultantes deste processo são solúveis em água e tornam estas lenhinas diferentes de outros tipos (Clark, 1985).

Este método é ainda é um método utilizado por diversas empresas, em particular na produção de pastas a partir de madeiras resinosas, embora o seu uso tenha decaído em detrimento do

processo Kraft.

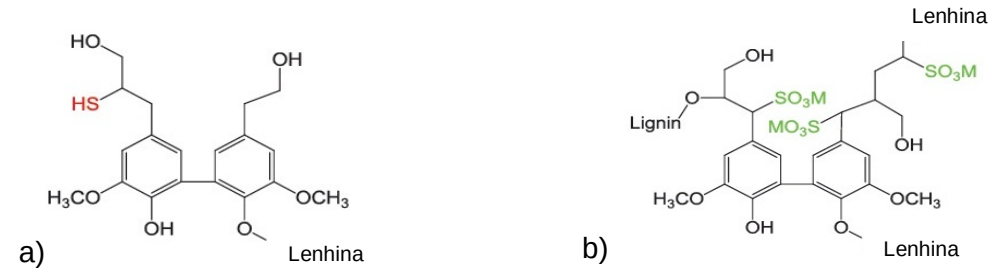


Figura 9 - Lenhina Kraft com grupo tiol (a) e lenhina do tipo lenhossulfonada com grupo sulfonato (b) (adaptado de *Gosselink, 2011*)

1.4.2.4 Métodos com solventes

Estes métodos utilizam os conceitos de solubilização diferencial e de fracionamentos dos vários componentes da biomassa. Existem vários métodos disponíveis sendo os processos organosolv e os baseados em líquidos iónicos dos mais atraentes.

Organosolv

O processo organosolv utiliza um único solvente ou misturas de solventes orgânicos juntamente com água para a remoção da lenhina e de forma a melhorar o processo de deslenhificação podem ser adicionados catalisadores e/ou variar a temperatura. Dependendo do processo utilizado pode também ocorrer alguma degradação dos compostos fenólicos (Buranov e Mazza 2008). Os solventes mais comummente usados são os álcoois e cetonas. As temperaturas utilizadas dependem dos solventes orgânicos escolhidos para o processo e da matéria-prima. Geralmente, temperaturas entre os 150-200ºC podem ser usadas com ou sem adição de catalisadores como os ácidos orgânicos ou inorgânicos (Bozell et al. 2011; Toledano et al. 2012).

O principal benefício do organosolv, como um pré-tratamento consiste na produção lenhina de alta qualidade e na perspetiva de diminuição dos custos associados ao uso de enzimas. Neste processo, de facto, consegue-se separar a lenhina da celulose de forma eficaz antes da hidrólise enzimática. No entanto, este processo também apresenta algumas desvantagens. O solvente por si só pode ser um inibidor para os passos subsequentes à hidrólise enzimática e fermentação (Harmsen et al. 2011). A remoção e recuperação do solvente são passos recomendados de forma a reduzir os custos e também o impacto ambiental que poderia originar (Sun e Cheng, 2002).

Os processos organosolv são processos alternativos aos métodos tradicionais e têm grandes vantagens do ponto de vista ambiental, para além de poderem ser usados para uma gama alargada de matérias-primas incluindo palhas e cascas (El Hage et al. 2010; Huijgen et al. 2010; Huijgen et al. 2012; Mesa et al. 2010; Mesa et al. 2011; Sindhu et al. 2012). Estes processos apresentam vantagens relativamente aos métodos convencionais também pelo facto de se conseguir lenhinas com menores percentagens de enxofre, menor teor em cinzas, maior pureza (devido ao menor teor de hidratos de carbono), e, em geral, menor peso molecular. Estas lenhinas são também, mais hidrofóbicas e menos degradadas resultando desta forma uma maior

possibilidade de poderem ser utilizadas posteriormente para produtos de valor acrescentado, principalmente, devido ao grau de pureza ser elevado (Bozell et al. 2007; Sidrach 2010).

O objetivo principal pode passar por solubilizar a lenhina e obter resíduos sólidos ricos em celulose, para uma hidrólise enzimática posterior. Na deslenhificação organosolv usam-se solventes orgânicos tais como álcoois (metanol, etanol, butanol), acetona e ácidos orgânicos (fórmico, acético) responsáveis pelas clivagens das ligações éter da lenhina e podem ou não ser utilizados juntamente com outros catalisadores como por exemplo HCl,H_2SO_4, $CaCl_2$, entre outros {Stewart, 2008; Lora, 2008). Obtém-se desta forma, uma lenhina chamada lenhina organosolv ou lenhina acetosolv (no caso em que se usa ácido acético). Os solventes utilizados neste processo apresentam, em geral, baixos pontos de ebulição podendo ser recuperados por destilação, são os casos do etanol ou acetona.

O processo Alcell® é um processo bastante conhecido em que se utiliza uma mistura etanol água 50:50 e pode ter os seguintes objetivos, extração de lenhina para produzir celulose, recuperação da lenhina do licor e obtenção de co-produtos (Sidrach, 2010). Um outro processo conhecido é o organocell que utiliza uma base como o hidróxido de sódio, carbonato de sódio e sulfito juntamente com uma solução de metanol e antraquinona (catalisador) de forma a melhorar a deslenhificação (Sidrach, 2010). Este método tem sido utilizado com bastante sucesso na produção de pasta para papel pois produz celulose com grande resistência e permite rendimentos mais elevados comparativamente ao processo Kraft. No entanto, não é habitualmente referido como processo para recuperação de lenhina.

1.4.2.5 Métodos enzimáticos

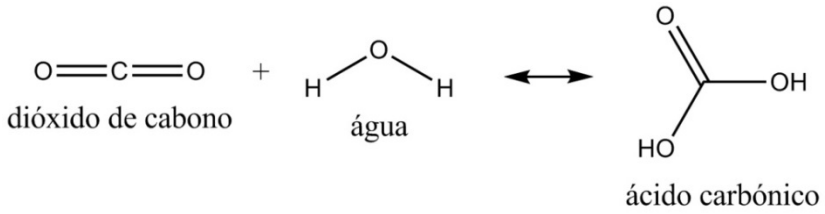
A degradação enzimática da lenhina pode ser levada a cabo com fungos filamentosos, os fungos de podridão castanha e branca. Os fungos da podridão branca, por exemplo *Phanerochaete chrysosporium*, *Cerípoŕia lacerata*, entre outros, são responsáveis pela produção de enzimas, lacases e peroxidases, que conseguem degradar a lenhina de uma forma bastante eficiente (Kneževic et al. 2013). O interesse por processos que utilizem este tipo de enzimas tem aumentado nos últimos anos. A grande diferença entre estes dois tipos de enzimas é o facto de as lacases, que são oxidases, usarem o oxigénio como aceitador final de eletrões, enquanto as peroxidases usam H_2O_2.

Os métodos enzimáticos apresentam diversas vantagens ambientais, na medida em que apresentam requisitos energéticos baixos e dispensam o uso de produtos químicos para além das enzimas. No entanto, as taxas de hidrólise não são, em geral, muito elevadas quando comparadas com outros processos. Além disso, as enzimas podem também utilizar os polímeros (lenhina, celulose e hemiceluloses) como fonte de carbono impedindo a sua recuperação e utilização noutros processos (Kamei et al. 2012). A aquisição destas enzimas e a sua produção pode, por outro lado, encarecer o processo e alguns dos produtos formados podem inibir a hidrólise (Chang et al. 2012).

1.4.3 Outros processos de fracionamento

1.4.3.1 Processos catalisados com CO_2

Como alternativa ao processo auto-hidrolítico convencional, tem sido proposta a hidrólise catalisada com CO_2. O CO_2 em solução aquosa dá origem à formação de ácido carbónico que nessas condições é instável, mantendo-se o equilíbrio de acordo com a equação:



Alguns autores descrevem este método como um processo que pode oferecer os benefícios catalíticos do ácido sulfúrico sem as suas desvantagens (Narayanaswamy et al. 2011;van Walsum 2001), ainda que o ácido carbónico dê origem a soluções com pH relativamente moderado, não oferecendo a mesma capacidade hidrolítica que o ácido sulfúrico. (Van Walsum e Shi 2004) referem que para temperaturas da ordem dos 200ºC, este ácido torna-se um catalisador importante, conduzindo a um aumento do rendimento em xilose, assim como à diminuição do grau de polimerização dos xilanos, em comparação com o uso de uma hidrólise só à base de água (van Walsum e Shi, 2004).

1.4.3.2 Fluidos supercríticos

Os fluidos supercríticos são compostos que se encontram acima de sua temperatura e pressão críticas, mas a uma pressão inferior à necessária para condensá-los (Clark 2002). Com o aumento da temperatura, o líquido torna-se menos denso devido à expansão térmica e à medida que a pressão aumenta o gás torna-se mais denso. Quando as densidades se tornam iguais, a diferença de fase entre líquido e gás desaparece e atingindo o ponto crítico. Os fluidos supercríticos mais utilizados são o dióxido de carbono (Tc= 31,0 °C, pc= 73,8 bar), água (Tc= 374,0 °C, pc = 221,0 bar) e propano (Tc= 96,7 °C, pc= 42,5 bar) (Jessop e Leitner 1999). Acima do ponto crítico, há apenas uma fase de gás. Os fluidos supercríticos demonstram vantagens importantes para utilização em processos químicos. Pequenas mudanças na temperatura ou pressão perto do ponto crítico podem resultar em alterações de solubilidade, o que simplifica o processo de separação.
Não existem ainda muitos estudos de pré-tratamento de materiais lenhocelulósicos por processos supercríticos, nomeadamente por água supercrítica (Miyafuji et al. 2005; Zetzl et al. 2011), ou por CO_2 supercrítico (Kim e Hong 2001). Água em condições supercríticas comporta- se de forma muito diferente da água sob temperatura e pressão normais. Os pré-tratamentos com água super-crítica podem ser bastante eficientes pois a hidrólise é facilitada pelo facto da água de apresentar características ácidas a temperaturas elevadas (Schacht et al. 2008). Assim, as hemiceluloses poderão ser completamente separadas da celulose e lenhina, aumentado a digestibilidade

enzimática de celulose (Kim e Lee 2006;Sasaki et al. 2003). Além disso, a quantidade de hemicelulose dissolvida aumenta com o aumento da temperatura e o tempo do tratamento. No entanto, a formação de pentoses monoméricas e produtos de degradação como o furfural ou HMF e outros subprodutos tóxicos deve ser também considerada.

O uso de CO_2 supercrítico, não origina mudanças significativas ao nível da estrutura microscópica dos ML (Ritter e Campbell 1991), mas pode aumentar significativamente a hidrólise da celulose (Kim e Hong 2001). A utilização de CO_2 geralmente reduz a temperatura do processo e diminui a degradação de xilose aumentando o rendimento da reação (Zheng et al. 1998). Além da fração hemicelulósica, a utilização de CO_2 pode também ser relevante para a separação lenhina ou compostos fenólicos (Persson et al. 2002; Schacht et al. 2008).

1.4.3.3 Hidrólise com sais inorgânicos

Os estudos recentes mostram que o uso de sais inorgânicos tem vindo a ganhar cada vez mais interesse para o pré-tratamento da biomassa. Esses estudos referem que os sais inorgânicos podem aumentar a taxa de hidrólise da celulose e das hemiceluloses durante o processo de pré-tratamento com ácido diluído. Esses sais podem ser, por exemplo KCl, NaCl, $CaCl_2$, $MgCl_2$, $MgCl_2$ ou $FeCl_3$. Em comparação com o processo de hidrólise com ácido diluído, o uso de sais inorgânicos tem demonstrado algumas vantagens como por exemplo uma maior velocidade de reação, a diminuição da corrosão e a possibilidade de recuperação dos sais (Liu et al. 2009). Nguyen e Tucker (2002) descrevem que o uso de uma mistura de ácido diluído com um sal inorgânico permite obter uma maior percentagem de hidrólise da hemicelulose e celulose do que usando só o ácido diluído. Foi também demonstrado que o pré-tratamento com sais facilitou a remoção da hemicelulose dos ML (Sun et al. 2011).

Os sais mais utilizados para o fracionamento dos ML são $FeCl_3$, $FeSO_4$, $Fe(NO_3)_3$, $Al_2(SO_4)_3$, $AlCl_3$ e $MgSO_4$, tendo alguns deles sido testados em vários tipos de materiais lenhocelulósicos como a palha de trigo e os resíduos de milho (*corn stover*). No entanto, de acordo com os estudos já efetuados, os que promovem um melhor fracionamento e consequente remoção das hemiceluloses são o $FeCl_3$ e o $Fe(NO_3)_3$ (Sun et al., 2011; Liu et al., 2009), originando rendimentos elevados de xilose monomérica e oligomérica da fração líquida. Contudo o mais importante não será só a remoção e recuperação da hemicelulose, mas também garantir que a celulose seja pouco afetada (em termos quantitativos) e que a sua digestibilidade enzimática aumente bastante. Este novo método tem vantagens que se sobrepõem à hidrólise com ácidos diluídos, tem ainda a vantagem do passo da neutralização dos hidrolisados ser dispensada, pelo facto destes apresentarem um pH próximo da neutralidade, e ainda a vantagem da redução dos problemas de corrosão do equipamento.

1.4.3.4 Líquidos iónicos

Os líquidos iónicos são compostos por iões, sais fundidos e frequentemente são definidos como um líquido a temperaturas inferiores a 100°C (em muitos casos, mesmo à temperatura ambiente). Os iões têm um grande volume, de modo que as forças de atração entre eles são menores do que

no caso dos sais convencionais não iónicos e, consequentemente, os seus pontos de fusão são baixos. No entanto, eles têm alta viscosidade que dificulta a sua utilização, por exemplo, os fenómenos de transporte são inibidos, o que é uma desvantagem para processos em que são importantes para a transmissão de calor ou de transferência de massa (Gericke et al. 2012; Maki-Arvela et al. 2010). Geralmente, o catião é de natureza orgânica e contém uma estrutura cíclica (Costa et al. 2010; Maki-Arvela et al. 2010). Entre os mais utilizados são o BMIM (1-butil-3-metil-imidazólio), EMIM (1-etil-3-metil-imidazólio) AMIM (1-alil-3-metilimidazólio) e DMEA (N, N dimetiletanolamonio). Os aniões utilizados são muitas vezes simples: os mais comuns são o ião cloreto e o ião acetato. Os líquidos iónicos são apresentados como uma alternativa aos processos de fracionamento convencionais, com a vantagem de dissolver rapidamente os ML, a temperaturas moderadas. Em geral, a dissolução total dos substratos não é possível, mas facilitam os tratamentos químicos ou enzimáticos subsequentes (Dadi et al. 2006; Girio et al. 2010). Os pré-tratamentos com líquidos iónicos têm como principais desvantagens a necessidade da sua reciclagem, essencial devido aos preços elevados que ainda não permitem a sua utilização em grande escala e à toxicidade de alguns deles.

1.4.4 Processos enzimáticos com celulases

A hidrólise enzimática é um dos processos mais utilizados para a hidrólise da fração celulósica, pois pode permitir a conversão quase quantitativa da celulose a glucose (Alvira et al., 2011; Rossberg et al. 2014). No entanto, uma vez que a celulose não está facilmente acessível à ação das enzimas é necessário que os ML sejam sujeito a pré-tratamentos adequados de modo a facilitar a despolimerização. Este processo requere a ação sinergética de três enzimas: endoglucanase, exoglucanase (celobiohidrolase) e β-glucosidase.

As endoglucanases quebram as ligações glucosídicas das cadeias de celulose criando novos terminais; exo-1,4-β-D-glucanases ou celobiohidrolases, responsáveis pela ação nos terminais levando à formação de celobiose; e 1,4-β-D-glucosidades que hidrolisam a celobiose em glucose. As endo-1,4-β-glucanases ou 1,4-β-D-glucana-4-glucano-hidrolases (EC 3.2.1.4) atuam aleatoriamente nas regiões amorfas da celulose, hidrolisando ligações glucosídicas β-(1,4) (Lynd et al. 1999). As celobiohidrolases (exo-1,4-β-D-glucanases, EC 3.2.1.91) atuam nos terminais redutores das cadeias de celulose, ocorrendo a formação de D-celobiose. As "β-D-glucosidases" ou β-D-gluco-hidrolases (EC 3.2.1.21) catalisam a hidrólise em unidades monoméricas de D-glucose a partir da celobiose e celodextrinas solúveis.

Entre os microrganismos produtores de celulases incluem-se principalmente bactérias e fungos. O sistema da celulase de fungos, em particular o de *Trichoderma reesei* tem sido bastante estudado. Este foi o primeiro fungo a ser utilizado na produção industrial de celulases, permanecendo ainda como a fonte mais utilizada. Muitos estudos subsequentes abordaram a sua mutação/seleção para a produção de enzimas comerciais (Den Haan et al. 2001; Szengyel e Zacchi, 2000). O fungo *T. reesei* produz duas celobiohidrolases, cinco endoglucanases e duas β-glucosidades. A configuração molecular das endoglucanases e celobiohidrolases têm um papel importante nas respetivas atividades catalíticas (Lynd et al. 1999).

A hidrólise pode ser facilitada pela redução do teor de lenhina (Vinzant et al. 1997), mas também pela redução do teor de hemiceluloses (Moniz et al, 2013; Rossberg et al. 2014). Neste caso, o principal efeito da remoção das hemiceluloses parece ser o aumento do volume dos poros (Grethlein e Converse, 1991) e o consequente aumento da área superficial disponível para as enzimas. Por este motivo a utilização de processos de pré-tratamento que conduzam à redução da lenhina/hemicelulose e que permitam a exposição da celulose à ação das enzimas, contribuem para a melhoria do rendimento do processo (Jiang et al. 2012; Kumar et al. 2012; Sindhu et al. 2012).

1.5 Valorização e aplicações das hemiceluloses

Para a valorização da fração hemicelulósica da biomassa os tratamentos hidrotérmicos encontram-se entre os métodos de fracionamento mais adequados por serem muito seletivos para esta fração. Dependendo da severidade do tratamento, os produtos resultantes dessa solubilização são oligossacáridos (OS), monossacáridos, produtos resultantes da degradação dos monossacáridos e ácido acético.

Em condições moderadas, os OS são, em geral, o principal produto obtido, sendo as proporções relativas de OS/monossacáridos/produtos de degradação variáveis de acordo com as condições operacionais e com o tipo de tratamento hidrotérmico. Entre os diversos tratamentos hidrotérmicos, a auto-hidrólise permite, em geral, obter rendimentos em OS mais elevados, ao contrário da explosão com vapor, em que os rendimentos em monossacáridos são mais elevados. Esta é, aliás, uma característica típica que distingue estes dois tipos de tratamentos (Bouchard et al. 1991; Carvalheiro et al. 2008;Taherzadeh e Karimi 2008).

1.5.1 Xilo-oligossacáridos

Os oligossacáridos (OS) são compostos massa molecular baixa ou média que apresentam unidades de monossacáridos unidas por ligações glucosídicas. Alguns autores consideram que os OS contêm, em geral, entre 3 a 10 unidades de monossacáridos (Crittenden e Playne 1996), embora sejam muitas vezes considerados nesse grupo, OS com um grau de polimerização mais elevado (Van Loo et al. 1999). O limite entre oligossacárido e polissacárido não está claramente definido.

Os xilo-oligossacáridos (XOS) são OS constituídos por cadeias lineares de xilose, podendo possuir resíduos de arabinose ou outras oses, grupos acetilo e ácidos urónicos como substituintes laterais. Alguns destes XOS têm sido referidos como produtos prebióticos que apresentam propriedades semelhantes ou até melhoradas em relação a outros prebióticos já reconhecidos, como por exemplo os fruto-oligossacáridos (FOS) (Aachary e Prapulla 2011;Gullon et al. 2011;Rivas et al. 2012). O efeito prebiótico define-se como o aumento, induzido pela dieta, do número e/ou atividade de bifidobactérias ou bactérias lácticas no intestino. Por este motivo os microrganismos pertencentes aos géneros *Bifidobacterium* e *Lactobacillus* são os alvos preferenciais em estudos de avaliação do potencial prebiótico. Em termos de microflora intestinal,

é desejável uma proliferação seletiva destes géneros microbianos, que se consideram benéficos para a saúde do hospedeiro.

Os XOS comerciais são obtidos pelo tratamento enzimático de materiais ricos em xilanas, caracterizando-se apresentar baixo grau de polimerização (principalmente constituídos por xilobiose e xilotriose). Uma alternativa económica para a obtenção de XOS com características diversas, consiste no fracionamento seletivo dos materiais lenhocelulósicos por processos hidrotérmicos, como é o caso da auto-hidrólise.

A produção de XOS é feita a partir de carolo de milho, à escala industrial (Suntory 2001), ainda que a produção deste tipo de OS possa ser feita a partir de outros ML ricos em xilanas. Para a hidrólise das xilanas, de modo a obter compostos de menor grau de polimerização poderão ser utilizadas várias abordagens. A primeira será a hidrólise enzimática direta da matéria-prima; alternativamente pode ser efetuado um tratamento químico que vise o fracionamento do ML de modo a isolar (ou solubilizar) as xilanas, seguido de uma hidrólise enzimática dos polímeros a XOS. Pode ainda promover-se a degradação hidrolítica das xilanas a XOS utilizando processos aquosos (água ou vapor) ou soluções de ácidos diluídos (Carvalheiro, 2005).

A produção de XOS pela combinação de processos químicos e enzimáticos consiste, numa primeira etapa, na obtenção dos fragmentos de xilana solúveis, por exemplo, através de uma extração alcalina, da hidrólise com ácido diluído (HAD) ou tratamentos aquosos. Destes, o processo mais utilizado é a extração alcalina, ainda que o custo elevado dos reagentes e tempos de hidrólise, associados à necessidade de uma neutralização posterior, limitem a sua aplicação a nível industrial (Gírio et al. 2012). A HAD tem a desvantagem de conduzir à formação de uma quantidade elevada de monossacáridos e também de produtos de degradação.

Alternativamente, os licores de XOS podem ser obtidos exclusivamente através da reação dos ML com água ou vapor, por um processo de auto-hidrólise, seguido de um processo de purificação. O objetivo da purificação é a remoção de substâncias indesejáveis e/ou a separação de OS com diferente DP. Consoante o(s) método(s) de hidrólise utilizado(s), poderão ser usadas diferentes estratégias de purificação. Essa purificação pode ser feita utilizando processos cromatográficos ou de membrana. A separação simultânea de compostos indesejáveis e de várias categorias de XOS pode ser feita, por exemplo, através de processos de exclusão molecular (Cara et al. 2012; Ho et al. 2014; Moura et al. 2007), cromatografia aniónica (Kabel et al. 2003a) ou combinação de ambos (Kabel et al 2003b). Alguns exemplos de estudos de produção de XOS a partir de diferentes materiais lenhocelulósicos são apresentados no Quadro 14.

A purificação de OS (XOS ou outros) por processos de membrana começou por ser mais utilizada naqueles obtidos por via enzimática. Podem ser utilizadas a ultrafiltração (UF) (Dhara 1991;Kim et al. 2003) ou a nanofiltração (NF), que tem vindo a suscitar um interesse crescente (santos et al., 2011; Gomez et al. 2014; Gonzalez-Munoz et al. 2013; Goulas et al. 2004; Jeon e Kim 2000; Mountzouris et al. 1999; Rivas et al. 2012;). Estes processos apresentam, no entanto, algumas limitações, nomeadamente pelo facto de os produtos obtidos, por exemplo após UF, poderem também conter monossacáridos.

Quadro 14 - Exemplos de estudos de produção de XOS a partir de diferentes matérias-primas.

Material	Tipo de Oligossacáridos	Grau de Polimerização	Referências
Carolo de milho	XOS	2-6	(Moura et al. 2007)
Cascas de arroz	XOS	n.d.	(Vegas 2006)
Farelo de trigo	AXOS	4-37	(Swennen et al. 2006)
Dreche cervejeira	XOS	3-25	(Carvalheiro 2005)
Eucalipto	XOS	n.d.	(Vazquez et al. 2005)
Sementes de algodão	1,4-β-D-XOS	2-15	(Sun et al. 2002)

n.d. – Não descrito

Embora ainda pouco estudados, comparativamente a outros OS comerciais, os XOS apresentam um conjunto de propriedades tecnológicas e efeitos para a saúde, que os tornam adequados para incorporação em muito produtos alimentares. São estáveis numa vasta gama de temperaturas (até 100ºC) e de pH (2,5 - 8) e apresentam um tempo de prateleira elevado, permanecendo estáveis após 2 meses a 37ºC (pH 2,5 - 8) (Suntory 2001), o que é uma vantagem, em particular na gama ácida, comparativamente a outros OS, tais como os FOS, atendendo ao baixo valor de pH do suco gástrico. Além disso, a estabilidade naquela gama de pH é também vantajosa do ponto de vista do processamento de alimentos, permitindo a sua incorporação em produtos com baixo valor de pH.

Como ingredientes alimentares, os XOS, são ligeiramente doces (cerca de 40% do poder adoçante da sacarose), não-cariogénicos e apresentam um baixo valor calórico (Carvalho et al. 2013; Suntory 2001; Vázquez et al. 2000), podendo ser utilizados em alimentos para diabéticos e em produtos alimentares de baixo valor calórico em geral.

Do ponto de vista das suas propriedades funcionais, os efeitos benéficos dos XOS mais estudados e comprovados, tanto *in vitro* como *in vivo,* relacionam-se com o seu efeito bifidogénico (Campbell et al. 1997; Crittenden et al. 2002; Jaskari et al. 1998; Moreno et al. 2014; Okazaki et al. 1990; Rastall 2010; Rastall e Hotchkiss 2003; Rycroft et al. 2001; Suntory 2001; Suwa et al. 1999; Van Laere et al. 1997; Van Laere et al. 2000), conduzindo a um crescimento das bifidobactérias mais elevado do que aquele que se obtém, por exemplo, com OS derivados do frutano (Carvalho et al. 2013).

Os XOS são substratos que proporcionam também um crescimento relativamente elevado de diversas estirpes de *Bacteroides* (Crittenden et al. 2002; Jaskari et al. 1998; Van Laere et al. 2000). Quanto à sua utilização por estirpes de *Clostridium*, parece claro que são assimilados por *C. beijerinckii* (Crittenden et al. 2002; Van Laere et al. 2000), uma estirpe não-patogénica, mas não existe consenso relativamente à sua utilização por microrganismos patogénicos como *C. difficile* (Crittenden et al. 2002; Jaskari et al. 1998; Okazaki et al. 1990).

Os estudos realizados demonstram que a ingestão de XOS, tal como acontece com outros OS, conduz a um aumento da produção de SCFA (principalmente acetato) e lactato (Campbell et al. 1997; Imaizumi et al. 1991), aos quais está associado o abaixamento de pH, o aumento a biodisponibilidade de alguns minerais tais como cálcio e magnésio (Broekaert, et al., 2011) e alguns autores relacionam ainda com a redução do crescimento de bactérias patogénicas (Gibson

e Roberfroid 1995). Os estudos em modelos animais indicam também que estes compostos têm potencialidades para utilização como agentes anti-ulcerosos (Gírio et al. 2004).

Outras aplicações não-alimentares dos XOS incluem a sua utilização como agente de tratamento da dermatite atópica (Yoshinari 2004), agente profilático (Retsuo et al. 2004) e terapêutico para a osteoporose (Retsuo e Schoichi 2004) e como promotor da produção de ácido hialurónico (Hisaya e Shoichi 2004) e de colagénio (Hisaya e Schoichi 2004).

Além das aplicações na indústria alimentar e farmacêutica têm sido estudas outras possíveis aplicações para compostos derivados das xilanas. Os XOS de elevado peso molecular podem ser usados como compostos termoplásticos para o fabrico de plásticos biodegradáveis, filmes solúveis, revestimentos (Ruiz et al. 2013a), e também na formulação de hidrogéis (Gabrielii et al. 2000).

Outros oligossacáridos

À exceção dos OS de soja, que são obtidos por extração direta, e da lactose que é produzida através de uma reacção catalisada por uma base, a produção dos outros OS de grau alimentar envolve processos enzimáticos, nomeadamente reações de transglucosilação a partir de açúcares simples como a sacarose ou lactose (Nilsson 1988; Prenosil et al. 1987), ou hidrólise (enzimática) controlada de polissacáridos como o amido (Nakakuki 1993), inulina (Nilsson 1988) ou xilana (Kaisha 2004; Keiichi et al. 1986; Okazaki et al. 1990).

No Quadro 15 são apresentados alguns dos OS mais representativos existentes no mercado e o seu modo de produção, assim como as respetivas fórmulas químicas (aproximadas). O mercado mundial é dominado pelos OS derivados da lactose, dos quais os principais são os galacto-oligossacáridos (GOS) e a lactulose. Seguem-se os fructo-oligossacáridos (FOS), os isomalto-oligossacáridos e os malto-oligossacáridos.

Para além da procura elevada, os FOS constituem o grupo de OS para os quais existem mais estudos relacionados com os seus efeitos fisiológicos, e também mais evidências do seu efeito prebiótico em humanos (Crittenden 1999). A este tipo de OS aparecem frequente associadas duas designações: oligofrutose, para os OS obtidos a partir da hidrólise da inulina, e FOS para os obtidos por síntese enzimática a partir da sacarose (Quadro 15).

Quadro15- Oligossacáridos mais representativos existentes no mercado e o modo de produção (adaptado de Crittenden e Playne, 1996)

Tipo	Modo de produção	Tipo de ligação e composição
Galacto-oligossacáridos	Síntese enzimática a partir da lactose (transglicosilação pela β-galactosidase)	α-D-Glc-(1→4).(β-D-Gal-(1→6-)$_n$, *n*=2-5
Lactulose	A partir da lactose (isomerização alcalina)	β-D-Gal-(1→4-)-β-D-Fru
Lacto-sacarose	Síntese enzimática a partir da lactose e sacarose (transglicosilação pela β-frutofuranosidase)	β-D-Gal-(1→4-)-α-D-Glc-(1→2)-β-D-Fru
Fruto-oligossacáridos	Síntese enzimática a partir da sacarose (transglicosilação pela β-frutofuranosidase)	α-D-Glc-(1→2)-(β-D-Fru-(1→2-)$_n$, *n*=2-4
Oligofrutose	Hidrólise enzimática da inulina (inulinase)	β-D-Fru-(1→2-)-(β-D-Fru-(1→2)$_n$, *n*=1-9 α-D-Glc-(1→2-)-(β-D-Fru-(1→2)$_n$, *n*=2-9
Oligossacáridos de soja	Extracção a partir do soro de soja	(α-D-Gal-(1→6)-)$_n$,-α-D-Glc-(1→2)-β-D-Fru, *n*=1-2
Xilo-oligossacáridos	Extracção do xilano de carolo de milho Hidrólise enzimática (endo-1,4-β-xilanase)	(β-Xyl-(1→4)-)$_n$, *n*=2-9

1.5.2 Outros produtos derivados das hemiceluloses

Tal como nos OS, o tipo de monossacáridos presente nos hidrolisados depende da origem das hemiceluloses. No caso de madeiras resinosas, os açúcares hemicelulósicos são essencialmente glucose e manose, enquanto a xilose é o principal monossacárido obtido a partir da hidrólise das hemiceluloses de folhosas. Nos materiais de natureza agrícola, para além da xilose, a arabinose pode também representar uma percentagem importante dos açúcares hemicelulósicos. Para além dos monossacáridos e do ácido acético proveniente da hidrólise dos grupos acetilo das hemiceluloses, os hidrolisados contêm também produtos resultantes da degradação dos monossacáridos, nomeadamente furfural e HMF, que são originados pela desidratação das pentoses e hexoses, respetivamente. A degradação do HMF, por sua vez, dá origem à formação de ácido levulínico (Ulbricht et al. 1984). O ácido fórmico é outro ácido alifático que pode estar presente nos hidrolisados e que se forma pela degradação do furfural e do HMF (Dunlop 1948) ou ainda a partir dos grupos metoxilo das hemiceluloses (Puls et al. 1985;Taherzadeh 1999). Dos ácidos alifáticos referidos, o acético é, quantitativamente, o mais importante. Em geral, são as madeiras folhosas que dão origem a uma maior libertação de ácido acético, seguidas dos materiais de natureza agrícola e das madeiras resinosas.

Durante a hidrólise, uma parte da lenhina pode também ser solubilizada dando origem a uma vasta gama de compostos fenólicos. No caso de reações catalisadas por ácidos sujeitas a calor, a formação de compostos aromáticos de baixa massa molecular pode também decorrer da degradação das pentoses e dos ácidos urónicos (Popoff e Theander 1972). Outra fonte de compostos fenólicos são os extrativos (Alén 2000;Pereira et al. 2003;Sjöström 1981).

Os licores provenientes da auto-hidrólise são especialmente ricos em OS, sendo descritos rendimentos em XOS relativamente elevados, da ordem dos 65-70% (Moura et al. 2007; Garrote et al. 2002). Estes licores de oligossacáridos para além de poderem ser utilizados para a produção de OS de grau alimentar, podem também ser utilizados como meios de cultura para bioconversões, nomeadamente para a produção de combustíveis (*e.g.*, etanol) ou outros produtos de valor acrescentado como os polióis (*e.g.*, xilitol ou arabitol).

1.5.2.1 Xilitol

Nos hidrolisados hemicelulósicos provenientes de materiais agrícolas, a xilose é o monossacárido presente em maior quantidade. Uma das possibilidades bastantes atrativas para a valorização destes hidrolisados consiste na sua utilização para a produção xilitol.

O xilitol é um poliol ($C_5H_{12}O_5$) com poder adoçante semelhante ao da sacarose e superior ao de outros polióis como o sorbitol ou o manitol. É encontrado como intermediário normal do metabolismo de hidratos de carbono em mamíferos e também em pequenas quantidades em algumas frutas, legumes (Hyvönen et al., 1982) e árvores de fruto (Winkelhausen e Kuzmanova, 1998).

O principal foco de interesse do xilitol provém do seu potencial como edulcorante alternativo, já que apresenta um baixo valor calórico (4,0 kcal/g) e um poder adoçante semelhante ao da sacarose e superior ao de outros polióis. Trata-se de um pó branco, cristalino, sem odor, e

altamente solúvel em água. O valor comercial do xilitol, assim como o seu mercado-alvo, que ao longo dos tempos tem vindo a crescer, provém de um conjunto de propriedades físicas, químicas e tecnológicas vantajosas deste produto para a utilização nas indústrias alimentar, cosmética e farmacêutica.

A concentração elevada de xilose presente em muitas espécies de biomassa vegetal e consequentemente nos hidrolisados hemicelulósicos resultantes de pré-tratamentos dessas espécies de biomassa, poderá ser aproveitada para a obtenção de produtos de valor acrescentado como xilitol.

A produção biotecnológica de xilitol consiste em converter a xilose em xilitol mediante a utilização de microrganismos. Existem bactérias, fungos e leveduras que apresentam essa capacidade. As leveduras das espécies *Candida guillermondii*, *Candida tropicalis* e *Debaryomyces hansenii* são referidas na literatura como as melhores produtoras de xilitol (Chen et al. 2010;Wang et al. 2011).

1.5.2.2 Butanodiol

O 2,3-butanodiol, também conhecido como 2,3-butileno glicol (2,3-BD) é um composto químico com várias aplicações, tais como combustível líquido, solvente e precursor de vários polímeros sintéticos e resinas (Saha 2003). O butanodiol é produzido por fermentação, em condições de limitação de oxigénio (Kosaric 1992). Potencialmente, todos os açúcares vulgarmente presentes nos materiais lenhocelulósicos podem ser convertidos em butanodiol. São vários os microrganismos, com capacidade de levar a cabo essa bioconversão entre os quais os dos géneros *Aeromonas* (Willetts 1984), *Paenibacillus* (Nakashimada et al. 2000) e *Klebsiella* (Qureshi et al. 2008).

O butanodiol começou por ser produzido pela fermentação de melaços (Afschar et al. 1993) ou da glucose resultante da hidrólise do amido (Willetts 1984). Têm sido efetuados vários estudos com vista à utilização de outros substratos para a produção deste composto. Um exemplo, é o estudo apresentado por (Qureshi et al. 2008) para a produção de butanodiol por *Klebsiella oxytoca* a partir da fermentação de hidrolisados de carolo de milho.

1.5.2.3 Furfural

O furfural é um aldeído heterocíclico e aromático de fórmula molecular $C_5H_4O_2$, também conhecido como 2-furanocarboxialdeído, furaldeído, 2-furanaldeído, fural e furfuraldeído. É produzido comercialmente a partir de materiais lenhocelulósicos por um processo que envolve a hidrólise ácida das xilanas ou pentosanas, presentes nas hemiceluloses de resíduos agrícolas ou madeiras. As xilanas são hidrolisadas em xilose e outras pentoses e por desidratação destes monómeros ocorre a formação de furfural (Mamman et al. 2008). A utilização de ácidos convencionais como o H_2SO_4 ou o HCl, para a produção de furfural causa alguns problemas de corrosão dos equipamentos e problemas ambientais. Para ultrapassar estas desvantagens é ainda necessário desenvolver tecnologias de produção de furfural mais favoráveis do ponto de vista ambiental (Vazquez et al. 2007).

O furfural embora possa ser utilizado como produto final, por exemplo como solvente, a sua

aplicação principal é como precursor nomeadamente para a preparação de outros solventes orgânicos, como o álcool furfurílico e o tetra-hidrofurano (THF). O THF é um solvente industrial obtido através da hidrogenação do furano que, por sua vez, é obtido por descarbonilação catalítica do furfural. O álcool furfurílico é produzido pela hidrogenação catalítica do furfural e apresenta uma série de aplicações na indústria química, tais como, precursor do álcool tetra-hidrofurfurílico, na produção de resinas e como intermediário na produção de fragrâncias e vitamina C (Nicholsa et al. 2008).

1.6 Valorização e aplicações da lenhina

Para o aproveitamento integrado de todas as frações dos ML é importante valorizar todas as frações, nomeadamente a lenhina (Octave e Thomas 2009). A lenhina faz parte, por exemplo, da composição dos subprodutos da indústria da pasta para papel, estando por isso disponível em grandes quantidades. Atualmente, a maior parte desta lenhina é usada como fonte energética e apenas uma pequena parte para a obtenção de químicos (Zakzeski et al. 2010). São produzidas cerca de 40 a 50 milhões de toneladas por ano de lenhinas (www.ili-lignin.com).

A razão principal da lenhina ter merecido menor atenção comparativamente à celulose deve-se à sua complexidade química e estrutural embora nos últimos 10-15 anos tenha havido um aumento na investigação de processos e produtos de valor acrescentado relacionados com este biopolímero. Quando separada e purificada, a lenhina origina compostos de peso moleculares inferiores relativamente ao da lenhina nativa (Toledano et al. 2010) e com características distintas, nomeadamente a hidrofobicidade e propriedades antioxidantes (dependem do número e posição dos grupos hidroxilo e do peso molecular) (Boeriu et al. 2004), apresentando desta forma uma grande potencialidade para a produção de compostos aromáticos (Pilon e Lavoie 2011) e biocombustíveis (Acar e Ayanoglu 2012). Outras formas alternativas para o uso da lenhina consistem na sua incorporação noutros materiais poliméricos, nomeadamente em polímeros condutores, poliuretanos, termoplásticos, vedantes, placas de bateria, agentes sequestrantes, gesso, entre outros e também na produção de resinas fenólicas, ligantes e colas (Bozell et al. 2007; Toledano et al. 2010; Toledano 2012). Na Figura 10 estão apresentadas algumas aplicações possíveis relacionadas com a valorização da lenhina.

Estão já disponíveis algumas formas de valorização da lenhina à escala industrial, como é o caso dos lenhossulfonatos provenientes da indústria da pasta para papel. Por exemplo, o processo Lignoboost produz sulfito de lenhina que pode ser utilizado como um dispersante produzido pela Inventia (www.inventia.com). Também a Borregard (www.borregard.com) produz lenhossulfonatos e derivados de lenhina, tais como vanilina. A diversidade de grupos funcionais da lenhina permite usar este polímero como dispersante em cimentos e gessos (Matsushita et al. 2008), como emulsionante (Boeriu et al. 2004) ou agente quelante para a remoção de metais pesados em efluentes industriais (Sena-Martins et al. 2008).

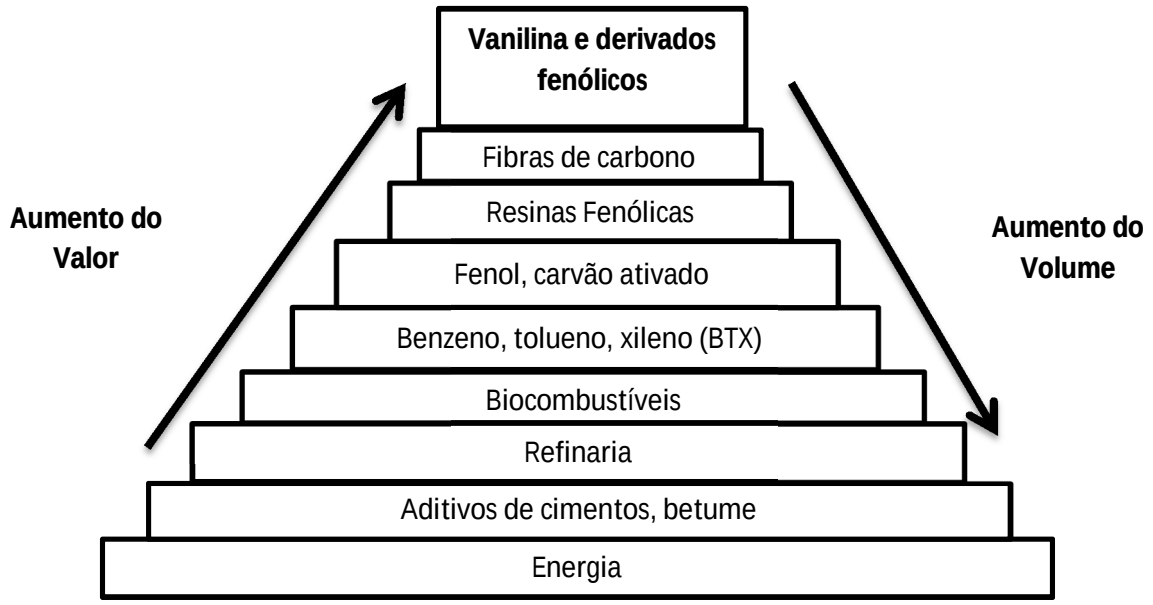


Figura 10 - Aplicações possíveis relacionadas com a valorização da lenhina (adaptado de *Gosselink, 2011*)

A complexidade da organização estrutural da lenhina, bem como a sua elevada porosidade sugerem que a lenhina tenha excelentes propriedades como agente adsorvente, mesmo em comparação com os carvões ativados (Mohan et al. 2006). Estas resinas podem ser obtidas utilizando tratamentos de carbonização da lenhina e a subsequente ativação pela gaseificação (Rodriguez-Mirasol et al. 2005).

A lenhina é a fonte mais abundante de compostos fenólicos na natureza, podendo gerar uma grande quantidade de químicos ou adesivos para substituir alguns produtos que são produzidos a partir de derivados do petróleo. Um exemplo são as resinas de fenol-formaldeído (Mansouri et al. 2005;Tejado et al. 2007a; Tejado et al. 2007b), em que parte do fenol pode ser substituída por lenhina, resultando num produto menos tóxico e mais económico do que o fenol.

O alto teor de enxofre (1-2% p/p) da lenhina kraft é um dos motivos pelos quais a sua aplicação principal tem sido na geração de energia (Doherty et al. 2011). Os lenhossulfonatos têm sido descritos como não adequados para posterior conversão em produtos químicos de valor acrescentado devido à presença de substituintes de enxofre e sais na estrutura destas lenhinas (Sannigrahi et al. 2011; Zhang et al. 2011). Na Figura 11 pode-se observar as relações entre os mercados potenciais de lenhina, a produção e os valores reais que os produtos derivados de lenhinas sem enxofre podem atingir.

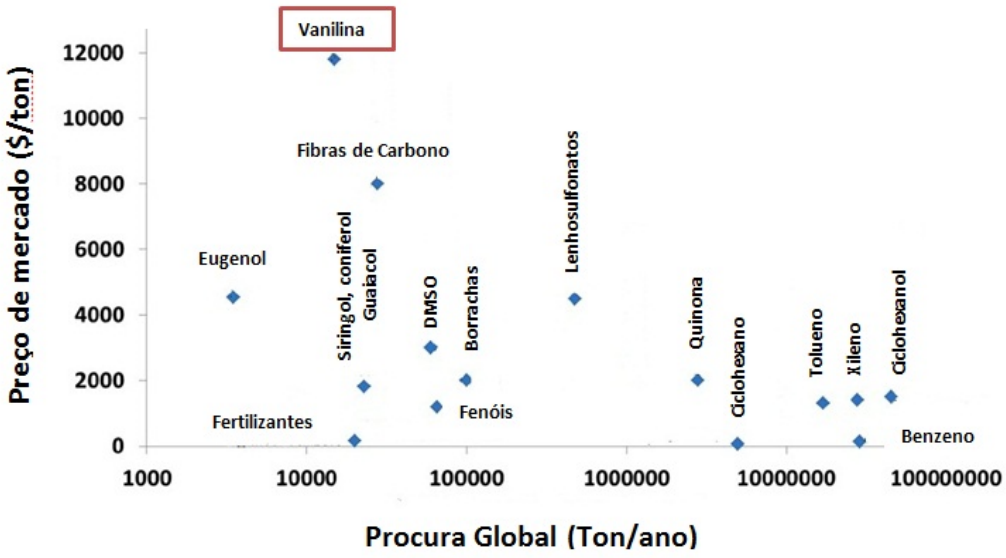


Figura 11 - Preços de alguns derivados da lenhina e a sua procura global (adaptado de *Varanasi et al. 2013*)

De entre os compostos fenólicos presentes na lenhina destacam-se alguns deles com valor comercial elevado como a vanilina, ácido ferúlico, ácido cumárico, ácido *p*-hidroxibenzóico, ácido vanílico, ácido siríngico, siringaldeído e *p*-hidroxibenzaldeído (Buranov e Mazza 2008b;Clark et al. 2009). O ácido ferúlico, por exemplo, está presente em concentrações elevadas em plantas como o arroz e milho e ultimamente tem sido muito estudado devido às suas grandes potencialidades de utilização como aditivo alimentar (como conservante e percursor de sabor), na área da saúde (como antioxidante, agente antimicrobiano e anti-inflamatório) e ainda em cosméticos como agente protetor da pele (Conde et al. 2011;Max et al. 2009). A vanilina em particular tem também propriedades muito interessantes e é usada como aromatizante em produtos alimentares, perfumes, desodorizantes, entre outros. Inicialmente a vanilina comercial era extraída dos grãos de baunilha mas com o aumento da procura começou a produzir-se também vanilina sintética chamada também de baunilha artificial (etil vanilina). Esta última é muito mais barata comparativamente à vanilina natural que pode custar até cerca de 10 000€/ton. Hoje em dia apenas 5% da vanilina comercializada é obtida a partir da lenhina (Mishra et al. 2013; Staniek et al. 2014; Zamzuri e Abd-Aziz 2013).

O aproveitamento da lenhina pode ser dividido em três categorias principais: i) fonte de carbono por uma quebra agressiva da sua estrutura polimérica; ii) aproveitamento da sua estrutura macromolecular para aplicações de elevado peso molecular e iii) quebra da estrutura macromolecular da lenhina mantendo a sua natureza aromática (Bozell et al. 2007).

Na primeira categoria podem ser obtidos como produtos os chamados *"green fuels"*, produzidos principalmente a partir de lenhinas lenhossulfonadas (Fig. 12); na segunda categoria estão inseridos produtos como as resinas e as fibras de carbono e na última categoria podem obter-se produtos de valor acrescentado como por exemplo os compostos fenólicos (Fig. 13).

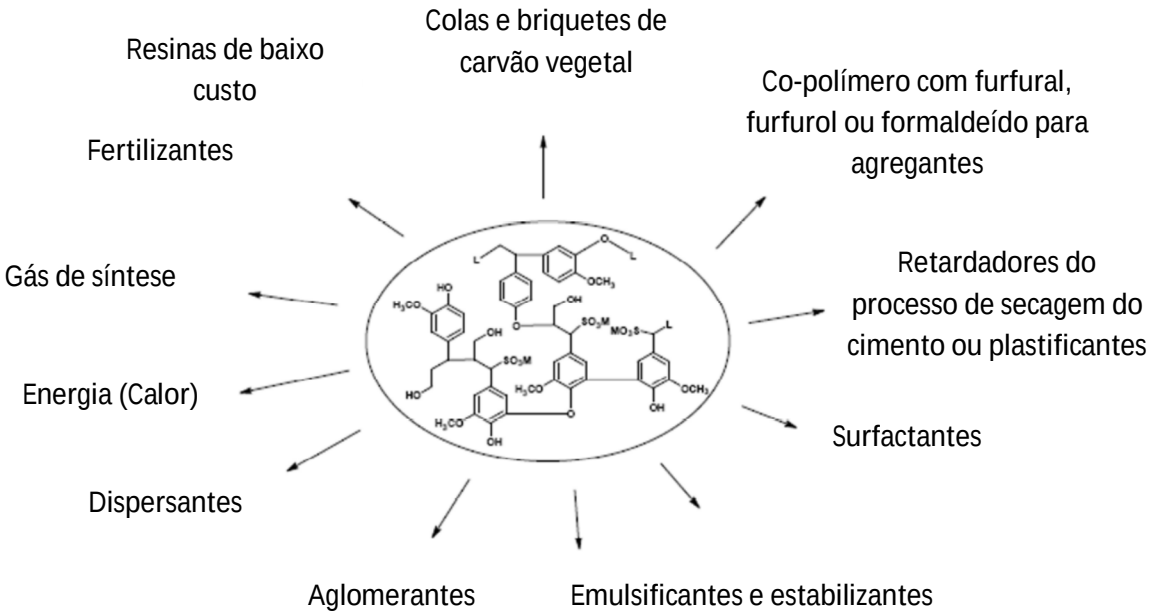


Figura 12 – Produtos produzidos a partir de lenhinas lenhossulfonadas. (adaptado de *Bozell, 2007*)

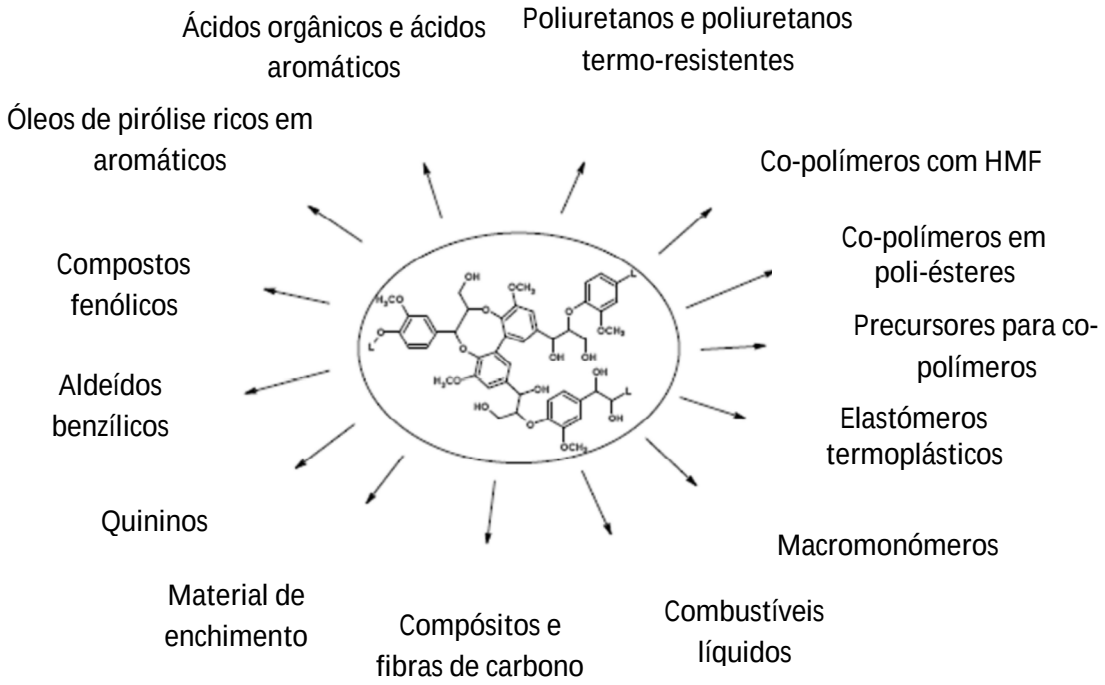


Figura 13 – Categorias de potenciais produtos de valor acrescentado que podem obter-se a partir da lenhina. (adaptado de *Bozell, 2007*)

Após deslenhificação é possível recuperar a lenhina solubilizada na forma sólida. O processo convencional consiste na acidificação dos licores. A precipitação pode ser intercalada com etapas de filtração e lavagem.

O interesse pela obtenção de frações definidas da lenhina, constituídas por compostos fenólicos de diferentes pesos moleculares e com potencial valor acrescentado, tem também vindo a aumentar. Tal deverá passar pelo estudo da sua separação, nomeadamente através da utilização de sistemas de membranas, por exemplo nano filtração, resistentes a solventes (Koncsag e Kirwan 2012; Toledano et al. 2010; Toledano et al. 2013). Alternativamente estes compostos poderão também ser separados e/ou purificados recorrendo a técnicas cromatográficas por processos semelhantes aos descritos para a purificação de oligossacáridos (Bozell et al. 2011; Javor et al. 2000).

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Capítulo II. Characterisation and hydrothermal processing of corn straw towards the selective fractionation of hemicelluloses

Abstract

Corn straw was chemically and anatomically characterized. Hydrothermal processing (autohydrolysis) was used for the selective solubilisation of hemicelluloses. The raw material was treated under non-isothermal conditions (150-240ºC) and the effects on the composition of both liquid and solid phases were evaluated.

The yields and composition of the solid fraction and soluble products are presented and interpreted using the severity factor (log R_0). The operational conditions for the maximum yield of xylo-oligosaccharides (XOS) of 53% of initial xylan, were established for log R_0 of 3.75. Under these conditions 72% of xylan was hydrolysed while cellulose and lignin were not substantially affected although an increase in the enzymatic digestibility of cellulose was attained. For the severest condition (log R_0=4.51) the solids contained 61.7% glucan and 31.0% lignin.

The XOS rich liquors and the glucan and lignin enrichment of the solid phase make corn straw a suitable raw material in a biorefinery framework and the hydrothermal treatment a favourable first step in the processing.

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Introduction

Plant biomass has the potential to play an important role in the global energy future because it can be grown in a sustainable manner and converted into a variety of products, namely biofuels and chemicals, using biochemical, thermochemical or catalytic conversion processes (Tyndall et al. 2011). In this context, agricultural residues are an interesting and potentially low-cost biomass source. Among them, corn (*Zea mays* L.) residues contribute with the largest quantities in the USA, and are also abundant in China, Brazil and EU. Their chemical composition and low-cost make it an attractive feedstock to be used in a biorefinery framework.

Before they can be converted into biofuels and different added-value products, lignocellulosic materials need to be fractionated into their main macromolecular components, cellulose, hemicelluloses and lignin (Carvalheiro et al. 2008). Among the promising biomass pretreatment options available, namely, autohydrolysis, steam explosion, and alkaline, organosolv and oxidation treatments, or their combinations, many produce a sugar-rich liquid stream derived from a selective hemicellulose solubilisation. Actually, the partial hydrolysis of hemicelluloses can enable a reduction both on energy requirements and especially on the formation of many relevant sugar degradation compounds, particularly of hydroxymethylfurfural (HMF) and furfural, that can inhibit the upgrade of both the liquid and solid fractions (Duarte et al. 2009). One approach is to perform an initial step by treating the lignocellulosic materials with a hydrothermal (or autohydrolysis) process which allows a high recovery of the solubilised hemicelluloses, both as oligosaccharides, and to less extent as monosaccharides, while cellulose and lignin can be recovered in the solid phase with minor losses (Carvalheiro et al. 2004;Garrote et al. 2003;Howard R.L. et al. 2003).

Since autohydrolysis only uses water as reagent, several advantages are associated with this process as compared to acid hydrolysis, such as low by-product generation, limited problems derived from equipment corrosion owing to the mild pH of reaction media and reduction of operational costs since further neutralisation can be omitted (Carvalheiro et al. 2004;Carvalheiro et al. 2008;Garrote and Parajó 2002) . The process can be oriented either towards the production of xylo-oligosaccharides (XOS), which are potential ingredients for the pharmaceutical and functional food markets, or to obtain hemicellulose-free solids for further valorisation (Carvalheiro et al. 2009;Garrote et al. 2007;Nabarlatz et al. 2007).

Several biological activities of XOS have been described such as prebiotic effect, antioxidant activity, protective effect against lipid peroxidation, and anti-inflamatory activity, among others (Garrote et al. 1999;Moure et al. 2006).

The aim of this work is to study the selective fractionation of hemicelluloses from corn straw using hydrothermal treatments (autohydrolysis). The effects of pre-treatment on the yield of XOS, monosaccharides and by-products, together with the effects on both cellulose and lignin recovery, were evaluated and interpreted using the severity factor (log R_0). In addition, anatomical studies were conducted to provide detailed anatomical characterization of corn straw, which is useful to assess potential structural requirements on polymers recovery.

Material and methods

Raw material

Corn straw was supplied by Estação Nacional de Melhoramento de Plantas (Elvas, Portugal) as an heterogeneous sample containing stalks and leaves as obtained as field agricultural residue. As described in the literature, stalks represent about 70% and leaves 30% (Pordesimo et al. 2004) of this type of raw material. Upon arrival, the raw material had a moisture content of approximately 10%, and was ground with a knife mill (Fritsh Industriestr, Germany) to particles smaller than 6 mm. Granulometric separation was performed using a shaker and seven sieves (Retsch, Germany) with different pore sizes (0.25, 0.355, 0.5, 1, 2, 4 and 6 mm).

After granulometric characterization of all fractions, particles smaller than 250 μm were discarded. The remaining material was then homogenised in a combined feedstock, and stored in plastic containers at room temperature. All experiments were carried out using this feedstock.

Anatomy

Corn stalks were cut as small cross-sectional discs and after inclusion in polyethylene glycol 1500, thin transverse and longitudinal sections (±17 μm thickness) were cut with a Reichert sliding microtome, stained with chrysodine and astral blue and mounted in Euparal. Samples were also prepared for maceration with a solution of glacial acetic acid: 20% hydrogen peroxide 1:1, at 60 ºC for approximately 48 h.

The light microscopic observations were made using a Leica DMLA microscope and photomicrographs were taken with a Nikon Microphot – FXA.

Scanning Electron Microscopy

Samples of corn straw were gold coated and observed using different magnifications with a Hitachi Scanning Electron Microscope (SEM) (S-2300; Hitachi, Japan) using 20kV accelerating voltage.

Both the corn raw material and the solid residues obtained after autohydrolysis treatments at 215ºC and 240ºC were observed.

Hydrothermal processing of corn straw

Autohydrolysis treatments of the corn straw lot, containing all the granulometric fractions but one (< 0.25 mm), were performed in a stainless steel reactor (Parr Instruments Company, USA) with a total volume of 600 ml. Temperature was controlled through a Parr PID controller (model 4842). The raw material was mixed with water in the reactor in order to obtain a liquid-to-solid ratio (LSR) of 10 (g water/g dry raw material). The agitation speed was set at 150 rpm and the reactor heated to reach final temperatures ranging between 150ºC and 240ºC (non-isothermal conditions). The effects of temperature on corn straw autohydrolysis were interpreted based on the severity factor, log R_0 (Overend and Chornet 1987):

$$R_0 = \int_0^t \exp\left(\frac{T(t) - Tref}{14.75}\right) dt$$

where the temperature T (ºC) is a function of time t (min) and Tref is the reference temperature (100ºC). The value 14.75 is an empirical parameter related with activation energy and temperature.

When the desired temperature was attained, the reactor was rapidly cooled down and the liquid and solid phases were recovered by pressing (up to 190 bar) using a hydraulic press (Sotel, Portugal). The liquid phase was filtered (Whatman filter paper no. 1) and the solid phase was washed, filtered again, dried at 40ºC and the composition and enzymatic digestibility was determined as described below.

Analytical Methods

Chemical characterisation of raw material and processed solids

The materials were ground in a knife mill (IKA, Germany) to a particle size smaller than 0.5 mm and the moisture content was determined by oven-drying at 100°C to constant weight. The ash content was determined at 550ºC using NREL/TP-510-42622 protocol (Sluiter et al. 2005). For extractives determination, the samples were successively extracted with dichloromethane, ethanol, and water (each for 4.5 h), with an adapted Soxtec extraction system (Gominho et al. 2009).

The samples were analysed for glucan, xylan, arabinan and acetyl groups after quantitative acid hydrolysis with 72% (w/w) H_2SO_4 followed by hydrolysis with 4% (w/w) H_2SO_4. The acid insoluble residue was considered as acid insoluble lignin, after correction for ash. Monosaccharides (glucose, xylose, arabinose) and acetic acid in the hydrolysates were analysed by high-performance liquid chromatography (HPLC). A sample of the hydrolysate was injected in HPLC (Waters, Milford, MA), equipped with an refractive index (RI) detector (Waters 2410), an ultraviolet (UV) detector (Waters 486) and an Aminex HPX-87H column (Bio-Rad, Hercules, USA) in combination with a cation H^+-guard column (Bio-Rad). Elution took place at 50ºC with 5 mmol/L H_2SO_4 at a flow rate of 0.4 mL/min.

Chemical characterisation of liquors and hydrolysates

The liquors were directly analyzed by HPLC. Elution took place at 50ºC with 5 mmol/L H_2SO_4 at a flow rate of 0.6 mL/min. Glucose, xylose, arabinose and acetic acid were detected with the RI detector; furfural and HMF were detected with the UV detector at 280 nm.

Another sample was hydrolysed with 4% (w/w) H_2SO_4 to convert soluble hemicelluloses into their constituent sugar monomers. The oligosaccharides concentrations were calculated from the increase in sugar monomers, as analyzed by HPLC, after acid post-hydrolysis. The term XOS has been used to name the hemicelluloses-derived oligosaccharides made up of xylose and arabinose units.

Total phenolic compounds were determined by the Folin-Ciocalteu colorimetric method according to Singleton and Rossi (Singleton et al. 1999). Briefly, 100 µL of the sample was mixed with 5 ml of the 1/10 (v/v) diluted Folin-Ciocalteu reagent and 4 ml of 7.5% Na_2CO_3. Absorbance was measured at

765 nm after 15 min incubation at 45ºC. Total phenolic compounds are expressed as mg GAE/ml (gallic acid equivalents).

FTIR Analysis

For FTIR spectra acquisition the extractive-free samples were first dried in an oven at 60ºC overnight and further dried over P_2O_5 in a vaccum desiccator.

The spectra were recorded with standard KBr pellet technique in a Nicolet 740 spectrometer with a dTGS/KBr detector at 4 cm^{-1} resolution. The pellets were prepared by mixing 0.5 mg of the material powder with moisture free KBr, spectroscopic grade. The spectra were rationed against pure KBr, and 64 scans were taken per sample in wavenumber range 4000 to 500 cm^{-1}.

Enzymatic hydrolysis

The enzymatic digestibility of the untreated and pretreated corn straw was evaluated based on the NREL/TP-510-42629 protocol (Selig et al, 2008). The results were expressed as the percentage of glucose released after 72 h enzymatic hydrolysis in relation to initial glucose. All assays were performed at least in duplicate and the results are given after correction for enzyme and biomass blank tests.

Results and discussion

Raw material characterisation

Figure 1 represents the anatomical structure of the stalk of corn (*Zea mays* L.) samples as observed by optical microscopy. It consists of epidermis, cortex and the ground parenchyma tissue with embedded vascular bundles (Fig. 1a).

The epidermis forms the outermost layer as a continuous uniseriate layer of closely packed epidermal cells covered at the outside by the cuticle and a cutin layer, showing frequent stomata openings (Fig. 2).The cortex is a thin layer with thickened parenchyma cells under the epidermis. The ground parenchyma tissue shows large and thin walled parenchyma cells (Fig. 1c).

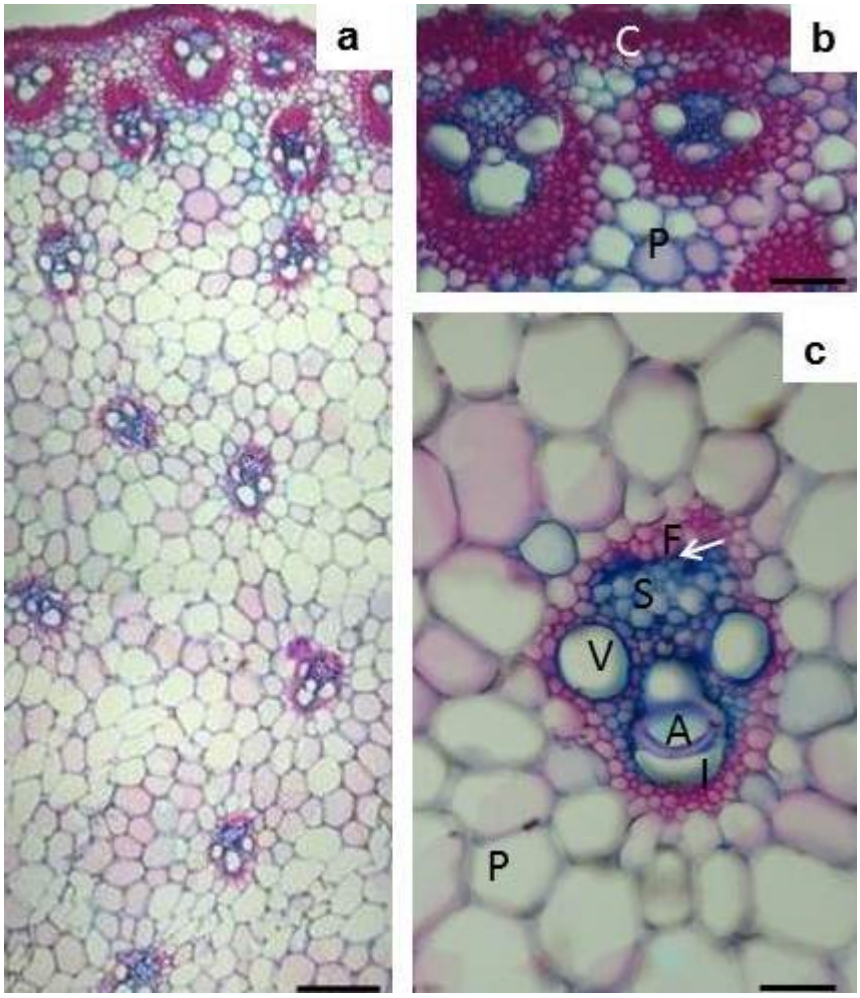

Figure 1 (a-c) - Anatomical structure of stalk of corn a: the general structure; b: epidermis/thin cortex (C) ; parenchyma tissue (P); c: vascular bundle with fibres of sclerenchyma (F), sieve tubes of the mataphloem (S), vessels of the metaxylem (V) vessel of the protoxylem (A) and intercellular space (I). Scale bars: a= 125 µm; b= 50 µm; c= 25 µm.

The vascular bundles are scattered in the ground tissue, densely packed in the periphery and loosely arranged in the central part (Fig. 1a). Each vascular bundle is composed by primary phloem and primary xylem surrounded by a sheath of sclerenchyma fibers (Fig.1b,c). The xylem includes narrow vessels with spiral and reticulate thickenings (Fig. 3a) (protoxylem), two wide pitted vessels with simple perforation (Fig. 3b) and some small diameter vessels (metaxylem), and parenchyma. The phloem is composed by sieve tubes (Fig. 1c) with associated companion cells. The fibers are long, wide and thick walled (Fig. 4).

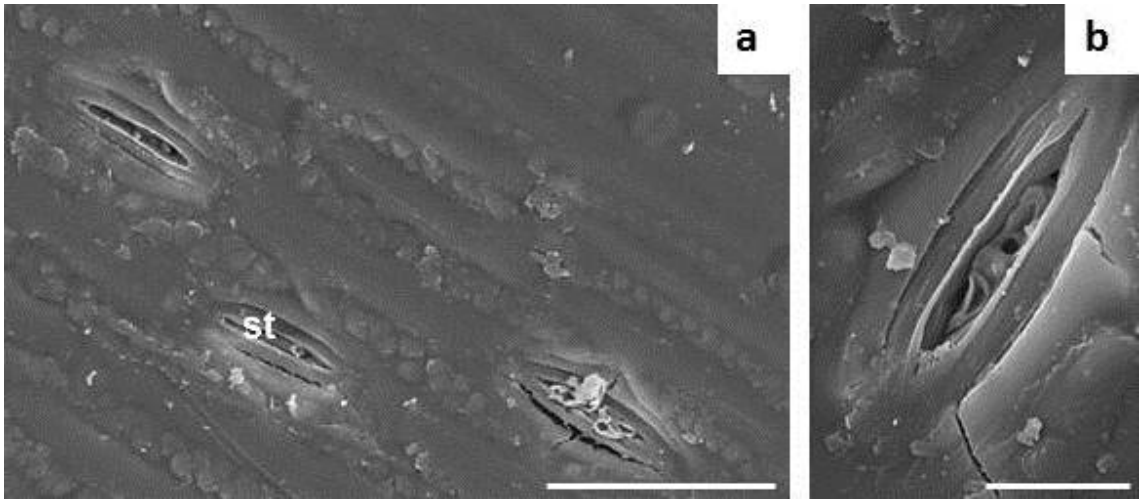

Figure 2– Epidermis of *Zea mays L*. a: general view; stomata (st) b: stomata. Scale bars: a= 100 µm; b= 10 µm

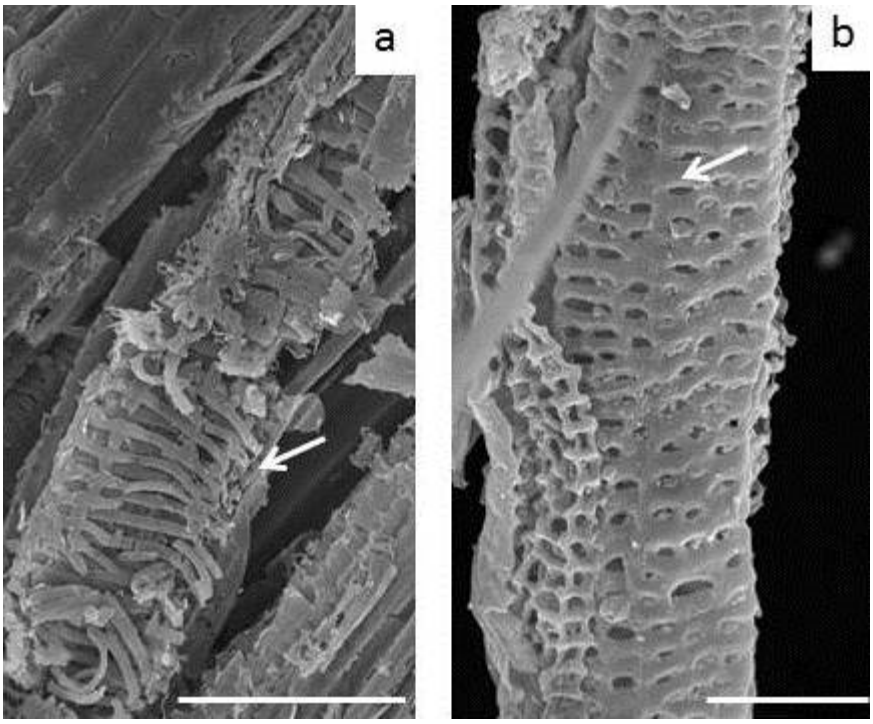

Figure 3– Wall structures of vessels in the vascular bundle of *Zea mays L*. a: Protoxylem vessel with annular spiral thickening (arrow); b: pitted metaxylem vessel (arrow) Scale bars: a= 100 µm; b= 30 µm

The analysed structure is in accordance with previous observations on this species (Cebrat et al. 2000) and similar grasses (Gominho et al. 2001;Quilho et al. 2004;Shatalov and Pereira 2002).

A granulometric separation was performed after size reduction of corn straw to particles inferior to 6 mm into seven fractions. Fractions with particle size ranging 0.5 and 2 mm corresponded to 77.3% of the total mass while larger fractions (≥ 2 mm) represented only 3.8% of total mass, and the smallest fractions (≤ 0.25 mm) 7.3 % of total mass (Fig. 5a). Biomass fractioning depends on the specific

structural characteristics and therefore is influenced by the material's anatomy (Miranda et al. 2012a.b). In this case, a rather homogeneous grinding was found with a low amount of fines, which is in accordance with the corn stalks anatomical features (Fig. 1).

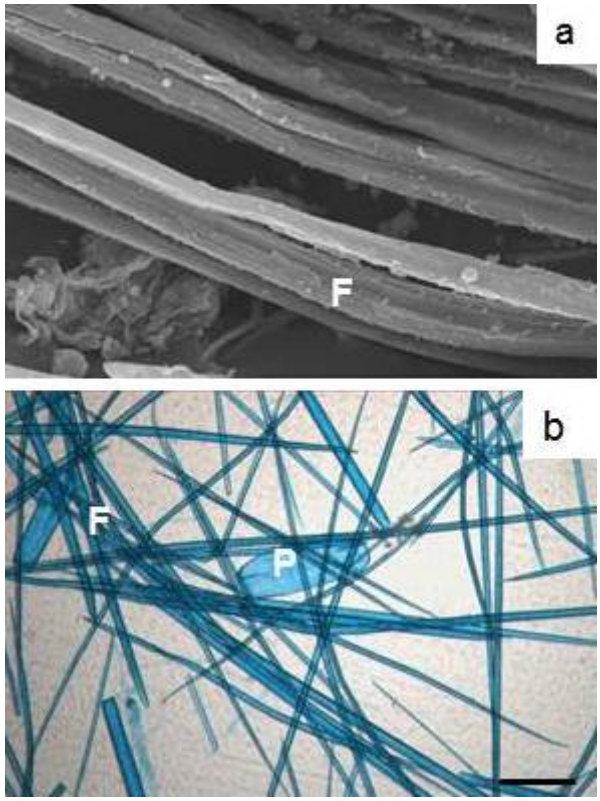

Figure 4 – Cells of corn straw. a: fibres (F). b: fibres (F) and parenchyma cells (P). Scale bars: a=30 µm; b= 50 µm

All the granulometric fractions were chemically characterized (Fig. 5b). The chemical composition was similar for all the fractions above 0.355 mm: glucan content ranging 40 and 45%, xylan 23%, arabinan 3%, acetyl groups 3.5%, lignin between 17 and 18% and ash between 4.2 and 6.1%. The fraction of fines (≤ 0.25 mm) revealed lower percentage of glucan (36.1%), xylan (21.4%) and lignin (17.6%) but a significantly higher ash content (9.7%). For this reason this fraction was discarded and the raw-material used for autohydrolysis was a thoroughly mixed sample of all the other fractions.

The enrichment in ash material in the fines after grinding coupled with a lower polysaccharides content has been reported for several types of biomass (Bridgeman et al. 2007;Miranda et al. 2012;Tamaki and Mazza 2010).

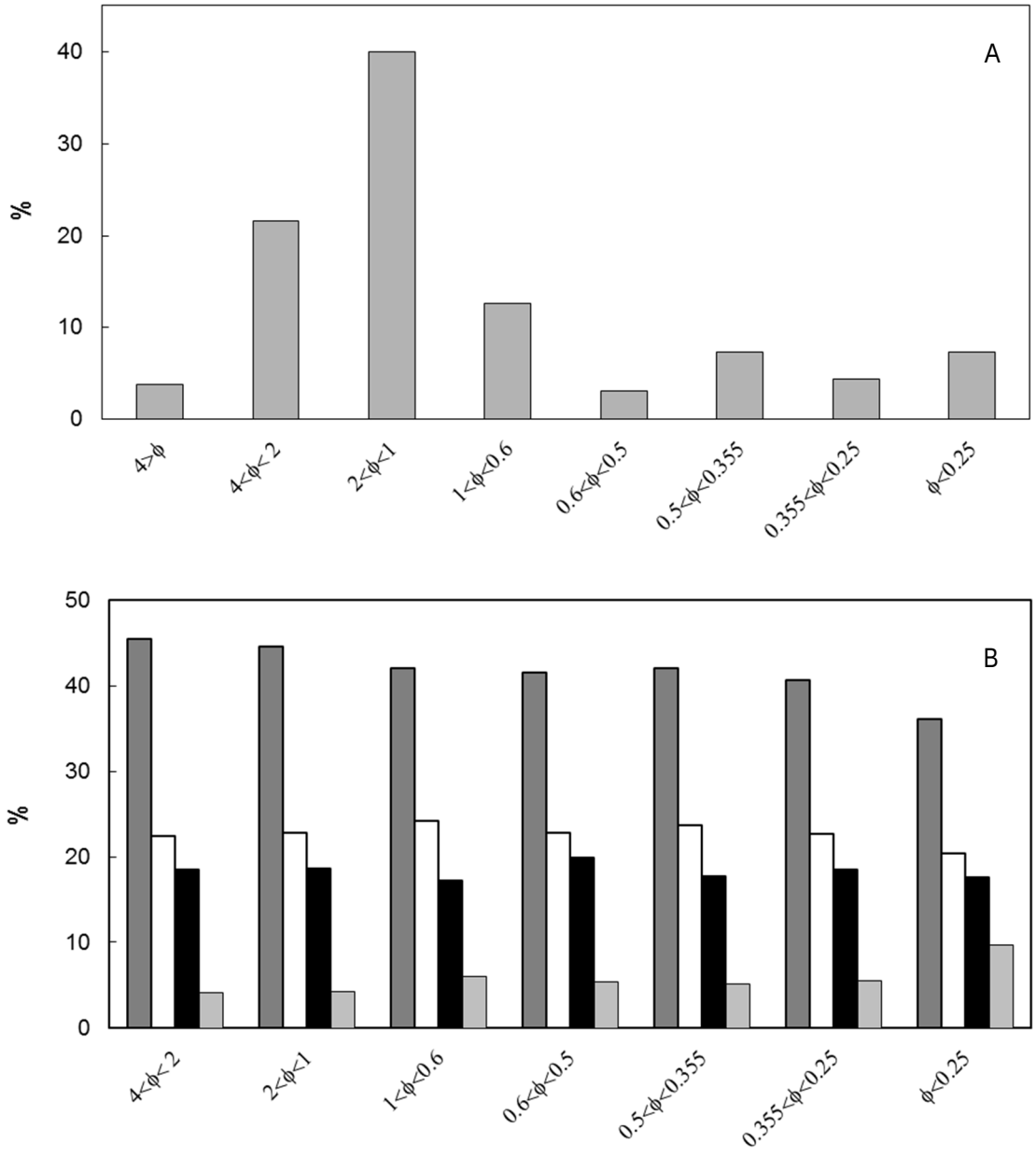

Figure 5 - Mass distribution of different granulometric fractions (A). Glucan, xylan, lignin and ash content in different particle size fractions (B). (■ Glucan; □ Xylan; ■ Acid insoluble lignin; ■ Ash)

The chemical composition of the raw material used in this work is shown in Table 1. The material presented very high polysaccharides content (71.3%) which favourably compares to previously reported values for corn stover (Mosier et al. 2005;Wyman et al. 2005). Cellulose, as estimated from the glucan content, was the major component, whereas hemicelluloses, estimated from xylan, arabinan and acetyl groups content accounted for 29.2% of the raw material. The amounts of arabinan and acetyl groups were similar to those reported for straws, e.g., wheat and barley (Nabarlatz et al. 2007) and corn stover (Wyman et al. 2005) while the xylan content was slightly higher, which favours the utilization of this material for XOS production.

Table 1 - Chemical composition of corn straw

Component	% of dry weight
Cellulose (as glucan)	42.1 ± 0.2
Hemicelluloses	29.2
Xylan	22.9 ± 0.1
Arabinan	2.9 ± 0.1
Acetyl groups	3.4 ± 0.02
Acid insoluble lignin	17.5 ± 0.4
Ash	4.2 ± 0.03
Extractives	9.8
Dichloromethane	1.1 ± 0.1
Ethanol	3.3 ± 0.02
Water	5.4 ± 1.5

Lignin content was in the range of values described for less lignified materials such as cereals and grasses (Carvalheiro et al. 2009; Garrote et al. 2007; Nabarlatz et al., 2007). Extractives content was similar to values described for corn stover (Chen et al. 2007) and higher than values reported for corn cobs (Garrote et al. 2002;Nabarlatz et al. 2007).

As for ash content it was inferior to the values described by other authors (Egues et al. 2012;Kaparaju and Felby 2010).

Figs. 6 and 7 show the variation of xylan and glucan in the solid fraction, and of oligosaccharides, monosaccharides and furan derivatives obtained in the liquid fraction, as a function of the severity factor of the autohydrolysis. As expected, autohydrolysis mainly affected the hemicellulosic components. Xylan hydrolysis became important for a severity factor above 2.97, and xylan content decreased sharply and showed a very significant solubilisation at the severest condition assayed (Fig. 6).

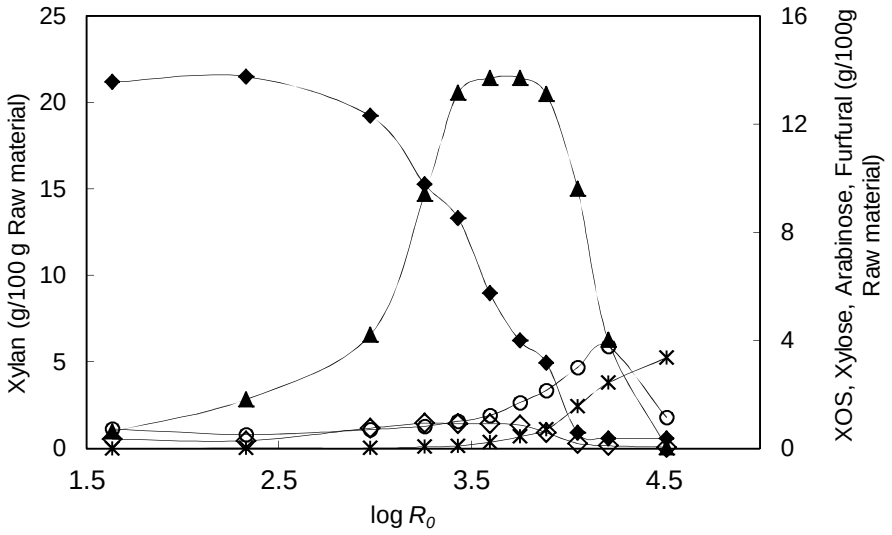

Figure 6 - Recovery of xylan (solid phase) and of xylo-oligosaccharides, xylose, arabinose and furfural (liquid phase) after autohydrolysis of corn straw, as mass yield in relation to raw-material in function of severity. (♦, Xylan; ▲, XOS; ○, xylose; ◊ arabinose; *, furfural)

The highest recovery of XOS was obtained by the hydrothermal treatment performed at 210 and 215ºC, corresponding to a log R_0 of 3.60 and 3.75, respectively. For the severity factor of 3.75, the recovery of XOS was 53.2 g/100 g feedstock xylan. In these conditions, 72.1% of the original xylan was solubilised and 63.2% of it was recovered as soluble saccharides (XOS, xylose and arabinose) in the liquor. The obtained XOS yield was slightly lower than the 65% reported for corn cobs autohydrolysis (Garrote et al. 2002;Moura et al. 2007) but higher than the reported values for other straws like wheat, barley and rye straw (Nabarlatz et al. 2007).

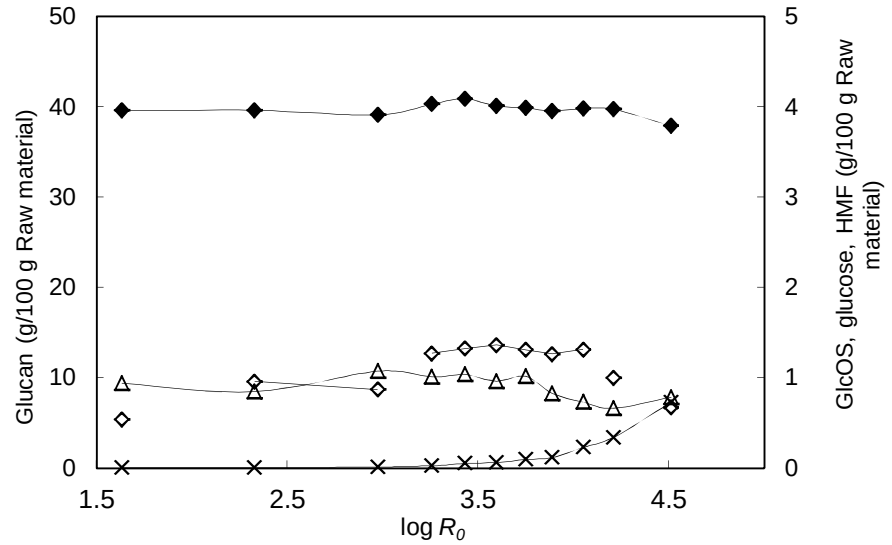

Figure 7 - Recovery of glucan (solid phase) and gluco-oligosaccharides, monomeric glucose and hydroxymethylfurfural yield (liquid phase) after autohydrolysis of corn straw, as mass yield in relation to raw-material in function of severity. (♦, Glucan; ▲, GlcOS; ○, glucose;*, HMF)

Xylose yield increased and reached a maximum value of 3.78 g/100 g feedstock for log R_0=4.21. For the severest condition tested there was a sharp decrease in the recovery of xylose and of other monomeric pentoses such as arabinose, due to degradation reactions. Arabinose recovery reached a maximum at log R_0=3.60 and then started to decrease, being almost negligible after log $R_0 \geq 4.05$. As a consequence, furfural concentration increased to reach up around 20% of the initial xylan.

These results are similar to those reported for wheat straw autohydrolysis (Carvalheiro et al. 2009) up to a severity factor of 3.89, although for higher severities a dramatic increase of furfural was observed here, which is consistent with the decrease of pentoses.

In contrast with xylan, glucan essentially remained in the solid phase. However, some oligosaccharides made up of glucose (gluco-oligosaccharides, GlcOS) were recovered in the liquor reaching a maximum yield of 1.36 g/100 g raw material for a severity of log R_0=3.60. The maximum yield of glucose was 1.08 g/100 g raw material, and it was achieved at log R_0=2.97 (Fig. 3). These results are similar to those obtained for wheat straw by (Carvalheiro et al. 2009), except for glucan recovery which was higher in this case (≥ 90%).

In all conditions, HMF concentrations were also low and similar to those reported for the autohydrolysis of other raw materials (Carvalheiro et al. 2004;Carvalheiro et al. 2009;Garrote et al. 2001;Nabarlatz et al. 2007).

Composition of the liquid phase

The composition of the liquors obtained from autohydrolysis of corn straw under the different conditions is presented in Table 2.

The total concentration of xylose either in monomeric or in oligomeric form, increased with the severity of the treatment until log R_0=3.89, while monomeric xylose kept increasing until log R_0=4.21. For the severest conditions, xylose concentration started to decrease and higher amounts of degradation products, namely furfural, were formed. Though the concentration of XOS was similar between log R_0=3.60 and 3.89, the condition log R_0=3.75 was considered the optimum since it had the highest concentration of XOS with relatively low furfural formation.

Arabinose and glucose had both the maximum concentration at log R_0=3.26.

Total phenolics concentration increased with severity, probably due to the solubilisation of some lignin compounds. This effect should however be small since there was no significant loss of lignin in the solid residues, even in the severest conditions (14% at the most).

The pH of the liquors for the mildest condition was about 5.5 and decreased with the severity of the treatment. This decrease of pH is due to the hydrolysis of the hemicellulosic acetyl groups which is higher in more severe conditions.

Table 2 - Composition (g/l) and pH of the liquors obtained from autohydrolysis of corn straw with different severity factors

	Severity factor, $\log R_0$								
	1.63 (150ºC)	2.33 (170º)	2.97 (190ºC)	3.26 (200ºC)	3.60 (210ºC)	3.75 (215ºC)	3.89 (220ºC)	4.21 (230ºC)	4.51 (240ºC)
pH	5.46	5.12	4.57	4.33	4.07	3.92	3.82	3.48	3.35
XOS	0.63	1.81	5.66	9.25	12.86	13.31	12.69	3.93	0.05
GlcOS	0.53	0.95	0.86	1.25	1.32	1.27	1.22	0.96	0.65
AcOS	0.10	0.25	0.34	0.77	1.24	1.25	1.29	1.00	0.56
Xylose	0.83	0.59	0.80	0.93	1.35	1.88	2.38	4.14	1.27
Arabinose	0.41	0.33	0.86	1.06	1.02	0.96	0.66	0.12	0.06
Glucose	1.04	0.94	1.18	1.19	1.04	1.10	0.89	0.71	0.84
Acetic acid	0.16	0.18	0.48	0.71	1.14	1.31	1.56	2.66	3.42
HMF	n.d.	n.d.	0.01	0.02	0.05	0.07	0.09	0.26	0.55
Furfural	n.d.	0.02	0.02	0.08	0.25	0.49	0.78	2.66	3.69
Total phenolics	0.57	1.52	1.27	1.89	2.96	2.93	3.24	5.12	6.97

XOS - xylo-oligosaccharides; GlcOS - gluco-oligosaccharides; AcO - acetyl groups linked to oligosaccharides, n.d. – not detected.

*Values in parentheses indicate the temperature of the pretratment.

Under the optimized condition (log R_0=3.75) sugars were recovered mainly in oligomeric form which is typical for autohydrolysis processes, as the conditions leading to the highest yield of soluble hemicelluloses do not lead to its complete hydrolysis to monomers (Duarte et al. 2009). Xylo-oligosaccharides were the main oligomeric components of the liquors at this severity condition. Gluco-oligosaccharides and acetyl groups linked to oligosaccharides were also obtained, although in much lower concentrations. Together, all the monosaccharides and acetic acid did not exceed 2 g/l, being xylose the main monosaccharide. In these conditions furfural content was low, and HMF appeared in almost negligible amounts.

Composition of the solid phase

Table 3 shows the composition of the solid phase obtained for the different severity conditions assayed. For the less severe conditions of autohydrolysis, the solid solubilisation was low and the composition of the solid residues remained quite similar to that of the initial raw material. However, for log R_0 higher than 2.33 the solid yield decreased sharply to reach about 61.5% for the severest operation condition assayed and the composition of the remaining autohydrolysed material changed significantly. This mass decrease can be mainly correlated to the solubilisation of hemicelluloses. The amount of solubilised xylan increased with severity to reach 95.4% of the initial xylan in the raw material and an almost complete solubilisation also occurred for the other hemicellulose components. These results show the efficiency of this treatment towards hemicelluloses.

Glucan was almost not affected by the hydrolytic treatment and therefore its content in the solid residue increased with severity in relation with the decrease of hemicellulose content. For instance the solid residue obtained for a severity of log R_0=4.21 had a glucan content of 64%. The increase of severity had only a minor effect on glucan solubilisation, with a maximum glucan loss of 9.9% for the severest condition. Glucan solubilisation that occurred for the less severe conditions (for instance 6.0% at severity 1.63) may be related with loss of water soluble non-structural sugars and not to cellulose hydrolysis (Chen et al. 2007). Low solubilisation of glucan has also been reported for corn cobs autohydrolysis (Garrote et al. 2001;Moura et al. 2007) and is an advantage for the integral utilization of this raw material in a biorefinery framework.

Under the present conditions of autohydrolysis no significant removal of lignin was expected to occur and in fact, up to severity factor log R_0=4.21, about 90% of the initial acid insoluble lignin was maintained in the solid. This is in accordance with the results previously reported for other materials (Garrote et al. 2002). For the most severe condition, lignin yield increased to over 100% of the initial lignin in the raw material.

Table 3 - Effect of severity factor on the solid yield (SY) and polymeric composition of processed solids obtained after autohydrolysis of corn straw

(%)	Severity factor, $\log R_0$								
	1.63 (150º)	2.33 (170ºC)	2.97 (190ºC)	3.26 (200ºC)	3.60 (210ºC)	3.75 (215ºC)	3.89 (220ºC)	4.21 (230ºC)	4.51 (240ºC)
SY[a]	92.4	91.2	85.0	76.4	69.6	67.8	64.9	62.1	61.5
Xylan[b]	23.0	23.6	22.6	18.9	12.9	9.2	7.7	1.0	1.0
Arabinan[b]	3.7	3.8	3.0	2.2	1.6	1.4	1.4	1.0	1.0
Glucan[b]	42.9	43.5	46.0	49.9	57.6	58.8	61.0	64.0	61.7
Acid insoluble lignin[b]	18.0	17.9	18.9	20.2	21.8	23.6	21.8	27.1	31.0

[a] (g/100 g raw material); [b] (g/100 g processed solids); SY – Solid yield
*Values in parentheses indicate the temperature of the pretratment.

This increase can been associated to the condensation of lignin with sugar and/or sugar degradation products, such as furfural to give insoluble reaction products that cause an increase in apparent acid insoluble lignin (Ramos 2003).

The compositional changes of the autohydrolysed material were also followed by FTIR spectroscopy. The FTIR spectra of the raw material and of the solid residue after autohydrolysis under optimized condition at 215ºC (log R_0=3.75) are illustrated in Fig. 8. FTIR spectroscopy can be used for approximate identification of polysaccharides and lignin in plant materials when combined with chemical analyses data (Yuan et al. 2010).

The most conspicuous differences were seen at the intensity of peaks at 1265 cm^{-1} and 1740 cm^{-1} which decreased in the autohydrolysed samples. The intensity of the 1265 cm^{-1} peak is attributed to the cleavage and/or alterations of acetyl groups (Kaparaju and Felby 2010;Sun et al. 2005;Windeisen et al. 2007). This is direct relation with the increase of acetic acid in the liquid phase with the observed pH decrease (Table 2). It is a feature typical of the autohydrolysis pretreatment and also in agreement with the increase of the 1740 cm^{-1} band, that corresponds to C=O stretching vibration, which is almost exclusively due to the carbonyl bonds of the acetyl group in xylan (Michell and Higgins 1999).

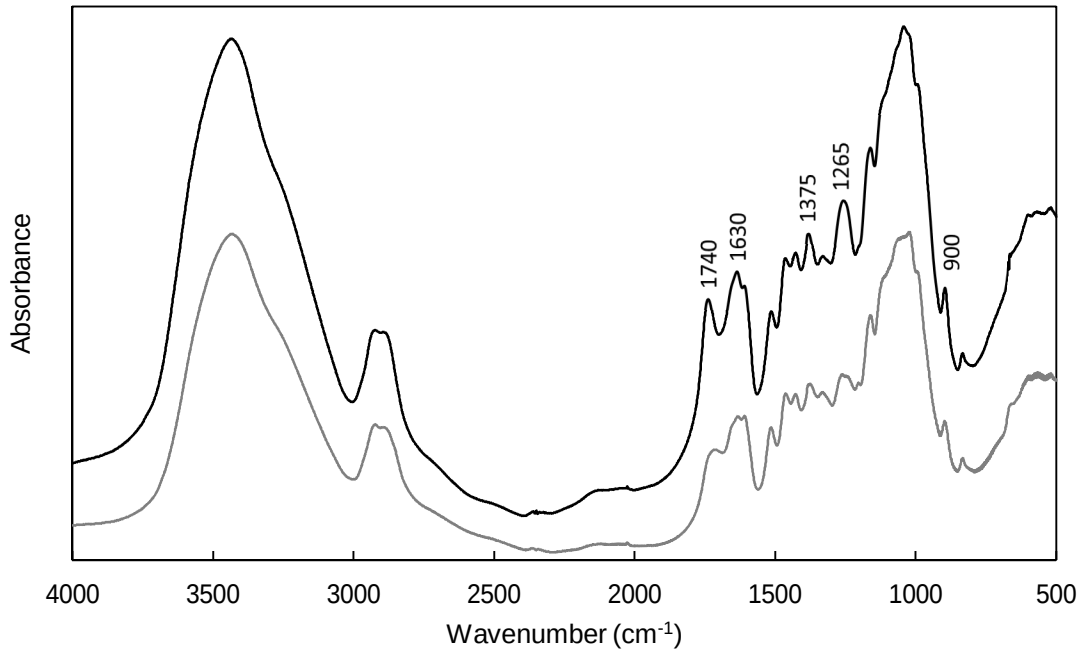

Figure 8 - FTIR spectra of corn straw samples. (Corn straw; Autohydrolysis treated corn straw (log R_0=3.75)

The band at 1630 cm^{-1} has been attributed to the increase of lignin condensation reactions at the expense of C=C double bonds in conjugated carbonyl groups in lignin, (Kaparaju and Felby, 2010). The intensity of this peak was higher in the untreated material compared with the pretreated materials, indicating a decrease in surface lignin after pretreatment.

Autohydrolysis pretreatment can alter the ratio of amorphous to crystalline cellulose associated

with the ratio of intensities of the 900 cm^{-1} and 1098 cm^{-1} bands (Laureano-Perez et al. 2005). However, the removal of cellulose from the solid residue should be very low or not occur (Kumar et al. 2009), in agreement with the present results for the solid residues chemical composition (Table 3).

The hydrothermal pretreatment previously discussed is conducted mostly by chemical considerations to solubilize the hemicelluloses, and leaving lignin and cellulose for further applications or for a subsequent enzymatic hydrolysis step (Zeng et al. 2012b).

In order to assess the effect of hydrothermal process conditions on the cellulose digestibility, enzymatic hydrolysis of insoluble solids was carried out. Table 4 shows enzymatic digestibility on both untreated and pretreated corn straw biomass for the condition leading to the highest XOS yield (log R_0 = 3.75) and for the severest condition (log R_0=4.51). The increase of pretreatment severity led to a further increase in digestibility. The maximum glucose yield was 90.1% and it was obtained at 240ºC. This susceptibility may be a consequence of the composition and structure of the raw material, namely the reduction of cellulose cristalinity imposed by the severity of the pretreatment. These results are in agreement to those obtained after hot water pretreatment of both corn fibre and corn stover for which an enzymatic digestibility of 85 and 94% were reported (Dien et al. 2006) (Liu and Wyman 2005).

Table 4 - Enzymatic digestibility of untreated and pretreated corn straw biomass.

Condition	Enzymatic digestibility (%)
Untreated	31.5 ± 1.7
log R_0 = 3.75 (215ºC)	54.5 ± 1.9
log R_0 = 4.51 (240ºC)	90.1 ± 4.7

The structural features of the autohydrolysed corn straw was observed at the microscopic level to see if the treatment besides affecting the chemical composition, as shown in Table 3, also altered the cell structure.

Figure 9 shows scanning electron micrographs (SEM) of corn straw after pretreatment at 215°C and 240ºC. No major structural changes were found, and cells remained aggregated, in agreement with the chemical data, thereby showing that the structural backbone of lignin and cellulose remains mostly intact.

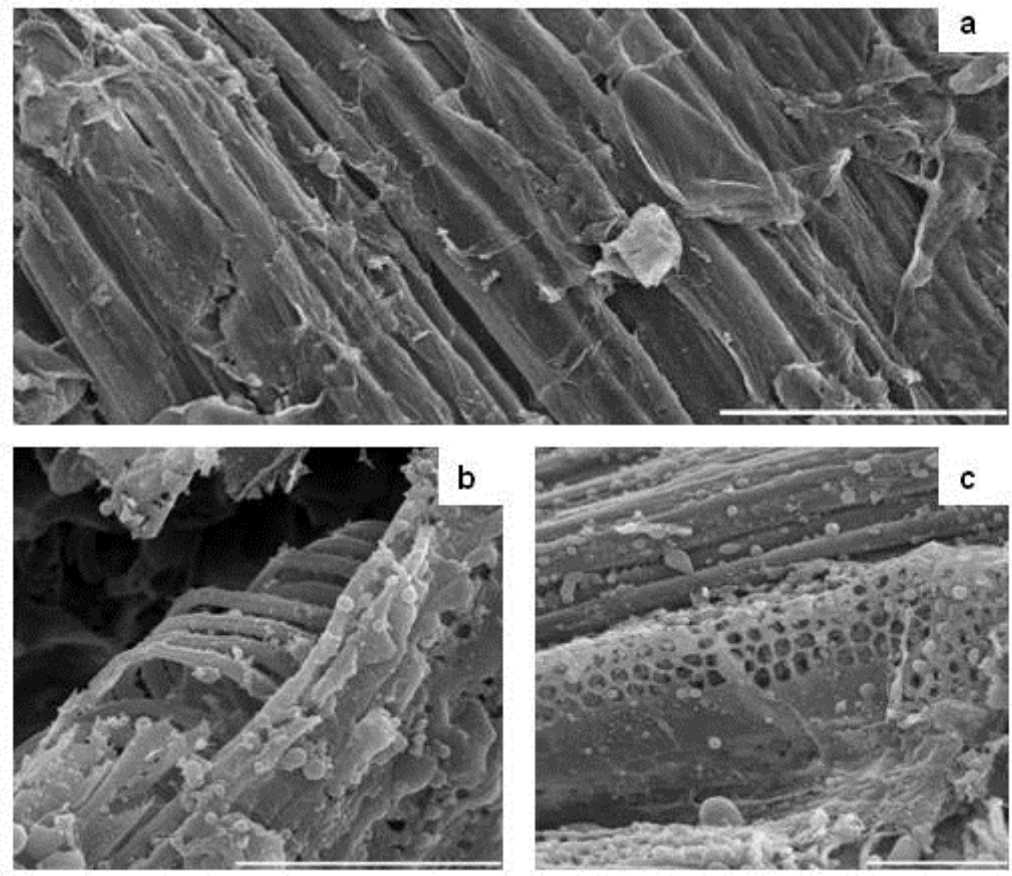

Figure 9- SEM photomicrograph of corn straw preated by auto hydrolysis at 215ºC (a) and 240ºC (b,c) showing the fibre aggregates with separated and collapsed parenchyma cells (a) and wall separation in vessels exposing the wall thickenings (b,c) Scale bars: a=100 µm; b= 30 µm; c= 30 µm

The fiber bundles remained as such (Fig 9a) although some differences in the wall ultrastructure should not be excluded due to hemicelluloses removal. The only effects that could be traced back to the hydrothermal treatment (and not to the eventual effects of comminution of the material) were cell separation of the more fragile cells such as the parenchyma cells that collapsed on the fibre aggregates (Fig. 9a) and cell wall separation in vessels that exposed more the wall thickenings (Fig. 9b,c). These may increase the enzyme-accessible surface area for further enzyme digestibility of corn straw (Zeng et al. 2012a).

Conclusions

Autohydrolysis was highly selective towards hemicelluloses. The extent of xylan depolymerisation depended on the severity of the autohydrolysis. A high recovery of XOS, that corresponded to 53% of initial xylan, could be obtained under relatively mild conditions at 215 ºC at a log R_0=3.75. The concentration of possible inhibitor compounds such as HMF and furfural and total phenolics was very low.

Cellulose and lignin were not substantially solubilised by the autohydrolysis treatment although an increase of cellulose enzymatic digestibility was attained.

The XOS rich liquors and the glucan and lignin enrichment of the solid phase makes corn straw a suitable raw material to be used in a biorefinery framework and the hydrothermal treatment a favourable first step in the processing.

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Capítulo III. Hydrothermal production and gel filtration purification of xylo-oligosaccharides from rice straw

Abstract

Hydrothermal treatment (autohydrolysis) is an advantageous alternative to fractionate biomass that was not yet explored for rice straw. In this work, the process was optimised and proved to be highly selective towards hemicellulose. Hydrolysates containing a mixture of oligomeric compounds (mainly xylo-oligosaccharides, XOS), could be obtained under relatively mild operation conditions (210ºC, log R0=3.59), yielding a maximum of 40.1g/100g of initial xylan. The produced XOS were separated by molecular mass using gel filtration chromatography (GFC). Different fractions of purified XOS were obtained ranging from small and high DP oligosaccharides (DP≥ 23), to medium and low DP oligosaccharides (DP ≥ 3), and separating these from by-products (acetic acid, furan derivatives and phenols) as well as di-, and monosaccharides. GFC was an efficient purification method enabling the recovery of interesting categories of XOS that can have potential applications to the pharma, food and feed industries.

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Introduction

Agricultural residues and by-products have gained increased attention as potential substrates for the production of fuels, chemicals and bio-materials (Kamm and Kamm 2007).

Rice is one of the major grain crops worldwide and its residues (straw and husks) are produced in large quantities. Rice straw is usually considered a residue or even a waste material although it may be used as mulching material, feed, compost substrate, cattle house flooring, as well as for straw handicraft and combustion (Kadam et al. 2000;Matsumura et al. 2005). But, conversely to other similar materials, the use of rice straw for feeding or for combustion for energy production may cause problems due to its high silica content (Domínguez-Escribá and Porcar 2009) and thus most of this material is left unused in the fields.

An alternative for rice straw valorisation can be the production of oligosaccharides within the biorefinery framework along with the production of biofuels. Rice wastes can be a major single feedstock for bioethanol production as both straw and husks have high polysaccharide and low lignin contents, with the straw presenting higher hemicellulose content (Binod et al. 2010;Domínguez-Escribá and Porcar 2009). Prior to bioethanol production, several pretreatments have to be applied to rice straw in order to increase its upgradability. Previously studied pretreatments mainly include dilute acid, alkali (including ammonia) and enzymatic methods (Binod et al. 2010). However such pretreatments are expensive and/or time-consuming, not selective, require neutralisation, or result in the formation of by-products that can inhibit subsequent fermentation. Furthermore, none of these methods is suitable for oligosaccharides production from biomass, as either they degrade oligosaccharides (dilute acid hydrolysis), are not selective (alkaline treatments), or present very low yields (enzymatic processes).

An alternative option for the valorisation of rice straw under the biorefinery approach is the hydrolysis of hemicellulose by hydrothermal processing (autohydrolysis), which is an effective pretreatment enabling a high recovery of soluble saccharides in oligomeric form. Since no chemicals other than water are added, hydrothermal processes have important advantages over the processes referred above. Nevertheless, as demonstrated by reported data in the literature for other materials, e.g. corn straw (Moniz et al. 2013), corn cobs (Moura et al. 2007), wheat straw (Carvalheiro et al. 2009), brewery's spent grains (Carvalheiro et al. 2004), or eucalypt wood (Garrote and Parajó 2002), the careful optimisation of the operational conditions is of paramount importance to achieve a high yield and the balanced distribution of oligosaccharides degree of polymerization (DP) for each material. XOS are potentially bioactive compounds, as they can be used as food ingredients and classed as nutraceuticals. In fact, XOS are receiving substantial attention due to their functional properties and health benefits as active ingredients in functional foods (Carvalho et al. 2013;Moura et al. 2008;Nabarlatz et al. 2007;Vazquez et al. 2005). As compared to the many existing oligosaccharides in the market, XOS are the only oligosaccharides that can be produced from lignocellulosic biomass residues. Other oligosaccharides are produced from sugars used in food such as sucrose, lactose, inulin and starch or from soy (Moreno et al. 2014;Rastall 2010) and are mainly produced by enzymatic processes synthesis/hydrolysis, making XOS a particular case among oligosaccharides.

Chemically, XOS are oligomers containing two to ten xylose molecules linked by β-1–4 glycosidic bonds (IUPAC), but molecules with DP higher than 20 have also been considered as oligosaccharides (Moure et al. 2006;Vazquez et al. 2005). The biological activity of XOS depends on its molecular weight (DP) distribution and prebiotic proprieties, i.e. those associated to the proliferation of beneficial microorganisms in human gut such as bifidobacteria (Hughes and Kolida 2007). Low/medium DP oligosaccharides have been described as promising fermentable substrates (Moura et al. 2007;Moure et al. 2006).

Besides the selective hydrolysis of hemicellulose aiming at oligosaccharides production, the autohydrolysis process also enables the recovery of cellulose and lignin in a solid phase in advantageous conditions for further processing, i.e., towards an integrated valorisation in a biorefinery approach.

Several strategies may be used for XOS purification. These include membrane separation or chromatographic techniques (Cara et al. 2012;Gonzalez-Munoz et al. 2013;Moure et al. 2006;Vegas et al. 2004). Membrane technologies have had an increased interest in recent years since they may be less expensive although the separation of XOS can be affected by the structural characteristics of the oligosaccharides and even by their solubility (Pinelo et al. 2009). Sequential membrane-based steps for concentration and fractionation to achieve XOS fractions free from monosaccharides and by-products have been employed in multistage purification processes (dos Santos et al. 2011;Gonzalez-Munoz et al. 2013). Conversely, to membrane processes, the less explored chromatographic separation has the potential advantage to yield XOS with high purity and separated by molecular weight and/or chemical structure.

In this work, rice straw was subjected to autohydrolysis at different final temperatures (150-240ºC) and the optimal operational conditions leading to the maximal recovery of XOS were established. The yields and composition of both liquid and solid phases were evaluated, and the yields of the solubilised products, namely oligosaccharides, monosaccharides, acetic acid and degradation compounds, such as furfural and HMF were determined and interpreted using the severity factor (log R_0) (Overend and Chornet 1987) . The XOS-rich hydrolysates were further purified by GFC and the target fractions were characterised in terms of XOS content and degree of polymerisation.

Material and methods

Raw material

Rice straw was supplied by Orivárzea (Salvaterra de Magos, Portugal) as a heterogeneous sample containing stalks and leaves as the field agricultural residue after crop harvest. The raw material was air dried and milled with a knife mill (Fritsh Industriestr, Germany) to particles smaller than 6 mm. The material was homogenised in a combined lot, and stored in plastic containers at room temperature.

Hydrothermal processing of rice straw

Autohydrolysis treatments of rice straw were performed in a stainless steel reactor (Parr Instruments Company, USA) with a total volume of 600 ml. Temperature was controlled through a Parr PID controller (model 4842). The raw material (25 g) was mixed with water in the reactor to reach a liquid-to-solid ratio of 10 (g water/g dry raw material). The agitation speed was set at 150 rpm and the reactor was operated under non-isothermal conditions, i.e. heated to reach final temperatures and rapidly cooled down. Several treatments were performed to study different final temperatures ranging between 150ºC and 240ºC. Typically, the average heating rate (from 100°C onwards) was 4°C.min^{-1} and the average cooling rate (down to 100°C) was 25°C.min^{-1}. The liquid and solid phases were separated by pressing (up to 200 bar) using an hydraulic press (Sotel, Portugal). The liquid phase was filtered (Whatman filter paper no. 1) and the solid phase was washed with twice the amount of water, filtered, dried at 40ºC and stored, at room temperature, until further use.

The effects of temperature on rice straw autohydrolysis were interpreted based on the severity factor, log R_0 (Overend and Chornet 1987) defined as:

$$R_0 = \int_0^t \exp\left(\frac{T(t) - Tref}{14.75}\right) dt$$

where the temperature T (ºC) is a function of time t (min) and Tref is the reference temperature (100ºC). The value 14.75 is an empirical parameter related with activation energy and temperature.

Gel filtration chromatography (GFC)

The preparative gel filtration chromatography for the purification of XOS was carried out in an Amersham Pharmacia Biotech system (Sweden) equipped with a refractive index (K-2401 Knauer, Germany) detector. A 400 mL sample of rice straw hydrolysate obtained under the optimized condition of 210ºC (see results and discussion) was eluted with deionized water at a flow rate of 25 mL min^{-1} through a BPG 100/950 column (Amersham Pharmacia Biotech, Sweden) with a Superdex 30^{TM} gel bed volume of 4.2 L, suitable to separate the target XOS from the lower molecular weight carbohydrates, phenolic compounds, acetic acid, and furan derivatives (HMF and furfural). The sample was fractionated into 28 fractions of 125 mL, and collected every 5 min. using a Super-frac^{TM} collector (Amersham Pharmacia Biotech, Sweden). All fractions were freeze-dried (Labconco, Missouri, USA) and weighted for mass quantification. After analytical determination it was verified that the sample started to elute only after a 30 min. running period, corresponding to an elution volume of 1250 mL. The carbohydrates were separated according to their molecular size. The smallest molecules, such as the monosaccharides, were the last to be eluted, after the disaccharides, whereas the higher molecular weight compounds were the first to elute.

Analytical Methods

Chemical characterisation of raw material and processed solids

The materials were ground in a knife mill (IKA, Germany) to a particle size smaller than 0.5 mm and the moisture content was determined by oven-drying at 100°C to constant weight. The ash content was determined at 550ºC using NREL/TP-510-42622 protocol (Sluiter et al. 2005). For extractives determination, the samples were successively extracted with dichloromethane, ethanol, and water (during 8h, 8h and 16h, respectively), with a Soxtec extraction system.

The samples were hydrolysed for determination of glucan, xylan, arabinan and acetyl groups using acid hydrolysis with 72% (w/w) H_2SO_4 followed by hydrolysis with 4% (w/w) H_2SO_4. The acid insoluble residue was considered as acid insoluble lignin, after correction for ash. Monosaccharides (glucose, xylose, arabinose) and acetic acid produced were analysed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad, USA) in combination with a cation H^+-guard column (Bio-Rad). Elution took place at 50ºC with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.4 mL min^{-1}. It was used an Agilent Technologies Liquid Chromatographer 1100 Series System (Santa Clara CA, USA), equipped with a diode array detector (DAD) and a refractive index detector (RI).

Chemical characterisation of liquors and hydrolysates

The liquors obtained with the autohydrolysis treatments were directly analysed by HPLC. Elution took place at 50ºC with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Glucose, xylose, arabinose and acetic acid were detected with the RI detector; furfural and HMF were detected with the UV detector set at 280 nm.

An aliquot sample of the liquors was hydrolysed with 4% (w/w) H_2SO_4 to convert soluble hemicelluloses into their constituent sugar monomers. The oligosaccharides concentrations were calculated from the increase in sugar monomers, as analysed by HPLC, after acid post-hydrolysis. The term XOS has been used to name the hemicelluloses-derived oligosaccharides made up of xylose and arabinose units.

Total phenolic compounds were determined by the Folin–Ciocalteu colorimetric method according to (Singleton et al. 1999). Briefly, 100 µL of the sample was mixed with 5 mL of the 1/10 (v/v) diluted Folin–Ciocalteu reagent and 4 mL of 7.5% Na_2CO_3. Absorbance was measured at 765 nm after 15 min incubation at 45 ºC. Total phenolic compounds are expressed as mg GAE mL^{-1} (gallic acid equivalents).

Chemical characterisation of purified fractions

A portion of the freeze-dried samples was dissolved (up to 10 g L^{-1}) for chemical characterisation and evaluation of DP. The carbohydrate, acetic acid and furan derivatives composition of each faction was determined using the same method described for hydrolysates.

Degree of polymerisation

The degree of polymerization of oligosaccharides in the fractions recovered was estimated using a BIOSEP-SEC S2000 column (Phenomenex, USA) in a combination with a BIOSEP-SEC-S 4000 guard column (Phenomenex) using the HPLC equipment and RI detector described before. Mobile phase was 50 mmol L^{-1} $NaNO_3$, at a flow rate of 0.7 mL min^{-1} and a column oven temperature of 30ºC.

Results and discussion

Composition of raw material

The chemical composition of the raw material used in this work is shown in Table 1. The cellulose, estimated as the glucan amount, is the major structural component of rice straw with 40.9%, whereas the hemicelluloses, estimated by the amount of xylan, arabinan and acetyl groups, represent 24.3%, with xylan as the main component of hemicellulose. This raw material has a high percentage of polysaccharides (65.1%), which is in agreement with other studies on the composition of rice straw (Buranov and Mazza 2008). The cellulose content is similar although slightly higher than the values reported in previous works for rice straw (Yu et al. 2010) and rice hulls (Vegas et al. 2004). For hemicelluloses, especially for xylan and arabinan, the values are slightly higher than those previously reported for rice straw (Yu et al. 2010) and rice husks (Vegas et al. 2004). The acid insoluble lignin content obtained in this study is in the range of values reported by other authors (Sun and Cheng 2002;Yu et al. 2010).

Table 1 - Chemical composition of rice straw

Component	% of dry weight
Cellulose (as glucan)	40.9
Hemicelluloses	24.3
Xylan	20.5
Arabinan	3.4
Acetyl groups	0.4
Acid insoluble lignin	14.4
Extractives	18.5
Dichloromethane	2.4
Ethanol	5.3
Water	10.8
Ash	10.6

Ash content, which is known to vary depending on the origin of rice straw, was in this case lower than it is usually reported in the literature ranging between 9 and 19% (Yu and Chen 2010;Yu et al. 2010;Zhong et al. 2009). The extractives were mainly polar compounds, soluble in ethanol and water,

which is similar to the reported for *Cynara cardunculus* (Gominho et al. 2009), *Picea abies* and *Pinus sylvestris* (Miranda et al. 2012).

Autohydrolysis profile

The behavior of the three main macromolecular compounds, cellulose, hemicelluloses and acid insoluble lignin after the hydrothermal pretreatment is shown in Fig 1. This autohydrolysis profile shows the effect of this treatment on xylan and arabinan solubilisation that, as expected, is strongly affected by the severity of the treatment. The hydrolysis of xylan was very low, less than 10g/100g of raw material, for the lower severity treatments (log R_0 <3) but became considerable for severity values log R_0 ≥3.42. In the most severe condition of log R_0 = 4.21, xylan solubilisation was higher than 90% of its initial amount in the raw material. In contrast, glucan and lignin were almost not affected by autohydrolysis.

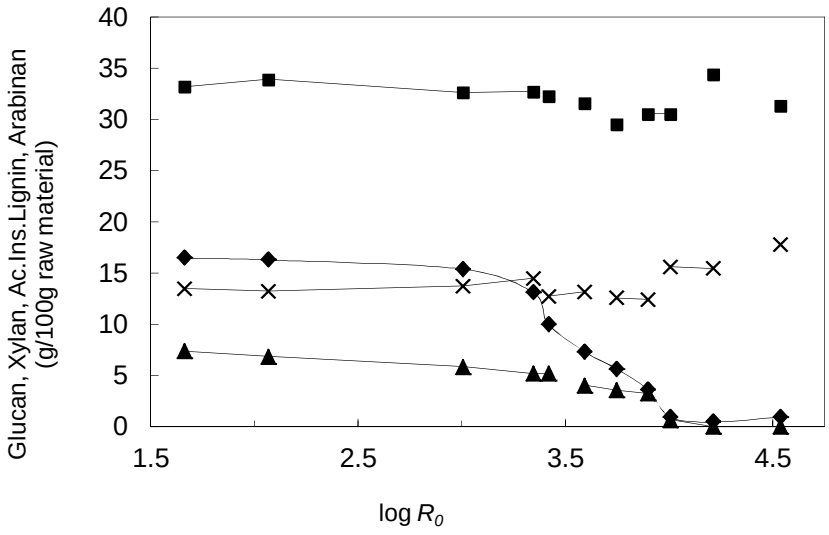

Figure 1 - Glucan, xylan, acid insoluble lignin and arabinan, in the solid phase after autohydrolysis of rice straw, as mass yield in relation to the raw material. (♦, Xylan; ▲, arabinan; ■, glucan; × acid insoluble lignin)

Figure 2 represents the variation of xylan derived compounds, xylo-oligosaccharides (XOS), xylose and furfural in the liquid phase as a function of the severity factor of the hydrothermal treatment. As the xylan begins to hydrolyse, there is an increased formation of XOS that reaches a maximum of 13.16g/100g raw material at log R_0=3.59. The amount of monomeric xylose was almost similar for all conditions and reached the highest value at log R_0 = 4.01, corresponding to 2.76 g xylose / 100 g raw material. This corresponds to a condition where most of the xylan was hydrolysed and XOS already started to decrease. The subsequent decrease of xylose and arabinose is due to their degradation and for this reason, the concentration of furfural increased with treatment severity.

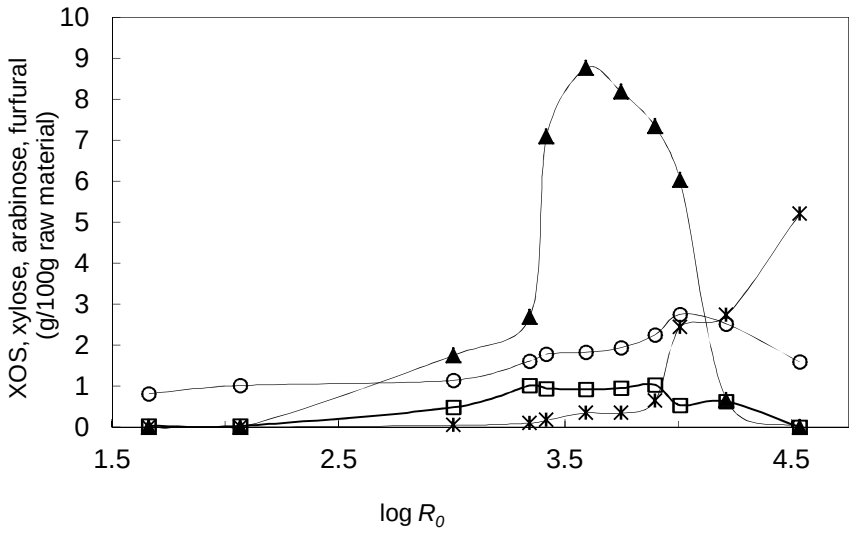

Figure 2 - Xylan soluble derivatives after autohydrolysis of rice straw, as mass yield in relation to raw material. (▲, XOS; ○, xylose; □, arabinose; × furfural)

The observed behavior of xylan during hydrothermal treatment was similar to that already described for the autohydrolysis of other straws (Carvalheiro et al. 2009;Moniz et al. 2013). The maximum amount of XOS obtained in this study (40.1g/100g of xylan) was higher than the 30% obtained for rice hulls (Nabarlatz et al. 2007), but lower than the 53 % and 63 % obtained for corn straw and corn cobs, respectively (Garrote et al. 2008;Moniz et al. 2013).

Composition of the solid phase

For milder conditions, the solubilisation of the solid is about 20% (data not shown), but increases with the severity of the treatment. The decrease in solid yield is mainly due to the solubilisation of hemicelluloses, which occurs particularly for the arabinan and xylan leading to an effective decrease of hemicellulose fractions in the solid phase.

These data demonstrate the efficiency of this process towards hemicelluloses recovery. The hydrothermal pretreatment allowed the separation of the hemicelluloses leaving also a cellulose and lignin rich solid. Glucan is little affected by these treatments, and in the most severe conditions, a solid phase containing about 60% of glucan was obtained. The increase in severity did not significantly change the solubility of glucan, and maximum solubilisation (about 20%) occurred at log R_0=4.21.

The concentration of lignin also increases with severity, being the highest value obtained for log R_0=4.01. For the conditions leading to the highest production of XOS in the liquor (log R_0=3.59), the solid is constituted by 55% cellulose and 22% lignin with only 15% of residual hemicelluloses. These values are in agreement with previous autohydrolysis treatments which were characterised to have low effect on the cellulose and lignin fractions (Carvalheiro et al. 2009;Moniz et al. 2013;Nabarlatz et al. 2007).

In agreement with the low hydrolysis of glucan is the low value obtained for the formation of glucose oligosaccharides (GlcOS), with a maximum yield of 9.98 g/100 g initial glucan (data not shown).

Composition of the liquid phase

The liquors resulting from the hydrothermal treatments contain a mixture of oligomeric compounds (XOS, GlcOS, AcOS and AOS), monosaccharides (xylose, arabinose and glucose), acetic acid and products resulting from decomposition of sugars (Fig. 3).

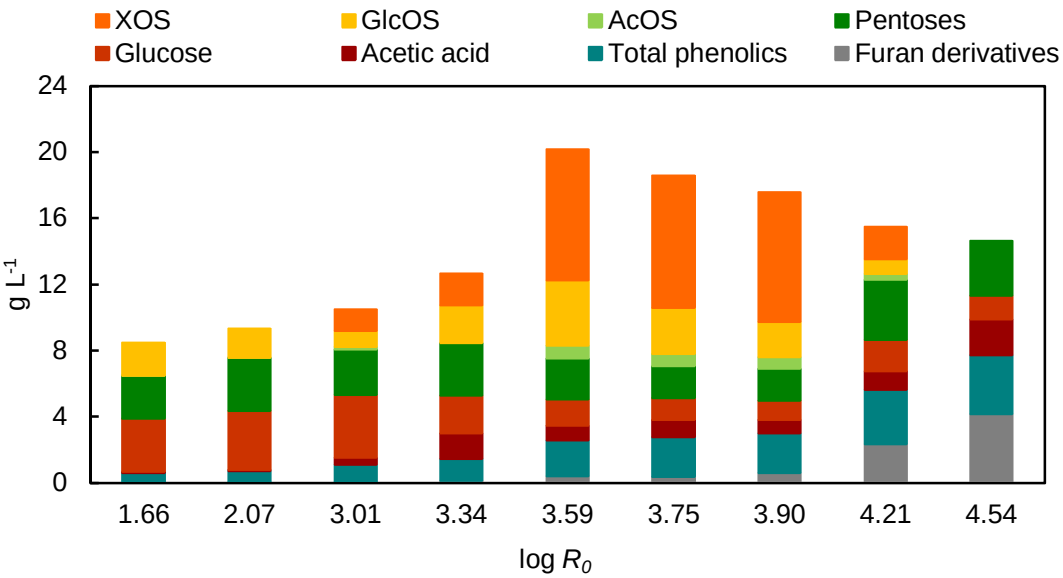

Figure 3 - Composition of the liquors obtained from autohydrolysis of rice straw for different severity factors

The composition of the liquors depended on the severity conditions of the treatment. Sugars were predominantly in oligomeric form as XOS, and these were the main components of liquors. XOS concentration increased with severity from log R_0=1.66 to 3.75, but for values of log R_0 higher than 3.90 concentrations started to decrease due to increased formation of monomeric sugars and degradation products. The concentration of GlcOS also increased slightly with increasing severity.

Increased severity of the process can facilitate some breaking of glucan structure leading to an increase of glucose concentration in the oligomeric form. Nevertheless, the obtained concentrations are low (less than 4g L^{-1}) which is in agreement to the low solubilisation of glucan (Fig. 2). In fact, the concentration of monomeric compounds was always low which makes autohydrolysis an adequate method for oligosaccharides production.

Low concentration of acetic acid was also observed during the treatments (below 2 g L^{-1}) corresponding to a concentration of free acetic acid of 1.58 g/100 g raw material, due to the low amount of this component in the composition of rice straw (Table 1).

For most of the conditions studied, there was a weak formation of degradation products, and the concentrations of furfural, HMF and phenolic compounds were very low, though there was a slight increase in concentration of these products for the most severe conditions. Previous studies have shown that the formation of these compounds is affected by the conditions of autohydrolysis, especially by temperature and reaction time (Carvalheiro et al. 2004;Nabarlatz et al. 2007;Vegas et al. 2004). The formation of degradation products was relatively low which can be eventually related to

a sub quantification of degradation products and monomeric compounds. In fact, the precipitation and/or condensation of these products with lignin present in the solid phase has been previously described by (Ramos 2003). It is also possible that due to their volatile character, some compounds (e.g., furfural) can be dissolved in the gas phase leading to miss quantifications.

Selection of the hydrolysates for further purification step was made for the autohydrolysis conditions that led to the highest concentration of XOS with lowest degradation products. The severity factor for this condition was log R_0=3.59, corresponding to a temperature of 210ºC and the composition of the hydrolysate is shown in Table 2. This hydrolysate, containing 17.83 g L^{-1} of oligosaccharides, 4.05 g L^{-1} of monosaccharides and 0.39 g $L^{-1}$of furan derivatives, was purified using preparative gel filtration chromatography.

Table 2 - Composition of the liquors obtained from autohydrolysis of rice straw at 210ºC, severity factor log R_0=3.59.

Components	Concentration (g L^{-1})
Xylo-oligosaccharides	12.86
Gluco-oligosaccharides	3.95
Acetyl groups linked to oligosaccharides	1.02
Xylose	1.64
Arabinose	0.82
Glucose	1.59
HMF	0.14
Furfural	0.25
Total phenolics	2.17

Fractionation by gel filtration chromatography

The rice straw hydrolysate produced at optimal conditions was subjected to GFC fractionation and 28 fractions were obtained. The elution profile, represented as a result of refractive index (RI) detection, and the collected fractions are shown in Fig. 4. The fractions collected between 1500 and 4750 mL of elution volume had an overall DP lower than 351. GFC separation enabled to obtain tree main categories of products: small polysaccharides and high DP-oligosaccharides (DP 351 to 23) that corresponded to 66% of the freeze dried sample mass, small oligosaccharides, which were the target XOS (DP 19 to 3), corresponding to 30% and di, monosaccharides (DP < 2) and by-products (acetic acid and degradation compounds, such as furfural and HMF, and phenolics) corresponding to 4%. This is an advantage as compared to membrane technology, as it was possible to separate the diverse classes of XOS present based on their DP, and to purify them from di-, and

monosaccharides, as well as by-products in a similar trend to the described before for GFC (Cara et al. 2012;Ho et al. 2014;Moura et al. 2007).

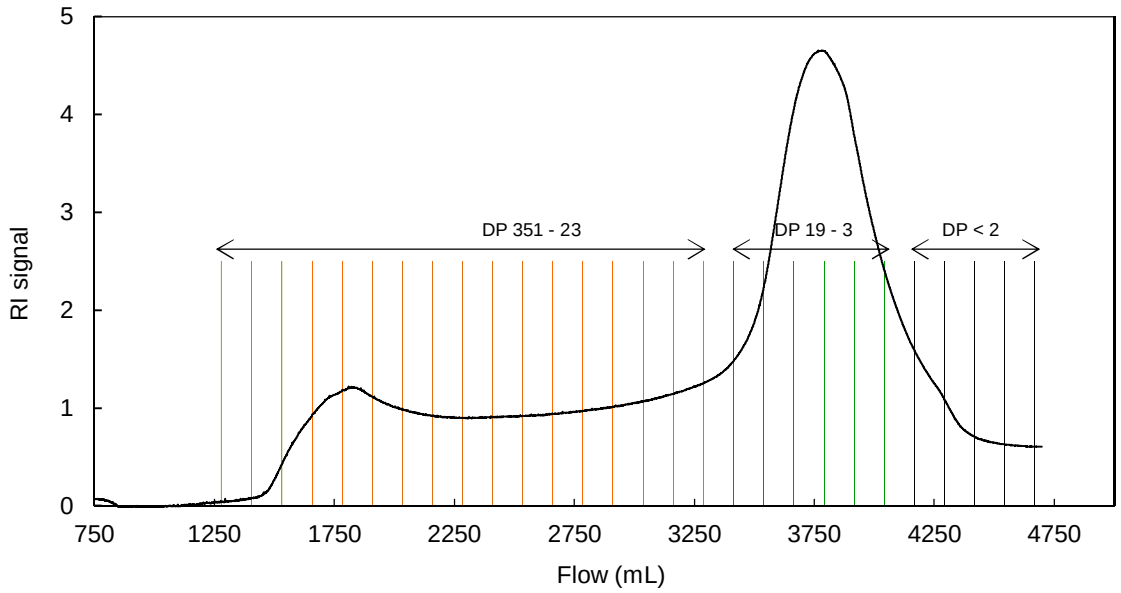

Figure 4 - Gel filtration chromatography (GFC) elution profile of the rice straw hydrolysate obtained from autohydrolysis at 210ºC (log R_0=3.59). Collected fractions are represented by vertical lines; DP intervals are indicated with arrows (RI, refractive index)

After analyzing all fractions, fractions 18 to 23 that correspond to small oligosaccharides are probably the most interesting concerning their eventual prebiotic effect, due to their low DP (Moura et al. 2008). Solid recovery after freeze-drying, average DP and oligosaccharide yield referred to raw material for these fractions is represented in Table 3. DP ranged between 19 to 3 and the amount of solids obtained after freeze-drying the liquid fractions, progressively increased up to a maximum of 0.76 g solids/100 mL hydrolysate, corresponding to fraction 19 (between 160 and 165 min elution time, data not shown). From this time on, solids yield was always inferior.

Purity, that was calculated taking into account the percentage of oligosaccharides (XOS, GlcOS and AOS) in total freeze dried solids, was in the range of 70–86% for fractions with average DP between 6 and 25. These results are in accordance with those from (Cara et al. 2012) that obtained a purity of 71% for autohydrolysis of olive tree prunings with average DP of 5 and 82–90% purity with average DP 7–25. The impurities in these fractions were mainly monosaccharides, since acetic acid, HMF and furfural were not detected.

Table 3 – Mass and purity of oligosaccharides in the GFC eluted fractions 10 to 23 of the liquors obtained from autohydrolysis of rice straw at 210ºC, severity factor log R_0=3.59

Fraction no.	DP	Recovery (g)	Purity %
10-17	23-54	3.18[a]	77.6[b]
18	19	0.59	70.4
19	15	0.76	81.9
20	10	0.60	78.9
21	6	0.31	81.4
22	5	0.43	85.6
23	3	0.16	76.5

DP – Degree of polymerisation; [a] Total mass of fractions 10 to 17
[b] Mean purity of fractions 10 to 17

Samples of all fractions were subjected to acid posthydrolysis, and the resulting hydrolysates were analyzed for structural units. The complete characterisation of the main fractions is presented in Table 4.

Table 4 - Oligomeric and monomeric composition (g L^{-1}) of the GFC eluted fractions 10 to 23 of the liquors obtained from autohydrolysis of rice straw at 210ºC, severity factor log R_0=3.59

Fraction no.	Oligosaccharides				Monosaccharides		
	XOS	GlcOS	AcOS	Total OS	Glucose	Xylose	Arabinose
10-17	6.24	2.03	0.08	8.4	n.d.	n.d.	n.d.
18	5.51	0.14	0.19	5.8	n.d.	n.d.	n.d.
19	6.05	0.15	0.18	6.4	n.d.	n.d.	n.d.
20	6.37	0.15	0.16	6.7	n.d.	n.d.	n.d.
21	5.37	0.11	0.09	5.6	n.d.	n.d.	n.d.
22	5.82	0.12	0.12	6.1	n.d.	0.07	0.01
23	5.78	0.11	0.09	6.0	0.01	0.12	0.04

XOS - Xylo-oligosaccharides; GlcOS - Gluco-oligosaccharides; AcOS - Acetyl groups linked to oligosaccharides; OS – oligosaccharides; n.d. – not detected

In all cases, XOS are the most abundant oligosaccharides, accounting for 80–90% of total OS. The highest total XOS concentrations were obtained in fractions 18–23. A progressive decrease in the concentration of XOS was found after fraction 23. Increasing amounts of monosaccharides and other monomeric compounds were detected beyond fraction 24.

Conclusions

Autohydrolysis was highly selective towards rice straw hemicellulose. A high recovery of XOS could be obtained under relatively mild operation conditions together with a low production of degradation compounds. The XOS rich liquors were fractionated by GFC into three main categories of products: small polysaccharides and high DP oligosaccharides, low and medium DP oligosaccharides, and to separate them from di-, monosaccharides and by-products. As such, the combined use of autohydrolysis to produce XOS and GFC to separate and purify them is a valuable strategy to upgrade rice straw. Furthermore, besides XOS recovery, a solid residue rich in cellulose and lignin was also obtained that can be used for further processing.

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Capítulo IV. Assessment on the bifidogenic effect of corn straw xylo-oligosaccharides

Abstract

The present work evaluates the prebiotic potential of xylo-oligosaccharides (XOS) obtained from a lignocellulosic feedstock (corn straw) within the biorefinery concept, foreseeing potential applications on food, feed and nutraceutical industries.

Autohydrolysis was used to selectively hydrolyse the xylan-rich hemicellulosic fraction and the soluble XOS-rich hydrolysates were further purified by gel filtration chromatography. Selected fractions of purified XOS within the desired ranges of polymerisation degree (4-6, sample S1 and 9-21, sample S2) were characterised and their befidogenic potential was investigated in *in vitro* fermentations using human fecal inocula. Parameters such as bacterial growth of selected microbial populations was assessed by fluorescent *in situ* hybridization (FISH), XOS consumption and short-chain fatty acid (SCFA) production were evaluated and compared with commercially available oligosaccharides (XOS and FOS). Under the tested conditions, all the substrates were utilized by the microbiota, and fermentation resulted in increased bifidobacteria populations. Samples S1 and S2 increased about 10% bifidobacteria and total substrate consumption was observed after 24h fermentation for both samples. The production profile of short chain fatty acids was similar for samples and commercial XOS and FOS. However, for longer fermentation times the highest concentration of SCFA was obtained for samples.

Este capítulo foi submetido para publicação pelos autores Patrícia Moniz, Ai Ling Ho, Luís C. Duarte, Sofia Kolida, Robert A. Rastall, Helena Pereira, Florbela Carvalheiro

Introduction

The scientific and commercial interest of oligosaccharides has increased significantly in the last two decades. This is mainly due to the identification of several health and technological properties benefited from these compounds. On the health aspect, the most recognised trait is their ability to stimulate the growth of beneficial microflora in the gut which has been associated to a prebiotic effect (Rastall, 2010). Certain classes of oligosaccharides have also demonstrated bioactive properties such as antioxidant activity, antimicrobial activity, immunostimulatory activity and anti-allergen activity, among others (Rastall, 2010). Furthermore, these compounds also have some advantageous technological properties, in particular their high pH and thermal stability which makes their use as food additives very interesting but also with potential to be used in the feed, food, pharmaceutical, chemical and biotechnological industries.

The commercial available oligosaccharides are mainly obtained by enzymatic reactions of lactose, sucrose or inulin (Moreno et al., 2014). The renewable lignocellulosic biomass is an emerging source that presents high potential to be used as feedstock for the production of novel oligosaccharides which is still almost unexplored. In fact, hemicellulosic oligosaccharides such as xylo-oligosaccharides (XOS) for example, are the only oligosaccharides in the market obtained from lignocellulosic materials and their market is still very small, particularly in the EU and US. The current production of the commercial XOS (Suntory Ltd, Japan.) is carried out using an alkaline extraction of hemicelluloses from biomass followed by enzymatic hydrolysis. XOS are xylose-based oligomers that show potential prebiotic effect by stimulating the growth of microflora in the gastrointestinal tract such as *Bifidobacteria* and *Lactobacillus*, that are related to the prevention and treatment of several gut health disorders and thus, can enhance health (Moure et al., 2006).

Amongst the biomass sources available, agricultural residues are interesting and potentially low-cost biomass. Corn (*Zea mays* L.) is one of the most produced crops worldwide and its residues, namely straws, contribute to large quantities of renewable lignocellulosic biomass. Currently, this biomass is mainly used for low value applications but, due to their chemical composition, availability and low-cost, they are an attractive feedstock.

As alternative to the processes already in use for the production of commercial XOS, the selective fractionation of hemicelluloses using mild hydrothermal pretreatments, such as autohydrolysis enables the selective hydrolysis of hemicelluloses, and a high recovery of soluble XOS (Kabel et al., 2002; Moniz et al., 2014). Besides hemicellulose-derived oligosaccharides, the hydrolysates from hydrothermal processing also contain monosaccharides and other compounds, which should be separated from target compounds in order to improve the purity. This can be achieved by membrane processing but sequential membrane-based steps are needed for concentration and fractionation to achieve high-purity XOS. This method has already been employed in a multistage purification process (dos Santos et al., 2011; Gonzalez-Munoz et al., 2013). In contrast to membrane processes, chromatographic separation has the advantage to yield XOS with high purity and separated by molecular weight and/or chemical structure (Gírio et al., 2003). Gel filtration chromatography (GFC)

was used for the purification of XOS-rich hydrolysates obtained by autohydrolysis of olive tree prunings, corn cobs and rice straw enabling the XOS fractioning by degree of polymerisation (DP), and excluding low molecular weight components, such as monosaccharides, and by-products such as furfural and hydroxymethylfurfural (HMF) (Cara et al., 2012;Ho et al., 2014;Moniz et al., 2014;Moura et al., 2007).

Different types of oligosaccharides have been studied by various *in-vitro* methods, animal models and human clinical trials. XOS for example, have shown intestinal improvement, hypolipidemic activities and antimicrobial activity against some bacteria (Christakopoulos et al., 2003). Furthermore, XOS also improved *in vitro* growth of *Bifidobacterium* spp (Moura et al., 2007, Kabel et al., 2002) which was as effective as raffinose and better than fructooligosaccharides (FOS) (Vazquez et al., 2000). The majority of the studies on prebiotics have focused on inulin, FOS, galacto-oligosaccharides (GOS), and lactulose. These groups of carbohydrates are the leading commercial prebiotics due to their efficacy (Moreno et al., 2014) in humans, and history of safe commercial use (Bouhnik et al., 2004;Macfarlane et al., 2008). Nevertheless, there is an increase in interest to develop potentially new prebiotic ingredients from both conventional and non-conventional sources, including wood or straws.

In this work, corn straw was subjected to non-isothermal autohydrolysis in order to obtain XOS. The oligosaccharides in the hydrolysates were purified and separated according to their molecular mass using gel filtration chromatography (GFC). The purification effects attained by GFC were measured, and two fractions of refined XOS were assayed for prebiotic properties (generation of SCFA and bifidogenic potential) by *in vitro* fermentations using fecal inocula.

Material and methods

Raw material

Corn straw was supplied by Estação Nacional de Melhoramento de Plantas (Elvas, Portugal) as a heterogeneous sample containing stalks and leaves. Upon arrival, the raw material was ground with a knife mill (Fritsh Industriestr, Germany) to particle size < 6 mm and set in a homogenized lot as described before (Moniz et al., 2013).

Hydrothermal processing of corn straw

Autohydrolysis treatments of the corn straw lot were performed in a stainless steel reactor (Parr Instruments Company, USA) with a total volume of 600 ml, under previously optimized conditions (Moniz et al., 2013). Briefly, the raw material was mixed with water in the reactor in order to obtain a liquid-to-solid ratio (LSR) of 10 (g water/g dry raw material). The agitation speed was set at 150 rpm and the reactor heated to reach final a final temperature of 215ºC, after which, the reactor was rapidly cooled down and the liquid and solid phases were recovered by pressing (up to 190 bar) using a hydraulic press (Sotel, Portugal). The liquid phase was filtered using Whatman filter paper no. 1 and the hydrolysates from several runs were combined in a single lot and used for further purification.

Purification of corn straw hydrolysates

Osligosaccharides purification was carried out using preparative gel filtration chromatography in an Amersham Pharmacia Biotech system (Sweden) equipped with a refractive index (K-2401 Knauer, Germany) detector. A 400 mL XOS-rich hydrolysate was eluted with deionized water at a flow rate of 25 mL min^{-1} through a BPG 100/950 column (Amersham Pharmacia Biotech, Sweden) with a Superdex 30^{TM} gel bed volume of 4.2 L, using a similar strategy reported for the separation of the rice straw oligosaccharides (Moniz et al., 2013). The sample was fractionated into 28 fractions of 125 mL, and collected every 5 min using a Super-frac^{TM} collector (Amersham Pharmacia Biotech, Sweden). All fractions were freeze-dried (Labconco, Missouri, USA), weighted for mass quantification, and analyzed for molecular weight and chemical composition.

Fermentation

Fecal inocula

Fecal samples were obtained from three healthy human volunteers who were free of known metabolic and gastrointestinal diseases (e.g., diabetes, ulcerative colitis, Crohn's disease, irritable bowel syndrome, peptic ulcers, and cancer). The samples were collected on site, kept in an anaerobic cabinet (10% H_2, 10% CO_2, and 80% N_2), and used within a maximum of 15 min after collection. Samples were diluted 1/10 (w/w) in anaerobic phosphate- buffered saline (PBS; 0.1 mol/L, pH 7.4) and homogenized in a stomacher (Stomacher 400, UK) for 2 min at normal speed.

In vitro fermentations

Sterile stirred batch culture fermentation systems (50 mL working volume) were set up and aseptically filled with a 45 mL volume of sterile, basal medium: peptone water (Oxoid, UK), 2 g/L yeast extract (Oxoid, UK), 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4$ $7H_2O$, 0.01 g/L $CaCl_2$ $6H_2O$, 2 g/L $NaHCO_3$, 2 ml Tween 80 (BDH, UK), 0.05 g/L hemin, 10 μl vitamin K1, 0.5 g/L cysteine HCl, 0.5 g/L bile salts, pH 7.0, and gassed overnight with oxygen-free nitrogen (15 mL/min). The samples of XOS mixtures (S1 and S2), commercial XOS (avDP2, Shandong Longlive Biotechnology Co. Ltd, China) and FOS (avDP4, Orafti®P95, Beneo, Tienen, Belgium), 1/100 [w/vol]) were added to the respective fermentation vessels just prior to the addition of the fecal inoculum. The temperature was kept at 37°C, and the pH was kept between 6.7 and 6.9 using an automated pH controller (Fermac 260; Electrolab, UK). Each vessel was inoculated with 5 ml of fresh fecal slurry (1/10, w/w). The batch cultures were run for a period of 36 h, and 5 mL samples were obtained from each vessel at 0, 10, 24, and 36 h for fluorescent *in situ* hybridization (FISH), short-chain fatty acid (SCFA) analysis and oligosaccharides consumption.

Before chemical analysis, a sample from each fermentation time point was centrifuged at 13,000 g for 10 min. Supernatants were filtered through a 0.22-μm filter unit (Millipore, Ireland) and used for HPLC analysis.

Analytical Methods

Chemical characterisation of XOS containing samples and fermentation samples

The autohydrolysis liquors and samples obtained after GCF purification and after fermentation were analysed for monomeric sugars, acetic acid and furan derivatives by HPLC (Agilent Technologies Liquid Chromatographer 1100 Series System, Santa Clara CA, USA) using an Aminex HPX-87H column (Bio-Rad, USA) in combination with a cation H^+-guard column (Bio-Rad) as described before (Moniz, 2013) Elution took place at 50ºC with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.4 mL min^{-1}. It was used an HPLC equipped with a diode array detector (DAD) and a refractive index detector (RI). For oligosaccharides quantification another sample was hydrolysed with 4% (w/w) H_2SO_4 (as described in Moniz (2013)).and analysed by HPLC under the conditions described above. The term XOS has been used to name the hemicelluloses-derived oligosaccharides made up of xylose and arabinose units.

The analysis of short chain fatty acids (SCFA) from each fermentation time point was carried out using the same HPLC system and column and similar operational conditions, excepting the flow rate (0.5 mL min^{-1}). Sample quantification was carried out using calibration curve standards for lactate, formate, acetate, propionate and butyrate.

The degree of polymerization of oligosaccharides in the GFC purified fractions was estimated by HPSEC on BIOSEP-SEC S2000 column (Phenomenex, USA) Elution took place at 30°C with 50 mM sodium nitrate and the elution was monitored using a refractive index detector as .previously described (Moniz et al., 2014). Calibration was performed using xylose, maltose, rafinose, stachiose and dextrans with molecular weight ranging 1 and 71 kDa, as standards.

Bacterial enumeration

Synthetic oligonucleotide probes targeting specific regions of the 16S rRNA molecule and labelled with the fluorescent dye Cy3 were utilized for the enumeration of bacterial groups (Table 1). Labelled cells were visualized using fluorescent microscopy.

Samples obtained from each vessel at each sampling time were fixed for 4 h (4°C) in 1,125 mL 4% (w/v) paraformaldehyde. Fixed cells were centrifuged at 13,000 g for 5 min and washed twice in 1 mL filter-sterilized PBS.

The washed cells were resuspended in 150 μL filtered PBS and stored in 150 μL ethanol (99%) at 20°C for at least 1 h before further processing. Samples (10 μL) were diluted in a suitable volume of PBS to obtain 20 to 100 fluorescent cells in each field of view, and 20 μL of the solution was added to each well of a six-well polytetrafluoroethylene/poly-L-lysine-coated slide (Tekdon Inc., USA). The samples were dried for 15 min in a drying chamber (46°C). They then were dehydrated using a series of alcohol solution (50, 80, and 96% (v/v) ethanol) for 3 min in each solution. Slides were returned to the drying oven for 2 min to evaporate the excess ethanol before adding the hybridization mixture. Hybridization mixture (50 μL consisting of 5 μL probe and 45 μL hybridization buffer) was added to each well and left to hybridize for 4 h in a microarray hybridization incubator (Grant-Boekel, UK). After hybridization, slides were washed in 50 mL washing buffer for 15 min. They then were dipped in cold

water for a few seconds and dried with compressed air. A small drop of polyvinyl alcohol mounting medium with 1,4-diazabicyclo(2.2.2)octane (DABCO) was added to each well, and a coverslip was placed on each slide (VWR, 20 mm; thickness no. 1; UK). Slides were examined under an epifluorescence microscope (Eclipse 400; Nikon, UK) using a Fluor 100 lens. For each well, 15 different fields of view were enumerated.

Table 1 - Oligonucleotide probes used in this study

Probe	Specificity	Ref.
Chis150	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	(Franks et al., 1998)
Erec482	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	(Franks et al., 1998)
Bac303	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae*	(Manz et al., 1996)
Bif164	*Bifidobacterium* spp	(Langendijk et al., 1995)
Ato291	*Atopobium* cluster	(Harmsen et al., 2000)

Statistical analyses

Statistical analysis was performed using SPSS for Windows, version 15.0. Univariate analysis of variance (ANOVA) and post hoc Tukey's test were used to determine the significant differences of substrates used on bacterial group populations, SCFA production. Differences were considered significant at $p < 0.05$.

Results and discussion

Composition of corn straw hydrolysates

The composition of the liquor obtained from autohydrolysis of corn straw is presented in Table 2.The final temperature of 215ºC was considered the optimum since it enabled the highest concentration of XOS with relatively low formation of degradation products (Moniz, 2013).

Under this condition sugars were mainly recovered in oligomeric form, corresponding to 72.2% of total hydrolysate, which is typical for autohydrolysis processes of straws (Carvalheiro et al., 2009; Moniz et al., 2014). Xylo-oligosaccharides were the main oligomeric components representing 84% of total oligosaccharides. Gluco-oligosaccharides (GlcOs) and acetyl groups linked to oligosaccharides (AcOS) were also obtained, although in much lower concentrations. Together, all the monosaccharides and acetic acid accounted for 2 g/L, being xylose the main monosaccharide obtained. Furfural content was low, and HMF appeared in almost negligible amounts (≤ 0.5g/L). GFC was also used in the purification of hydrolysates from olive tree prunings and oil palm empty fruit

bunch and small amounts of monosaccharides, acetic acid and furans were present in the oligosaccharide fraction at average DP of 3–6 (Cara et al., 2012; Ho et al., 2014).

Table 2 – Composition of corn straw hydrolysate obtained using autohydolysis.

Compounds	Composition (g/L)
Total oligosaccharides	**15.82**
XOS	13.3
GlcOS	1.27
AcO	1.25
Total monosaccharides	**3.94**
Xylose	1.88
Arabinose	0.96
Glucose	1.10
Acetic acid	1.31
HMF	0.07
Furfural	0.49

Fractionation by gel filtration chromatography

Corn straw liquors were subjected to GFC fractionation enabling the separation of oligosaccharides co-products and different molecular weight fractions of oligosaccharides. The first fraction was collected at 1500 mL of elution volume and a total of 28 fractions were collected. With this technique carbohydrates with different degree of polymerization (DP) were recovered, and removing (totally or partially) monomers and low molecular weight compounds. The elution profile obtained (Fig. 1) has some similarities to the previously obtained for rice straw (Moniz et al., 2014).

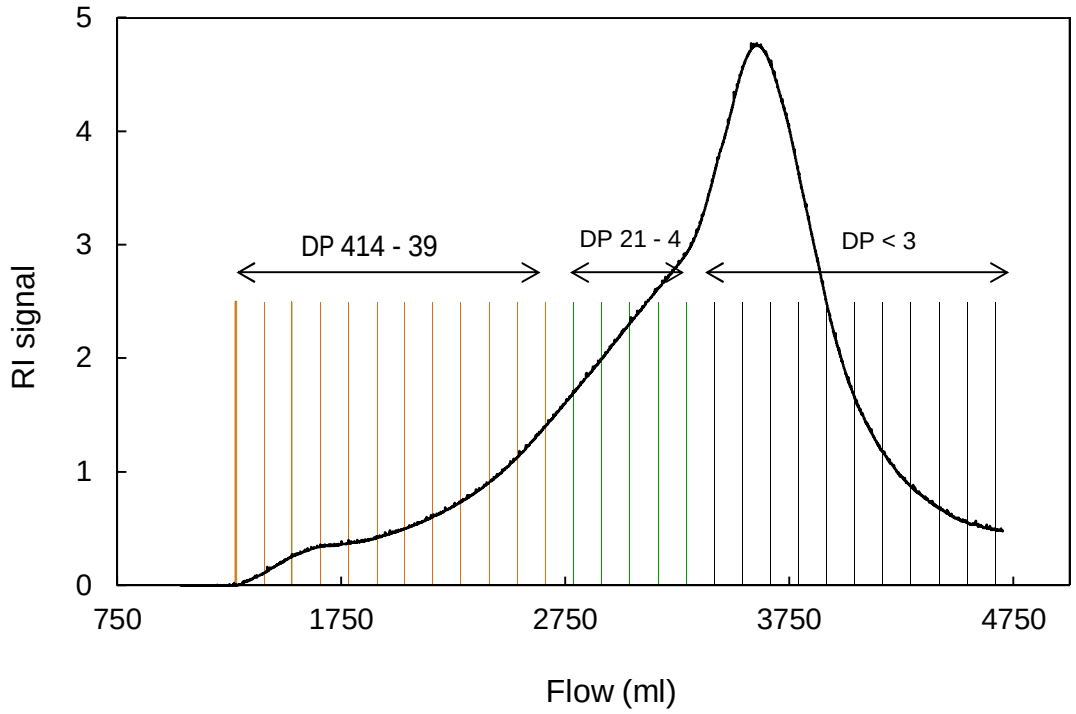

Fig. 1 - Gel filtration chromatography (GFC) elution profile of the corn straw hydrolysate obtained from autohydrolysis. Collected fractions are represented by vertical lines; DP intervals are indicated with arrows (RI, refractive index).

Table 3 shows the results for mass recovery after freeze-drying, average DP and oligosaccharide yield referred to raw material for fractions 4 to 17. The average DP of these fractions, as determined by HPSEC, ranged between 414 and 4. After analyzing all fractions, fractions 13 to 17 corresponded to medium/low DP oligosaccharides which were considered the most interesting for evaluation of prebiotic effect. For these fractions (DP of 4 to 21), the average freeze dried weight collected was 2.85 g, corresponding to about 35% of total mass recovered. The purity obtained was always higher than 72%, while samples with average DP 4-21 had 77-84% purity and of which at least 86% consisted of XOS (Table 3).

Samples of all fractions were analysed for structural units. The complete characterisation of the main fractions is presented in Table 4.

As expected, the purified samples consisted mainly of xylose oligomers. In all cases, XOS are the most abundant oligosaccharides, accounting for 77–85% of the total. The highest total XOS concentrations were obtained in fractions 13-17.

Table 3 – Mass recovered, purity and oligosaccharides concentration in the GFC eluted fractions 4 -17of the hydrolysate obtained from autohydrolysis of corn straw.

Fraction no.	DP	Recovery* (g)	Oligosaccharides (g/L)	Purity %	of which xylose oligomers %
4	414 - 63	0.07	7.29	70.82	41.52
5	189 - 49	0.09	7.36	72.51	41.04
6	81 - 44	0.09	8.07	77.94	45.01
7	51	0.09	7.11	70.42	50.91
8	54	0.04	8.27	79.16	54.33
9	51.	0.17	7.97	77.75	54.80
10	48	0.18	8.22	80.96	60.93
11	43	0.27	8.64	84.75	63.04
12	39	0.33	7.90	77.41	68.52
13	21	0.41	8.07	80.30	76.58
14	14	0.50	8.00	78.40	82.45
15	9	0.58	8.45	83.64	77.03
16	6	0.67	7.90	77.07	84.56
17	4	0.69	8.10	81.18	86.42

DP - Degree of polymerisation; * Fraction mass after freeze-drying

Monomeric glucose and arabinose, were not detected in these fractions, but only trace amounts of xylose and acetic acid. Increasing amounts of monosaccharides and other monomeric compounds were detected but only beyond fraction 19 (data not shown), which mainly contained the byproducts of oligosaccharides production. In fact, all oligosaccharides containing fractions showed similar concentrations regardless the DP range. As for acetyl groups linked to oligosaccharides (AcOS), the concentration increased with DP and there was a slight increase in acetyl groups to xylose unit ratio with higher DP, as expected.

Table 4 - Oligomeric and monomeric composition (g/L) of the target GFC eluted fractions 13 –17 of the hydrolysates obtained from autohydrolysis of corn straw.

Fraction No.	Oligosaccharides				Monosaccharides and acetic acid	
	GlcOS	XOS	AcOS	Total OS	Xylose	Acetic
13	0.94	6.18	0.94	8.07	0.07	0.19
14	0.66	6.59	0.75	8.00	0.08	0.21
15	0.50	6.51	1.44	8.45	0.07	0.21
16	0.57	6.68	0.65	7.90	0.12	0.33
17	0.56	6.72	0.92	8.20	0.10	0.29

XOS – xylo-oligosaccharides; GlcOS – gluco-oligosaccharides; AcOS – acetyl groups linked to oligosaccharides; OS – oligosaccharides.

Compared to commercial XOS, which consists mainly of DP 2-3 with a purity of 70-95 % (Moure et al., 2006), the XOS mixtures obtained in this work exhibited a suitable purity with the advantage that a wide range of DP oligosaccharides were obtained. For in vitro fermentations two samples were

selected: Sample 1 (S1) containing fractions 16 and 17, presenting an average DP ranging from 4 to 6 and Sample 2 (S2) containing fractions 13 to 15, presenting an average DP ranging from 9 to 20. By using these samples it is expected to evaluate the bifidogenic potential of both low and medium molecular weight of corn straw XOS.

In vitro fermentation

The selected fractions were used for *in vitro* fermentation followed by FISH analysis. For comparison, additional results were determined for media containing FOS as a positive control due to their well-established prebiotic properties (Rastall, 2010), a commercial XOS that has similarities with the test samples and a negative control (media without a carbon source).
Bacterial group counts are shown in Table 5.

Table 5 - Mean bacterial populations in pH-controlled batch cultures at 0, 10 and 24 hours

	Time (h)	S1		S2		XOS		FOS		Negative control	
Bif 164	0	8.07 ±	0.12	8.07 ±	0.12	8.07 ±	0.12	8.07 ±	0.12	8.07 ±	0.12
	10	8.65[1] ±	0.17	8.63[1] ±	0.13	8.65[2] ±	0.24	8.61[1] ±	0.20	8.10 ±	0.19
	24	8.83[1] ±	0.10	8.84 ±	0.26	8.78[2] ±	0.19	8.92[2] ±	0.23	8.45 ±	0.15
Bac 303	0	8.08 ±	0.12	8.08 ±	0.12	8.08 ±	0.12	8.08 ±	0.12	8.08 ±	0.12
	10	8.49[2] ±	0.03	8.64 ±	0.12	8.65[2] ±	0.12	8.68[2] ±	0.08	8.53 ±	0.15
	24	8.52 ±	0.18	8.97[2] ±	0.07	9.03[2] ±	0.21	8.85[2] ±	0.07	8.77 ±	0.11
Ato 291	0	8.21 ±	0.33	8.21 ±	0.33	8.21 ±	0.33	8.21 ±	0.33	8.21 ±	0.33
	10	8.22 ±	0.30	8.37 ±	0.11	8.54 ±	0.34	8.80 ±	0.19	8.39 ±	0.10
	24	8.30 ±	0.23	8.32 ±	0.16	8.31 ±	0.34	8.51 ±	0.20	8.29 ±	0.16
Erec 482	0	8.48 ±	0.10	8.48 ±	0.10	8.48 ±	0.10	8.48 ±	0.10	8.48 ±	0.10
	10	8.43 ±	0.28	8.41 ±	0.48	8.61 ±	0.16	8.35 ±	0.13	8.22 ±	0.35
	24	8.54 ±	0.22	8.46 ±	0.41	8.59 ±	0.12	8.36 ±	0.20	8.50 ±	0.13
Chis 150	0	7.24 ±	0.23	7.24 ±	0.23	7.24 ±	0.23	7.24 ±	0.23	7.24 ±	0.23
	10	7.46 ±	0.13	7.50 ±	0.09	7.43 ±	0.12	7.56 ±	0.17	7.56 ±	0.08
	24	7.49 ±	0.03	7.35 ±	0.07	7.42 ±	0.13	7.57 ±	0.18	7.49 ±	0.11

S1 - Sample 1 containing fractions 16 and 17, presenting an average DP ranging 4 to 6
S2 - Sample 2 containing fractions 13 to 15, presenting an average DP ranging 9 to 20
FOS – commercial fructo-oligosaccharides; XOS– commercial xylo-oligosaccharides, Negative control without carbon source
[1], significant difference from the 0-h value, $P < 0.01$
[2], significant difference from the 0-h value, $P < 0.05$
± standard deviations

There was an increase in *Bifidobacterium* and *Bacteroides* populations (enumerated by probe Bif164 and Bac 303) in response to both samples (S1 and S2) and well as commercial OS (XOS and FOS) tested, at all time-points. In negative controls there was no significant increase during fermentation, confirming the suitability of these substrates as carbon source for the metabolism of bifidobacteria. These results are in agreement with those obtained for low molecular corn cobs XOS which exhibit a potential bifidogenic capability similar to commercial XOS (Gullon et al., 2011b). Similar results were also obtained with oligosaccharides derived from wood mannan (Rivas et al., 2012), which demonstrated a similar performance to FOS to modify the *Bifidobacterium* population. The substrates tested (samples and commercial OS) showed a significant increase concerning *Bacteroides.* No significant changes were recorded in other bacterial populations with all substrates tested, namely in *Clostridium* population.

Overall, it can be seen that the XOS fractions obtained in this work led to an increase of the bifidobacteria population which was very similar to commercial XOS and FOS. After 24 hours of fermentation a 10% increase bifidobacteria population was recorded for S2 and FOS. For all other substrates the increase was similar. These results compare positively with those obtained for other substrates such as inulin and different molecular weight dextrans used by Sarbini et al., 2011.

Substrates consumption

Figure 2 shows the substrate consumption obtained for samples S1 and S2, XOS and FOS throughout the fermentation. Independently of the substrate tested, all substrates were exhausted at 36 h fermentation. For other fermentation times the substrates tested display a different trend, although in general, almost all substrates were consumed at 24 h fermentation. FOS, for example were consumed faster than any other oligosaccharides. The concentration of FOS dropped markedly to reach about 20% of the initial value during the first 5 h of fermentation. In contrast, XOS tend to be consumed slower. Sample S2, corresponding to higher DP XOS, presented a sugar consumption of about 13 % in the first hours of fermentation. Commercial XOS and FOS presented less than 10 % of initial sugars after 10 h, whereas the concentrations sample XOS decreased to 54.3% for S1 and 73% for S2 of their respective initial amounts.

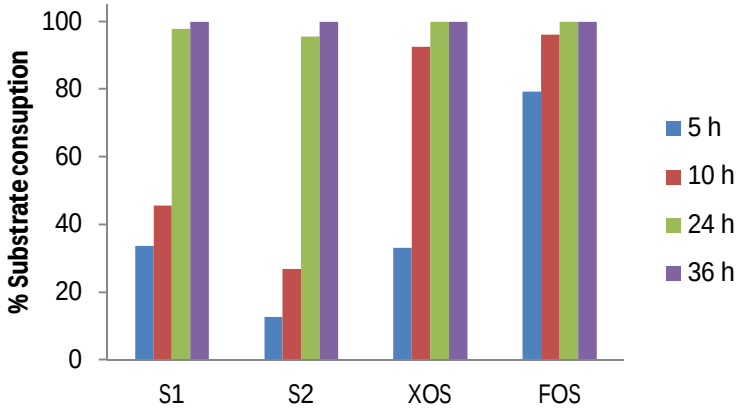

Fig 2 – Substrate consumption obtained in the *in vitro* fermentation at different fermentation times (5 h, 10 h, 24 h and 36 h).

The media inoculated with sample S2 presented the slowest fermentable substrate. The observed OS consumption pattern was in agreement with previous reported results (Gullon et al., 2011a) and the slow assimilation of S1 and S2 suggests that fermentation of these XOS in humans may proceed (at least, in part) in the distal part of the colon. Increased SCFA production in the distal colon is expected to result in protective effects (Yang et al., 2014). In fact, the chemical structure of oligosaccharides (defined by degree of polymerization, sugar composition, type of linkages, or degree of esterification) has influence in their fermentation rate (Cardelle-Cobas et al., 2012). This way, it was expected that these medium-high DP hemicellulose derived oligosaccharides exhibit a branched and complex structure as compared to low DP oligosaccharides leading to a slowest fermentation. Moura et al. (2008) proposed that medium chain XOS obtained from autohydrolysis of xylan-rich materials can be regarded as promising distally fermentable substrates.
Fermentation clearly resulted in microbial growth and formation of SFCA and lactate.

Short chain fatty acids

Lactate, acetate, propionate and butyrate formation was analysed throughout the fermentation in batch cultures (Fig. 3). Total SCFA concentrations increased in all substrates tested. Comercial XOS, FOS and sample S2 presentend a similar SCFA production profile. However, for longer fermentation times (i.e., 24 h) the highest concentration of SCFA was obtained for samples S2, and at 36 h of fermentation, the production of SCFA was considerably higher when compared to commerial OS, being S1, the substrat with lower DP, the one that presented the highest value. These results are in agreement with previous studies where SCFA concentrations incresed, with XOS fermentation (Campbell et al. 1997; Imaizumi et al. 1991).
Acetate was the main SCFA produced of all substrates tested and its production was higher for XOS samples, than for commercial OS. Acetate production (Fig. 3A) accounted for about 60% of total SCFA, followed by propionate (Fig. 3B) and butyrate (Fig. 3C). Substantial increases in acetate were found on all substrates tested, and the highest concentrations were observed in XOS samples (S1 and S2). The production profile of propionate was similar to acetate, although the concentrations attained were lower. This is in agreement with the significant increase in the *Bacteroides-Prevotella* group (Table 5), as these microorganisms are known to be propionate producers (Gomez et al., 2014). A number of other bacterial groups including *clostridia* (e.g. *Clostridium histolyticum*) and *clostridial* cluster IX could also be implicated in propionate production (Zigova et al., 1999). However, no significant increase of *C. histolyticum* and *Clostridium* cluster IX was observed. (Djouzi and Andrieux, 1997) also reported an increase of propionate production during the fermentation of FOS, GOS and GlcOS which was ascribed to *Bacteroides* populations.
Clostridium coccoides one of the major butyrate producers (Barcenilla et al., 2000), was not stimulated by the supplementation of the substrates used. Erec482 cover wide range butyrate producing bacteria groups. It is suspected that a specific species caused the increase of butyrate, otherwise no significant changes in this SCFA can be observed.

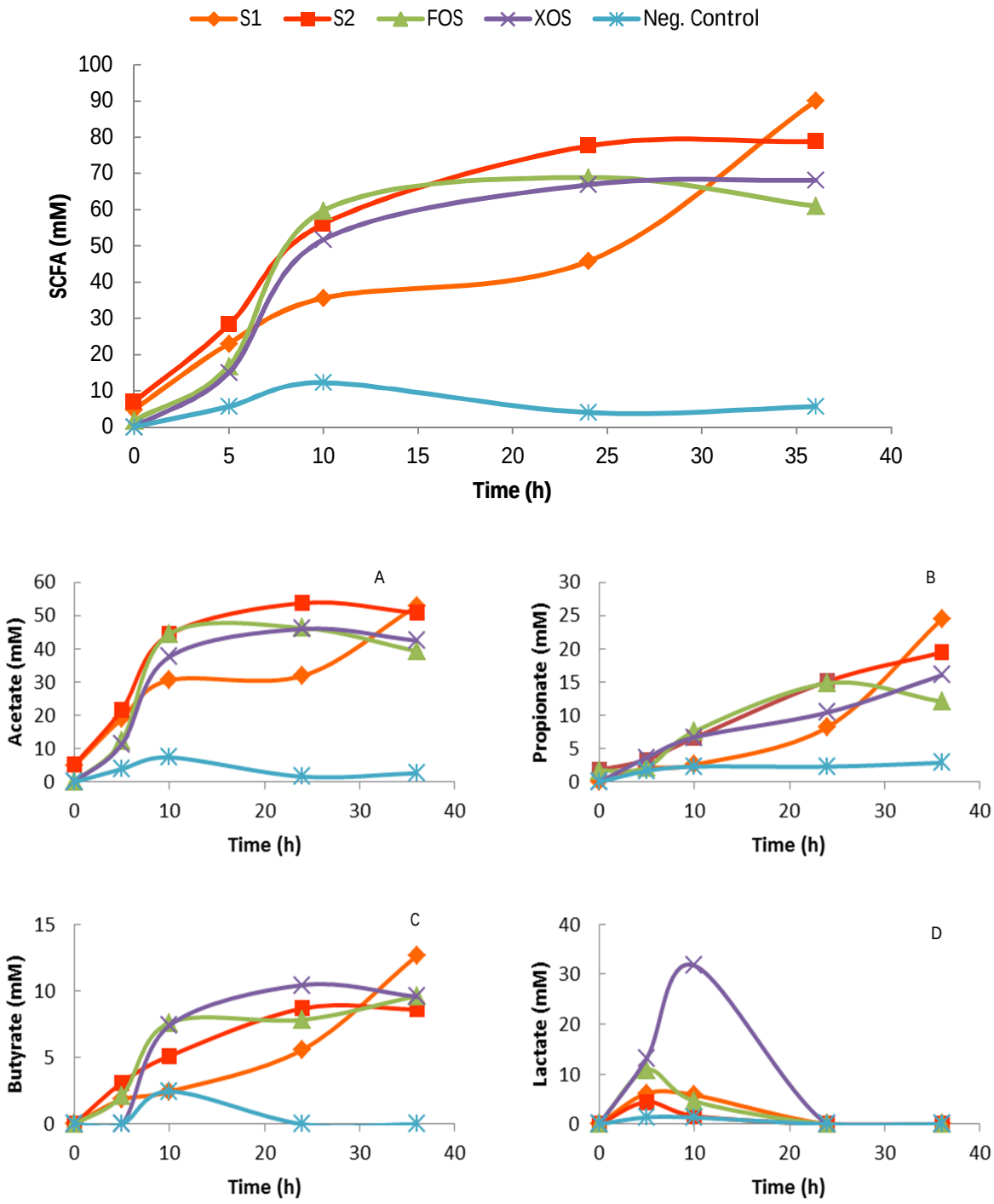

Fig.3 - Time course of total SCFA, Propionate (A), Acetate (B), Butyrate (C) and Lactate (D) production during fermentations.
S1 - Sample 1 containing fractions 16 and 17, presenting an average DP ranging 4 to 6; S2 - Sample 2 containing fractions 13 to 15, presenting an average DP ranging 9 to 20; FOS – commercial fructo-oligosaccharides; XOS – commercial xylo-oligosaccharides, Negative control - without carbon source

The fermentation of all substrates tested induced the production of lactate (Fig. 3D). This correlates with the significant increase of *Bifidobacterium* populations. *Bifidobacterium* spp. and lactic acid bacteria such as *Lactobacillus* and *Enterococcus* spp. produce lactate as a major product. The highest concentration of lactate was obtained for commercial XOS although this level declined beyond 10 h, as occurred for other substrates tested. This was expected, as lactate is utilized by other bacteria which then produce acetate, butyrate, and propionate (Duncan et al., 2004). In general, lactate does not accumulate in healthy subjects.

Conclusions

Autohydrolysis of corn straw under the condition employed in this work resulted in wide hemicellulose solubilisation, leading to a XOS rich hydrolysate. The purification process of the hydrolysates enabled to obtain high purity fraction with different molar mass distributions. In vitro fermentation experiments confirmed the ability of XOS samples to support the growth of bifidobacteria. Fermentation clearly resulted in microbial growth and formation of SFCA and lactate.

Acknowledgments

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Capítulo V. Fractionation of hemicelluloses and lignin from rice straw by combining autohydrolysis and optimized mild organosolv delignification

Abstract

An integrated strategy was followed to valorise rice straw, one of the most relevant biomass feedstocks available worldwide, to selectively recover solubilised hemicellulose and lignin. The pathway encompassed the use of autohydrolysis to hydrolyse the hemicelluloses and an ethanol-based organosolv process to solubilize lignin.

Several autohydrolysis conditions were assayed with the best results obtained at 210 ºC (log R_0 4.15), which enabled a high removal of hemicelluloses, yielding an oligosaccharide-rich hydrolysate and a treated biomass with low content of hemicelluloses and enriched in cellulose and lignin.

The effects of ethanol concentration (5-75%) and reaction time (0-24 h) on lignin removal under mild temperature (30ºC) conditions were studied. Under the optimal conditions the delignification yield reached 42 % whereas glucan solubilisation was always lower than 17%. Reaction time did not affect the process, conversely to ethanol concentration that favored the delignification up to 60.5% ethanol, after which delignification yield decreased. The organosolv liquors to contained economically interesting lignin-derived compounds such as vanillin, ferulic and coumaric acids. The chemical composition and enzymatic digestibility of the treated biomass from autohydrolysis and from autohydrolysis followed by delignification were compared, with the latter presenting an almost 10 % higher enzymatic digestibility than the former.

Este capítulo foi submetido para publicação pelos autores: Patrícia Moniz, João Lino, Luís C. Duarte, Luísa B. Roseiro, Carmen G. Boeriu, Helena Pereira, Florbela Carvalheiro

Introduction

Rice straw is usually considered a waste material, used only to a small extent for cattle beds, mulching and combustion. Notwithstanding, its potential for valorisation within the biorefinery framework has been demonstrated as a major single feedstock for bioethanol production (Matsumura et al., 2005).

Environmental friendly processes, yielding separate streams that can be further used for different product lines are preferential, and the selectivity of the processes used towards the target polymer is essential. One possible strategy is to carry out in a first step the hydrolysis of the hemicellulosic fraction by hydrothermal processing (autohydrolysis) before further processing within the biorefinery. This is an effective and selective pre-treatment enabling a high production of soluble hemicellulosic oligosaccharides from the liquid phase and a high recovery of cellulose and lignin in the solid phase (Carvalheiro et al., 2009; Moniz et al., 2013).

For the fractionation of lignin, processes based on organic solvents such as low boiling-point alcohols (e.g. methanol, ethanol), acetone, and/or organic acids (organosolv processes) (which can be easily recovered have shown promising results (Binod et al., 2010; Bozell et al., 2011; Girio et al., 2010). The products that can be obtained include sulphur-free lignin fragments, which are useful for the production of lignin-based high value products due to their high purity, low molecular weight, and easily recoverable organic reagents (Garrote et al., 2008; Toledano et al., 2012). If applied directly to the lignocellulosic material, the organosolv treatment will yield a liquid stream containing both lignin and hemicellulose derived products, requiring major purification (Harmsen et al., 2011). Furthermore it would induce some carbohydrate loss for the subsequent processes (Bozell et al., 2011; Huijgen et al., 2010; Toledano et al., 2013)

The use of sequential autohydrolysis and organosolv processes can be a relevant strategic pathway, especially if organosolv delignification is carried out under mild conditions. A proposal for the selective fractionation of hemicelluloses and lignin aiming at the production of valuable compounds is shown in Fig. 1.

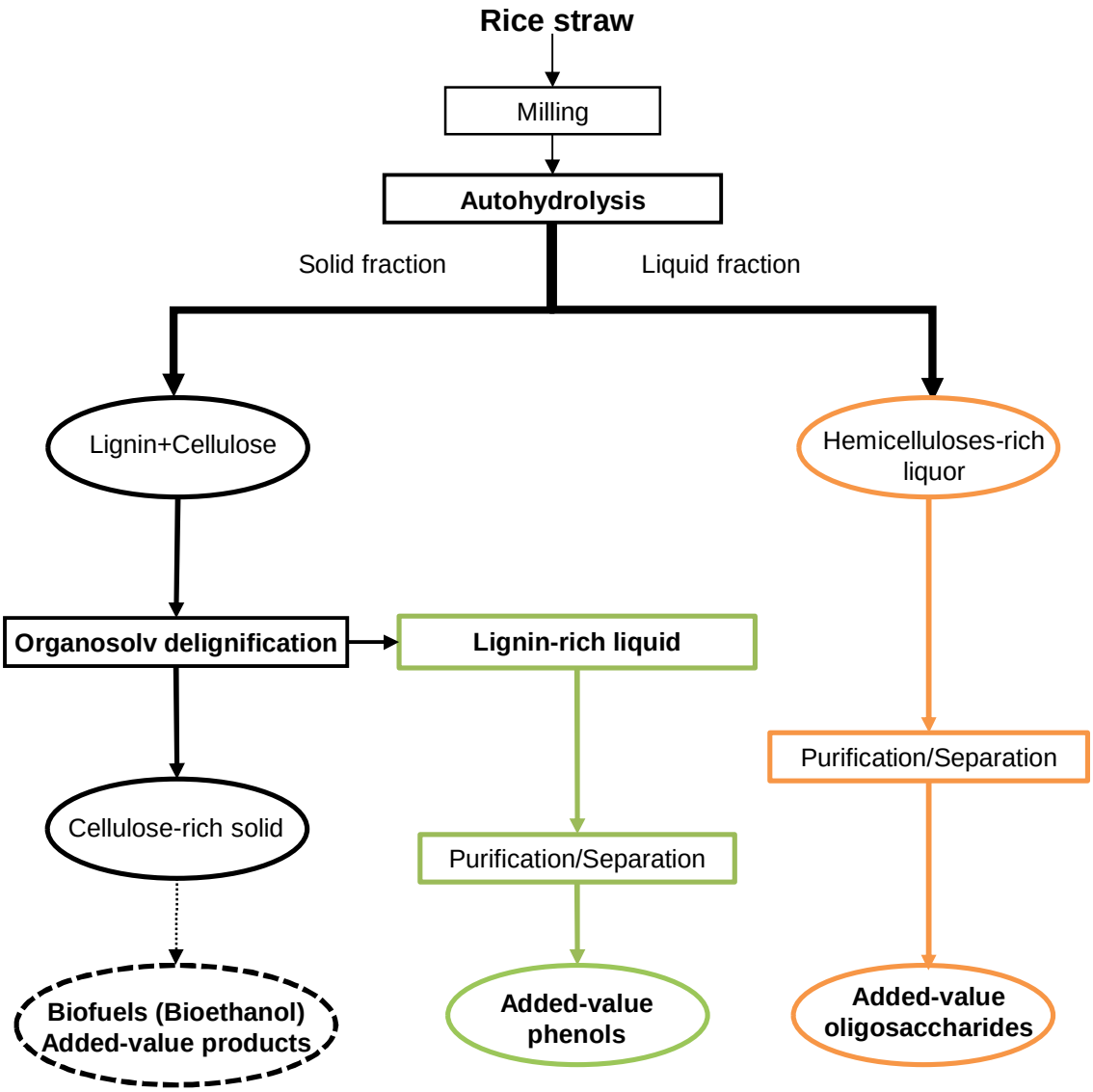

Fig. 1 – Proposed pathway for the selective fractionation of structural polymers from rice straw.

In this work, the potential upgrading of rice straw by sequential pre-treatments of autohydrolysis and organosolv delignification was investigated. The organosolv delignification was optimised in relation to reaction time and ethanol concentration by a experimental design for the maximum recovery of high quality lignin. The lignin-derived products were characterized and the upgrading potential of the remaining cellulose-rich solids (i.e. for bioethanol production) was also evaluated.

Experimental

Raw material

Rice straw was kindly provided by Orivárzea (Salvaterra de Magos, Portugal). The raw material was dried, milled to particles smaller than 6 mm, homogenised, combined in a single lot, and stored in plastic containers at room temperature as described before (Moniz et al., 2014).

Soda wheat straw lignin and P1000 soda lignin from mixed Sarkanda grass and wheat straw were obtained from Greenvalue SA (Lausanne, Switzerland). Organosolv lignin from mixed hardwoods (Alcell) was obtained from Repap Technologies Inc. (Valley Forge, PA, USA).

Autohydrolysis of rice straw

Autohydrolysis treatments were carried out in a stainless steel reactor (Parr Instruments Company, USA) with a total volume of 2 L. Temperature was controlled through a Parr PID controller (model 4842).The raw material was mixed with water in the reactor to a liquid-to-solid ratio of 10 (g water/g dry raw material). The reactor was heated to the final temperatures of 195, 200, 205, 210, 215 and 220ºC, corresponding to a severity factor (log R_0) ranging from 3.66 to 4.35. After reaching the desired temperature, the reactor was rapidly cooled down, the liquid and solid phases were separated, and the solid phase washed and dried (Moniz et al., 2013). After selection of the optimised conditions at 210ºC, several pretreatment batches were performed in order to produce solids to be used in organosolv studies, that were previously thoroughly mixed into an homogenized lot.

Organosolv delignification

The solids obtained after autohydrolysis under optimized conditions (210ºC) were subjected to organosolv delignification using different ethanol/water mixtures and duration periods, following the experimental design described in 2.5. A solid-liquid ratio of 1:10 (w/w) was used and all reactions were carried out using Schott flasks in an incubator set for 30ºC and 150 rpm. Upon reaction completion, the flask content was filtered and the solid phase was washed with twice the amount of ethanol/water solution and dried at 45ºC to be used both for chemical characterisation and saccharification assays. The solid yield of the organosolv delignifications was determined as g of solid per 100 g of the autohydrolsed material (oven dry mass).

The yields of lignin (KL_R), glucan (Gn_R) and xylan (Xn_R) were calculated according to the following equations:

$$KL_R = SY \cdot \frac{KL}{\cdot KL_i} \quad \text{(Eq. 1)}$$

$$Gn_R = \frac{Gn.SY}{Gn_i} \quad \text{(Eq. 2)}$$

$$Xn_R = \frac{Xn.SY}{Xn_i} \quad \text{(Eq. 3}$$

$$Y_{KL} = 100 - KL_R \quad \text{(Eq. 4)}$$

where SY is the solid yield (g of solid recovered after treatments per 100 g feedstock), KL, Gn, and Xn refer to the polymer content in the solid samples after each treatment (autohydrolysis/organosolv delignification), YKL is the delignification yield and Gni, Xni, and KLi, refer to the corresponding polymer content in the sample prior to the treatment. All data is reported on dry weight basis.

Experimental design

The experimental statistical design for optimisation of the organosolv delignification followed a distribution for two factors, adapted from (Doehlert, 1970; Ferreira et al., 2004). Twelve experiments (including four replicates near the center of the experimental domain) were carried out (Table 1). Besides seven experiments prescribed by the Doehlert matrix for two variables, two additional tests were carried out (11 and 12) to further explore extreme conditions of very low ethanol proportion. The factors studied were delignification time (X_1) that varied between 0 and 24 h, and ethanol concentration (X_2) that varied between 0 and 100% (w/w).

Table 1 - Experimental conditions of the experimental design

Exp.	Coded Variables		Real Variables	
	X_1	X_2	Time (h)	Ethanol (%, w/w)
1	0	0.050	12	52.5
2	0	0.050	12	52.5
3	0	0.050	12	52.5
4	0	0.050	12	52.5
5	1	0.050	24	52.5
6	-1	0.050	0	52.5
7	0.5	0.440	18	71.90
8	-0.5	-0.340	6	33.05
9	0.5	-0.340	18	33.05
10	-0.5	0.440	6	71.90
11	-0.5	-0.866	6	6.7
12	0.5	-0.866	18	6.7

The effect of each variable was determined according to the polynomial model (Eq. 5):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \qquad \text{(Eq. 5)}$$

Where Y is the response variable, X_1 is time (h) and X_2 is ethanol concentration.

β_0 is the intercept and represents the value of the response in the center of the experimental domain, β_1 and β_2 are the parameters directly associated with the time and the concentration of ethanol, respectively, representing the relative importance of each factor in the analysed

response. The interaction parameter, β_{12}, indicates the dependence of the effect of one factor in relation to the level of another factor. The quadratic terms (β_{11} and β_{22}) are model tuning parameters that provide information about the geometric trend of the response surface.
Data fitting and statistical analyses were carried out using Microsoft Excel®, v. 2010.

Analytical Methods

Chemical characterisation of raw material and processed solids
The materials were ground in a knife mill (IKA, Germany) to a particle size smaller than 0.5 mm and the moisture content was determined by oven-drying at 100°C to constant weight. The ash content was determined at 550ºC using NREL/TP-510-42622 protocol (Sluiter et al., 2005). The samples were hydrolysed for determination of glucan, xylan, arabinan and acetyl groups using acid hydrolysis with 72% (w/w) H_2SO_4 followed by hydrolysis with 4% (w/w) H_2SO_4. The acid insoluble residue was considered as acid insoluble lignin, after correction for ash and the quantification of macromolecular compounds was carried out as previously described (Moniz et al., 2013).

Chemical characterisation of the autohydrolysis liquors

The autohydrolysis liquors were analysed for monomeric sugars, acetic acid and furan derivatives by HPLC (Agilent Technologies Liquid Chromatographer 1100 Series System, Santa Clara CA, USA) using an Aminex HPX-87H column (Bio-Rad, USA) in combination with a cation H^+-guard column (Bio-Rad) as described before (Moniz, 2013) Elution took place at 50ºC with 5 mmol/L H_2SO_4 at a flow rate of 0.6 mL/min. It was used an HPLC equipped with a diode array detector (DAD) and a refractive index detector (RI). For oligosaccharides quantification another sample was hydrolysed with 4% (w/w) H_2SO_4 (as described in Moniz et al., 2013).and analysed by HPLC under the conditions described above.

Total phenolic compounds

Total phenolic compounds in the organosolv liquor were determined by the Folin–Ciocalteu colorimetric method according to (Singleton et al., 1999). Briefly, 100 µL of the organosolv sample was mixed with 5 mL of the 1/10 (v/v) diluted Folin–Ciocalteu reagent and 4 mL of 7.5% Na_2CO_3. Absorbance was measured at 765 nm after 15 min incubation at 45 ºC. Total phenolic compounds are expressed as mg GAE mL^{-1} (gallic acid equivalents).

Capillary Zone Electrophoresis

The organosolv liquors were analysed by Capillary Zone Electrophoresis (CZE) in order to obtain their phenolic profile using an Agilent System, with diode-array detector (DAD). ChemStation data software and a fused-silica uncoated i.d. 50 um and 62/56 cm effective

length, extended light path capillary also from Agilent was used. 30 kV voltage was applied and injection was done at 50 mbar for 6 s. A 15 mM borate in 10% MeOH was used as electrolyte adjusted to pH 9.1 and temperature was maintained at 25°C. The capillary was preconditioned between runs by flushing with 0.1 M NaOH (3 min) followed by buffer (3 min). Detection was at 200 and 280 nm and compounds were identified by electrophoretic comparison (migration times and UV spectra) with authentic standards.

Molecular weight characterisation

The molar mass distribution of the lignin fragments solubilised in the organosolv liquors was analysed by alkaline size exclusion chromatography (SEC) using a TSK gel Toyopearl HW-55F column, 0.5 M NOH as eluent, UV detection at 280 nm and calibration with sodium-polystyrene sulfonates, according to the procedure as described elsewhere (Gosselink et al., 2010). Calculations included Mp (peak molecular weight), Mn (number average molecular weight) and Mw (weight-average molecular weight and polydispersity (PD, Mw/Mn).

Enzymatic hydrolysis

The enzymatic digestibility of the untreated, the autohydrolysis pretreated and the organosolv delignified solids of rice straw was evaluated based on the NREL/TP-510-42629 protocol (Selig et al., 2008). The results were expressed as the percentage of glucose released after 72 h enzymatic hydrolysis in relation to initial glucose. All assays were carried out at least in duplicate and the results are given after correction for enzyme and biomass blank tests.

Results and discussion

Selective fractionation of rice straw hemicelluloses

In order to selectively fractionate hemicelluloses, six temperatures of an autohydrolysis pretreatment were studied ranging from 195 to 220ºC (corresponding to severity factors of log R_0 3.66 to 4.35), as previous studies carried out at a smaller scale demonstrated that optimal conditions for hemicellulose hydrolysis of rice straw ranged within this temperature interval (Moniz et al., 2014). Fig. 2 and Table 1 show the effect of autohydrolysis on the macromolecular components of rice straw. The variation of xylan, glucan, lignin and total pentoses (oligomeric and monomeric) as a function of the severity factor shows that autohydrolysis was a selective pretreatment for the hydrolysis of the hemicellulosic fraction (Fig. 2). The hydrolysis of xylan increased with the temperature and became significant for temperatures above 200ºC, for which more than 70% of the initial xylan was solubilised. A similar trend was obtained in other autohydrolysis studies using rice straw, corn straw and wheat straw as raw materials (Carvalheiro et al., 2009; Moniz et al., 2013; Moniz et al., 2014).

The amount of solubilised pentoses in the liquid increased in parallel with xylan hydrolysis to reach a maximum of 41% of the initial amount at 210ºC which corresponds to a concentration

11.6 g/L. The maximum production of pentoses obtained in this work was similar to that obtained in other studies with rice husk (Nabarlatz et al., 2007) but lower than those obtained with corn straw (Moniz et al., 2013). Contrasting with xylan solubilisation, glucan remains essentially on the solid phase: maximal solubilisation corresponded only to 20% of the initial glucan in the raw-material. These values are in agreement with the known behavior of autohydrolysis treatments that are characterized by a low solubilisation of the cellulose fraction.

Lignin content in the solid followed a similar profile which is also typical of autohydrolysis pretreatment. Up to 210ºC, the lignin that remained in the solid was always close to 100% of the initial amount, showing that the treatment did not substantially affect this component. From 210ºC onward, lignin in the solid decreased e.g. about 30% at 215º, and increased again for the most severe condition studied. This lignin increase is also typical of these processes and can be associated with condensation reactions of lignin with sugars and degradation products such as furfural, leading to the formation of insoluble compounds (Ramos, 2003) that are quantified as Klason lignin.

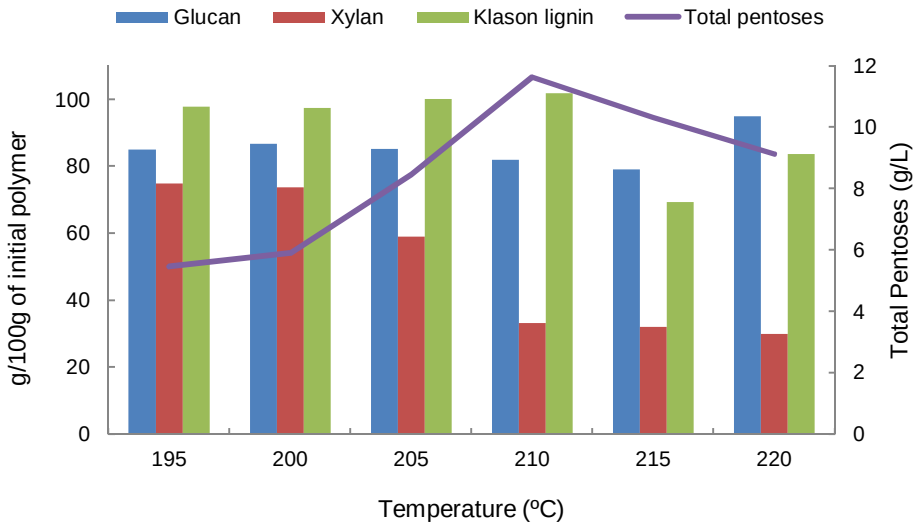

Fig. 2 – Effect of autohydrolysis temperature on glucan, xylan and Klason lignin recovery in the treated solids (bars) and total soluble pentoses concentration in the liquid phase (line).

The composition of the raw material and the solid phase resulting from the autohydrolysis of rice straw for different severity conditions is presented in Table 2. For the milder conditions (195ºC and 200ºC), the solubilisation of the solid phase was about 20%, but increased with increasing severity of the treatment e.g. 35% at 210ºC.

Glucan was practically not affected by the treatments and the processed solids obtained in the most severe condition contained 53.2% glucan; this corresponds to an enrichment in glucan of the solid as compared to the raw material by an increase of 30%.

Xylan content was highly affected by the pretreatment and decreased with increasing severity to reach 8% in the autohydrolysed solids at 220ºC. Lignin was almost not solubilized (Fig.1), and therefore lignin content generally increased with pretreatment severity, and the highest value

was obtained for 210ºC. The autohydrolysis temperature leading to small losses of glucan and lignin in the solid and to considerable xylan solubilisation with the highest amount of total pentoses in solution was 210ºC. This temperature was selected as the optimum autohydrolysis condition that enables both the production of pentose rich liquors (mainly oligosaccharides, (Moniz et al. 2014) and solids enriched in cellulose and lignin for further processing in the organosolv studies.

Table 2 - Raw material polymeric composition, solid yield and solids composition obtained after autohydrolysis at different temperatures.

		Temperature, ºC (log R_0)					
(%)	Raw material	195 (3.66)	200 (3.80)	205 (3.95)	210 (4.15)	215 (4.25)	220 (4.36)
Solid yield[a]	-	80.21	79.66	74.86	65.14	67.63	67.21
Xylan[b]	20.46	19.10	19.17	16.03	10.29	9.96	8.39
Glucan[b]	40.90	43.39	45.06	46.28	50.89	49.04	53.18
Klason lignin[b]	14.43	17.60	17.87	19.19	22.32	15.16	16.54

[a] (g/100 g raw material); [b] (g/100 g autohydrolysed solid)

Organosolv process optimisation

Table 3 presents the experimental conditions of the delignification experiments and the corresponding responses regarding the delignification yield (liquid phase) as well as the xylan and glucan yields (solid phase). The delignification yields i.e. percent of lignin solubilised ranged from 30.6% to 41.7% for experiments 1-10 and between 10.8% and 19.7% in the case of experiments 11 and 12. Reaction time favored delignification to only a small extent, and significant delignification was already obtained for very short reaction times. The highest lignin solubilisation corresponded to a delignification yield of 41.7% and was obtained in experiment 5 (52.5% ethanol and 24 h). The effect of ethanol content may be seen by comparing experiments 7 and 10 (71.9% ethanol) and 8 and 9 (33.05% ethanol), for which the delignification yields were 40% and 30%, respectively. This difference shows that ethanol concentration influence lignin removal.

An interesting result regards experiment 6 (52.5% Ethanol and 0 h) for which the very substantial delignification yield (38.3%) shows that there is a very rapid reaction of lignin with ethanol, probably of the more reactive moieties, and with immediate solubilisation of the lignin fragments. This shows that rice straw lignin is considerably reactive to ethanol, and over one third of the lignin solubilises at near ambient temperature (30ºC). This does not happen for wood lignin for which high temperatures (e.g. 165ºC) are needed to attain similar delignification degrees (Pereira et al. 1986). Also an important factor for the enhancement of lignin solubilisation is the solid matrix opening caused by the autohydrolysis treatment that the material underwent before the organosolv process. By dissolving the hemicelluloses during the

thermal hydrolysis, there is disruption of the lignin-carbohydrate linkages and of the macromolecular arrangement in the cell wall that will facilitate access and reactivity to subsequent processes. In fact, the lignins obtained showed very low molecular mass when compared to commercial lignins (see Table 5). The time of delignification is therefore not an important factor if only partial extraction of lignin is aimed. Conversely a minimum ethanol concentration is important for this process as shown by experiments 11 and 12 where the delignification yields were substantially lower (19.7% and 10.8%). .

Table 3- Experimental conditions of organosolv delignification and the corresponding responses

Experiments	Real Variables		Response		
	Time (h)	Ethanol (%)	Delignification yield (%)	Glucan yield (%)	Xylan yield (%)
1	12	52.50	32.95	83.52	99.82
2	12	52.50	37.84	87.99	102.25
3	12	52.50	39.74	83.82	100.61
4	12	52.50	38.98	84.61	101.41
5	24	52.50	41.69	85.16	104.56
6	0	52.50	38.28	85.44	107.41
7	18	71.90	39.69	84.14	105.77
8	6	33.05	30.60	86.37	101.97
9	18	33.05	33.03	89.02	101.08
10	6	71.90	38.28	82.72	99.31
11	6	6.70	19.69	96.28	95.34
12	18	6.70	10.75	101.84	107.38

The overall delignification yield obtained for the different experiments was lower than that obtained by (Romani et al., 2011; Sindhu et al., 2012; Sun and Sun, 2002) for rice straw organosolv delignification. This is the result of the very mild temperature used in this study (30ºC) in comparison with conditions used in other studies e.g. 70ºC and/or catalysts (e.g. H_2SO_4 or HCl) .

The amount of the polysaccharides (glucan and xylan) remaining in the solid was also analyzed. In contrast to lignin, the polysaccharides were almost not affected by this mild organosolv treatment demonstrating the selectivity of this process towards lignin. In fact both glucan and xylan yields after delignification were high with complete xylan recovery in almost all experiments. Higher xylan recovery was found in this work when compared to previous studies (El Hage et al., 2010). In the case of glucan, there was no solubilisation in experiments that used low ethanol concentrations for which glucan yield was close to 100%; in the other cases the glucan yields were about 85%. Glucan yields are in agreement with those obtained by (Huijgen et al., 2010) who studied the direct delignification of raw wheat straw. In other studies

(Caparros et al., 2007; Romani et al., 2011) with a pretreatment before organosolv delignification, glucan yields were inferior (ranging 70 to 87%) to the results obtained in this study.

The influence of time and ethanol concentration in solubilised lignin yield and on glucan and xylan yield was modelled and β parameters were calculated. Table 4 presents an estimate for the regression coefficients and respective significance levels for a polynomial model, along with the coefficient of multiple determination (R^2) for the different responses. The responses that can be correlated with the studied variables by the proposed equation (Eq. 1) are shown in Table 4.

Table 4 - Regression coefficients and statistical parameters measuring the correlation and significance of the experimental design

Parameters	Responses		
	Delignification yield (%)	**Glucan yield (%)**	**Xylan yield (%)**
β_0	38.86 ± 0.35 (0)*	84.31 ± 0.50 (0)*	100.68 ± 1.21 (0)*
β_1	1.83 ± 0.40 (0.01)*	0.42 ± 0.54 (0.47)	0.65 ± 1.45 (0.66)
β_2	11.81 ± 0.77 (0)*	-6.20 ± 1.07 (0)*	0.37 ± 2.88 (0.90)
β_{12}	-1.15 ± 1.69 (0.53)	0.70 ± 1.39 (0.63)	-1.47 ± 3.72 (0.71)
β_{11}	1.10 ± 0.62 (0.15)	1.30 ± 0.89 (0.21)	5.07 ± 2.30 (0.06)
β_{22}	-25.39 ± 1.55 (0)*	7.76 ± 1.69 (0*)	3.73 ± 5.43 (0.43)
R^2	0.998	0.988	0.714

All values are presented in the form "coefficient +/- standard error (*p*-value). * Coefficients significant at the 99% confidence level.

For delignification yield and glucan yield, R^2 values are greater than 0.9 showing that these compounds could be effectively correlated to the studied process variables by the proposed equation, giving statistically significant regressions. Xylan yield presented a R^2 of about 0.7.

According to the regression coefficients obtained, ethanol concentration (β_2) is the factor that impacts mostly, and positively, in the delignification yield. The parameter β_1, referring to delignification time, also affects delignification yield although to a much smaller extent. The quadratic term (β_{22}) is negative and statistically significant, suggesting that although the trend is the increased delignification with increasing ethanol concentration, this occurs only to a certain point after which the delignification yield is not favored by the increase of ethanol concentration (Fig 3). The coefficients β_1, β_2 and β_{22} are statistically significant at confidence level $p < 0.05$.

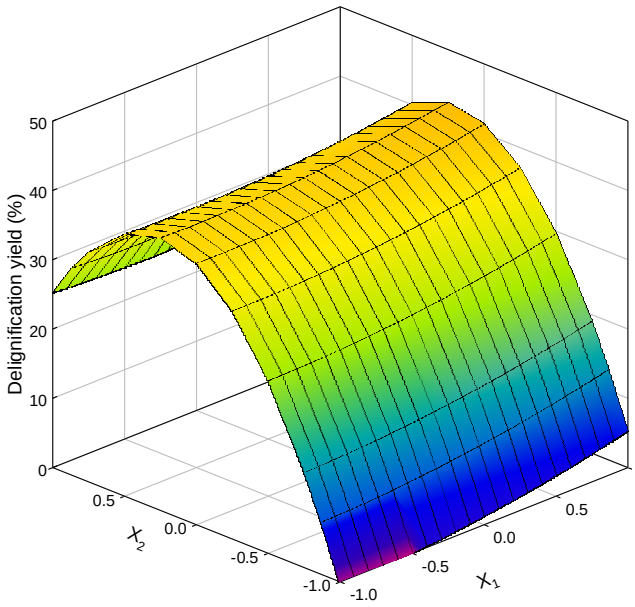

Fig 3 – Response surface for delignification yield in relation to time (X_1) and ethanol (X_2) concentration.

Glucan yield is negatively affected by the concentration of ethanol, and low concentrations lead to an enhanced recovery of glucan, as expected. The results obtained for β_{22}, also statistically significant, are therefore contrary to those obtained for the delignification yield. In the case of xylan yield, none of the factors studied were statistically significant and it can be considered that xylan was not degraded by this treatment (β_0 = 100.68%).

The response surface of delignification yield *versus* time (X_1) and ethanol concentration (X_2) (Fig 3) enabled to select the optimal conditions for delignification, which were 1 h time and 60.5% ethanol concentration for which a delignification yield of 42.4% can be predicted. In a previous study with rice straw, (Sun and Sun, 2002) obtained a delignification yield of 48% using more severe conditions (60% ethanol, 1% H_2SO_4 and 70ºC). Higher delignification yields were obtained using organosolv at high temperatures, for sugar cane bagasse at 195ºC and 30% ethanol (Mesa et al., 2011), and for wheat straw at 180ºC, 40% ethanol and 0.1% NaOH (Mesa et al., 2011; Ruiz et al., 2011).

Lignins molecular weight

The soluble lignin products obtained in the organosolv liquors of experiments 4, 5 and 6 (delignification times of 12 h, 24 h and 0 h, respectively) were analysed for molecular weight and compared with commercial Alcell lignin (organosolv hardwood lignin), P1000 (soda lignin from wheat straw and Sarkand grass) and soda wheat straw lignin (SodaWS). The rice straw lignins obtained in this work presented very similar and low mean molecular weight, showing that delignification time did not affect the molecular weight of the solubilised lignins. These lignins showed both substantially lower molecular weight and lower heterogeneity than all the commercial lignins tested (Fig.4).

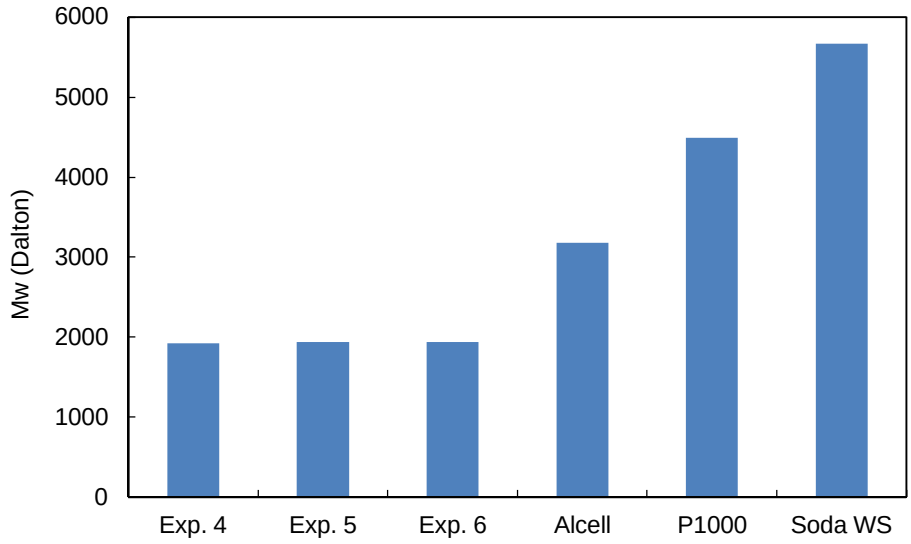

Fig. 4 - Molecular masses of lignins after organosolv delignification (Exp. 4. 5 and 6) and commercial lignins (Alcell, P1000, Soda WS).

Organosolv treatments produce more homogeneous lignin fractions with defined molecular mass distribution and chemical group functionalities (Gouveia et al., 2012) and low molecular weight (Lora, 2008). The results obtained here are in agreement with these studies.

Lignins of low molecular weight have been reported to be adequate as an extender or as component of phenolformaldehyde resins because of their high reactivity, in comparison with lignins with high percentages of high molecular weight molecules (El Mansouri and Salvado, 2007, Tejado et al., 2007).

Phenolic compounds

The total phenolic compounds in solution were analysed for all the experiments. Total phenolic concentration was relatively low ranging 1.1 and 2 g/L (data not shown) and was not in relation with organosolv conditions.

The phenolic compounds were separated using capillary electrophoresis and identification was done by comparison of spectra and migration times with the ones of authentic standards (Fig. 5). Some phenolic compounds present in the complex sample could be identified with a good matching: vanillin, ferulic and coumaric acids. However a major compound with 10.014 min migration time could not be fully identified and only indicatively suggested as a flavonoid by its spectrum. This is in accordance with its presence in rice straw (Karimi et al., 2014).

Ferulic and coumaric acids and vanillin have been identified in rice straw by other authors (Buranov and Mazza, 2008; Garrote et al., 2007; Sun et al., 2002) and also in corn, wheat and flax straws in different proportions being rice straw lignin the one that presents higher ferulic acid content (Buranov and Mazza, 2008). These phenolic compounds are well known for their bioactivities, particularly as antioxidants (Binod et al., 2010).

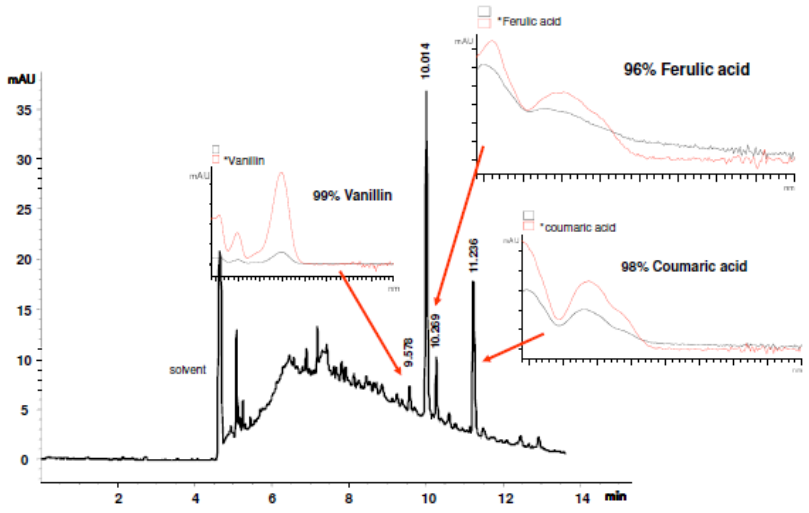

Fig 5 Electropherogram (200 nm) showing the phenolic profile for the sample obtained after organosolv delignification obtained for experiment 10. Matchings % were obtained by comparison with authentic standards run at the same conditions as sample

Enzymatic digestibility

The effect of the autohydrolysis pretreatment and organosolv delignification on the cellulose digestibility was evaluated by enzymatic hydrolysis of the remaining solid fractions. Table 5 shows the enzymatic digestibility of untreated rice straw, and on the solid fraction obtained after autohydrolysis pretreatment and organosolv delignification.

The pretreatment of autohydrolysis induces a high increase in digestibility and increase of pretreatment severity led to increased digestibility. This did not occur with the organosolv conditions tested and the enzymatic digestibility of the remaining solids after delignification was not affected significantly (68.5% vs. 62.8%, Table 5). The maximum glucose yield of 87.9% was obtained after an autohydrolysis at 220 ºC. These results are in range of those obtained after hot water pretreatment of rice straw at 200ºC for which an enzymatic digestibility of 82% was reported (Imman et al., 2013;Zhong et al., 2009) but lower than those obtained for rice straw after dilute acid hydrolysis (Kim et al., 2012) or AFEX (Zhong et al., 2009) and for wheat straw after autohydrolysis (Rossberg et al., 2014).

All the organosolv experiments were tested for enzymatic digestibility. Glucose yields ranged between 71.9 and 67.2% (data not shown), showing that neither time nor ethanol concentration had a significant influence on the enzymatic digestibility of the remaining solid. The maximum increase of glucose yield with the enzymatic hydrolysis in the organosolv solids when compared to the 210ºC pretreated solids was 14.5% (experiment 7).

Table 5 - Enzymatic digestibility on untreated, pretreated and organosolv solids.

	% Enzymatic digestibility
Raw material	32.78 ± 3.23
AH 210ºC	62.81 ± 0.05
AH 210ºC + Organosolv	68.54[a] ± 2.06
AH 220ºC	87.85 ± 3.83

AH 210ºC and AH 220ºC, autohydrolysis at 210ºC and 220ºC, respectively; [a]average of enzymatic digestibility obtained for the 12 organolsolv experiments according to the experimental plan.

Conclusions

The proposed strategy for the valorisation of rice straw using autohydrolysis followed by organosolv delignification showed interesting results for both the liquid and solid phases. The hydrolysate obtained from autohydrolysis is rich in pentoses (mainly in the oligomeric form) and the obtained solid fraction was enriched in lignin and glucan that showed a high enzymatic cellulose hydrolysis. A mild organosolv delignification could be optimized regarding maximal lignin solubilisation and glucan yield. In these conditions lignin extracts were obtained containing biological active compounds and glucan-enriched solid with a high enzymatic digestibility.

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Capítulo VI. Production, chemical characterisation and membrane separation of lignin-derived products from rice straw using a mild ethanol organosolv process after autohydrolysis

Abstract

An organosolv process using ethanol-water was carried out in order to recover high quality lignin from previously pre-treated solids of rice-straw by autohydrolysis at 210ºC. The organosolv delignification was studied at different temperatures (30ºC to 130ºC) and reaction times (1 to 3 h). The results showed a selective and appreciable lignin removal under very mild conditions and the highest delignification yield occurred at 30ºC. The lignin extracts were characterised using capillary zone electrophoresis, size exclusion chromatography and FT-IR, that enabled the identification of low molecular weight lignins, with a syryngyl/guaiacyl ratio of about 0.8 and containing phenolic compounds with potential bioactive properties.

In order to separate target compounds, membrane technology was used and value-added phenolics such as vanillin, ferulic acid and coumaric acid were obtained.

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Introduction

The efficient utilisation of lignocellulosic biomass within the biorefinery concept requires a selective fractionation of components to enable their separate utilization for defined purposes (Kamm and Kamm 2007). Extensive research has been carried out for separation of the three main structural components of biomass, hemicelluloses, cellulose and lignin, often in association with an initial removal of extractives (Harmsen et al. 2011).

Agricultural residues are one of the biomass types under consideration for biorefineries given their low cost and availability (Bhalla et al. 2013;Kolfschoten et al. 2014). For rice straw it was shown that it is possible to selectively obtain the three major fractions (hemicelluloses, cellulose and lignin) using a mild two-step strategy consisting of an autohydrolysis by which most of the hemicelluloses can be recovered in liquors as xylo-oligosaccharides and xylose, followed by a low intensity ethanol organosolv process, yielding a lignin-derived liquid stream and a cellulose-enriched solid phase (Moniz et al. 2014).

The organosolv solubilised lignin-derived products include several compounds, some of which known for their potential bioactivity, although separation and purification will be required prior to utilisation (Moniz, 2014b). . There are several potential methods for lignin separation depending on the delignification reactive medium. Organosolv lignins, for example, can be separated by evaporation and/or freeze-drying of the solvent. In soda or kraft pulping, lignin is precipitated with acids (e.g. sulphuric acid is the most common), although it results in a challenging recycling process for chemicals because of the formation of sodium sulphate. These processes have the disadvantage that they are not enough selective and/or may induce low recoveries (Girio et al. 2010;Harmsen et al. 2011).

The application of membrane technology for processing delignification liquors from a number of raw materials has been considered (Abels et al. 2013;Brodin et al. 2009;Gonzalez-Munoz et al. 2013;Toledano et al. 2013b). The use of membranes in ultrafiltration (UF), nanofiltration (NF) or both has been made mainly to separate other compounds from lignin-derived extracts. For example, the combination of UF-NF was studied for the separation of hemicelluloses or other products, such as salts and monosaccharides, from black liquor (Toledano et al. 2010). The efficiency of various membranes for the fractionation and purification of hemicellulosic oligosaccharides was also demonstrated (Gomez et al. 2014).

Membrane techniques can be particularly useful for the separation of lignin-derived compounds for added value applications due to their antioxidant and antifungal properties such as health, novel materials (e.g. with anti-fungal and antibiotic activity), UV-absorption (e.g. cosmetics), antioxidants (i.e. radical scavenger), as well as stabilisation of food and feed (Bozell et al. 2007). Other important compounds include monomeric phenolics such as vanillin and other bioactive phenolics e.g. with anti-carcinogenic and antibiotic activities (Boeriu et al. 2004;Egues et al. 2012;Soto et al. 2011;Toledano et al. 2013b). These applications present much higher added-value as compared to the traditional use as energy source or in leather tanning, and have been already reviewed (Bozell et al. 2007;Zakzeski et al. 2010).

In the present study, the upgrading potential of hydrothermally treated rice straw was evaluated using organosolv delignification and membrane separation of the complex liquor mixture using solvent resistant NF membranes to separate the phenolic compounds with high quality and maximum recovery. Their chemical characterisation and prediction of potential activities were made using FT-IR analysis that was shown to be a powerful tool for the chemical and functional characterisation of lignins (Boeriu et al. 2004), as already successfully applied in kraft, sulphite and soda lignins (Tejado et al. 2007;Toledano et al. 2013a).

Experimental

Hydrothermal processing of rice straw

Milled rice straw (Moniz et al. 2014) was subjected to autohydrolysis in a 2 L stainless steel reactor (Parr Instruments Company, USA) as described before (Moniz et al., 2014b). The raw material was mixed with water to a liquid-to-solid ratio of 10 (g water/g dry raw material) and the reactor heated to reach a final temperature of 210ºC as described (Moniz et al. 2014). The liquid and solid phases were recovered by pressing; the liquid phase was filtered (Whatman filter paper nº.1) and the solid phase was washed, filtered again, dried at 40ºC, homogenised in a combined lot and the composition was determined as described below.

Organosolv delignification

The solids obtained from the hydrothermal treatments were subjected to organosolv delignification process using an ethanol/water mixture of 60.5 % (w/w) ethanol concentration, as described by Moniz et al. (2014b). Several conditions were tested to evaluated the effect of temperature (30-130ºC) and time (1-3 h) using a solid-liquid ratio of 1:10 (w/w). All experiments were carried out using Schott flasks in a thermostatic bath (30-70ºC) or in autoclave (90-130°C). After reaction time had elapsed, the flasks were cooled down and the content of the flasks was filtered and the liquid fractions were recovered and stored at 4ºC until further use. The sample corresponding to 1 h delignification at 30ºC (OLRS30) was used for membrane separation. This sample and the sample corresponding to 1 h delignification at 110ºC (OLRS110) were also used for FTIR and SEC characterisation, after vacuum evaporation and freeze drying. The solid phase was washed with twice the amount of 60.5% ethanol (w/w) solution and then dried at 45ºC.

Membrane separation

Prior to the nanofiltration trials, the organosolv liquors extracted at 30ºC were filtered (Millpore 0.45 μm) and neutralized to pH 7 using $CaCO_3$, and filtered (Millipore® 0,45 μm). Nanofiltration was carried out in a tailor-made stainless steel dead-end filtration cell and using a semipermeable

membrane (Evonix Industries, UK) (PuraMem^{TM}) with a *cut-off* of 280 Da. The membrane was washed using pure ethanol (99.9%) at 25 bar. The membrane preconditioning was performed using an ethanol/water mixture of 60.5 % (w/w) at 45 bar, equal to the mixture used during the delignification process. In order to study the best separation conditions for the target compounds, a transmembrane pressure ranging from 15 to 40 bar was tested. The pressure was provided by compressed nitrogen gas and controlled by a regulator. All experiments were carried out at room temperature. The different permeates were analysed for total phenolics and composition by capillary electrophoresis (CZE). The membrane permeability was determined by calculating the slope of the linear regression between the sample permeation flux and the transmembrane pressure.

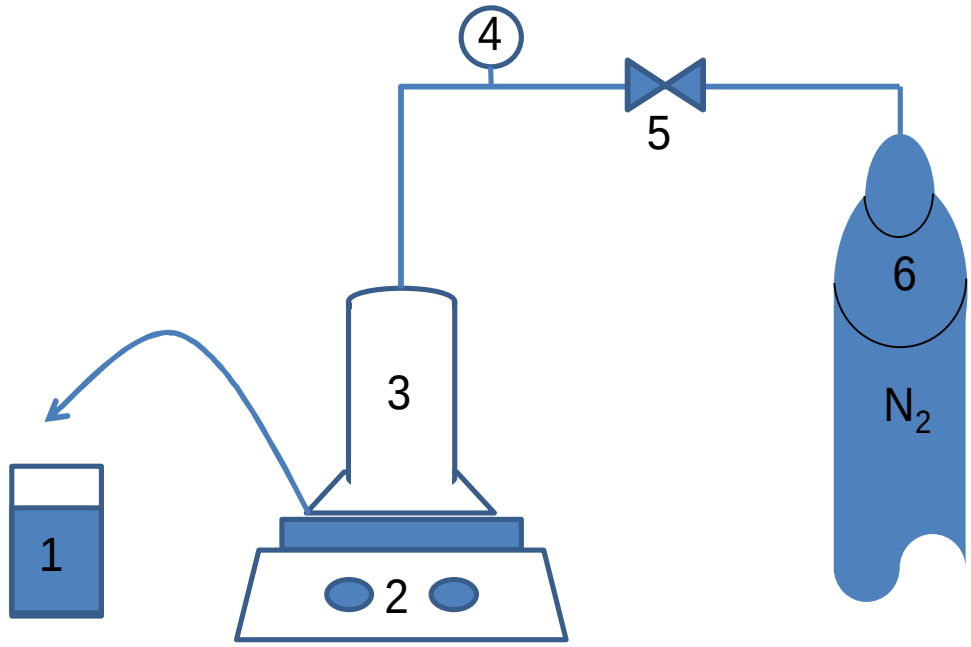

Fig 1 - Schematic representation of the experimental apparatus. (1) Permeate, (2) agitator, (3) nanofiltration dead-end cell, (4) pressure gauge, (5) pressure valve, (6) compressed N_2 gas.

Analytical Methods

Quantification of structural carbohydrates and lignin

The materials were ground in a knife mill (particle size < 0.5 mm) and the moisture content was determined by oven-drying at 100°C to constant weight. The ash content was determined at 550ºC using NREL/TP-510-42622 protocol (Sluiter et al. 2005). The samples were hydrolysed for determination of glucan, xylan, arabinan and acetyl groups using acid hydrolysis with 72% (w/w) H_2SO_4 followed by hydrolysis with 4% (w/w) H_2SO_4. The acid insoluble residue was considered as Klason lignin, after correction for ash. Monosaccharides (glucose, xylose, arabinose) and acetic acid were analysed by HPLC using an Aminex HPX-87H column (Bio-Rad, USA) as described before

(Moniz et al., 2014) in an Agilent Technologies Liquid Chromatographer 1100 Series System (Santa Clara CA, USA), equipped with a diode array detector (DAD) and a refractive index detector (RI). All calculations were made according to Moniz et al., 2014b

Phenolic compounds identification and quantification

Total phenolic compounds were determined by the Folin-Ciocalteu colorimetric method and their concentration expressed as gallic acid equivalents (GAE).
The phenolic profile was obtained by Capillary Zone Electrophoresis (CZE) using an Agilent System, with diode-array detector (DAD). ChemStation data software and a fused-silica uncoated i.d. 50 um and 62/56 cm effective length, extended light path capillary also from Agilent was used. 30 kV voltage was applied and injection was done at 50 mbar for 6 s. A 15 mM borate in 10% MeOH was used as electrolyte adjusted to pH 9.1 and temperature was maintained at 25°C. The capillary was preconditioned between runs by flushing with 0.1 M NaOH (3 min) followed by buffer (3 min). Detection was at 200 and 280 nm and compounds were identified by electrophoretic comparison (migration times and UV spectra) with authentic standards.

Molecular weight characterisation by size exclusion chromatography (SEC)

The molar mass distribution of lignins was analysed by alkaline SEC using a TSK gel Toyopearl HW-55F column, 0.5 M NaOH as eluent, UV detection at 280 nm and calibration with sodium-polystyrene sulfonates, as described elsewhere (Richard J.A.Gosselink et al. 2010). Mp (peak molecular weight), Mn (number average molecular weight), and Mw (weight-average molecular weight) and polydispersity (PD, Mw/Mn) were calculated.
Soda wheat straw lignin and P1000 soda lignin from mixed Sarkanda grass and wheat straw were obtained from Greenvalue SA (Lausanne, Switzerland). Organosolv lignin from mixed hardwoods (Alcell) was obtained from Repap Technologies Inc. (Valley Forge, PA, USA).

FT-IR spectroscopy

Fourier Transform Infrared (FT-IR) spectra of lignin samples were obtained on a Varian Scimitar 1000 FT-IR spectrometer equipped with a DTSG-detector and a PIKE MIRacle ATR and a diamond w/ZnSe lens single reflection plate. Spectra were collected in attenuated total reflectance (ATR) mode in the range 4000-650 cm^{-1} with a resolution of 4 cm^{-1} and with 128 co-added scans.

Results and discussion

Chemical composition of the feedstocks

The chemical composition of the raw material as well as the composition of the solids obtained after the autohydrolysis treatment are shown in Table 1.

Table1 - Chemical composition (g/100 g) of rice straw before and after autohydrolysis treatment at 210ºC

	Raw material	After Autohydrolysis
Glucan	40.90	53.63
Xylan	20.46	4.38
Arabinan	3.37	1.55
Acetyl groups	0.36	0.01
Klason lignin	14.43	21.45
Ash	6.41	10.32

The chemical composition of rice straw was already presented in a previous paper (Moniz et al., 2014). The major structural component of this raw material is cellulose (estimated as the glucan amount) followed by the hemicelluloses (estimated by the amount of xylan, arabinan and acetyl groups). As for Klason lignin it accounted for 14.4% of the raw material.

The autohydrolysis treatment mainly affected the hemicellulosic components (near to 73.7% solubilisation of the hemicelluloses), yielding 30.2% of xylo-oligosaccharides. In contrast, glucan was not much affected by the pre-treatment (recovery of about 80% of the cellulose, Moniz et al., 2014b), and the pre-treated material was enriched in cellulose in comparison with the raw material (53.6% vs. 40.9% glucan content respectively). Compared to other straws treated by autohydrolysis, the hydrolysis of glucan was slightly higher (Carvalheiro et al. 2009;Moniz et al. 2013), which can be mainly ascribed to the severity of the process.

The lignin content of the solid fraction after autohydrolysis was also higher when compared to the raw material (21.5% and 14.4% respectively), and corresponds to a high recovery of lignin in the solid phase (96% of the lignin, Moniz et al., 2014b). This autohydrolysis treatment does not solubilise lignin, which is an advantage for the purpose of this work, enabling its selective recovery in a second step.

Organosolv delignification

Table 2 shows the results obtained after organosolv delignification of the autohydrolysis pretreated rice straw. The delignification conditions were selected based on previous studies, which showed that when using a mild temperature of 30°C the delignification was higher with an ethanol concentration of 60.5% (Moniz, 2014b). The effects of temperature (30-130°C) and time (1-3 h) were studied here for this ethanol concentration.

Table 2 - Delignification yield (% of solubilised lignin in relation to initial lignin) and glucan yield (% of glucan in the solid in relation to initial glucan), as well as composition of the solid in relation to lignin and glucan (% of organosolv delignified solid) obtained for the different temperatures tested with 1 h reaction time.

Sample	Temp. (ºC)	Delignification Yield (%)	Glucan Yield (%)	Lignin (%)	Glucan (%)
OLRS30	30	32.3	93.2	16.2	43.4
OLRS50	50	27.8	94.0	16.9	44.5
OLRS70	70	28.0	91.8	15.9	46.6
OLRS90	90	29.5	86.7	17.0	51.5
OLRS110	110	31.4	83.8	16.7	47.7
OLRS130	130	29.5	89.4	16.9	53.5

No significant increase of delignification occurred with the increase of temperature: the delignification yields obtained ranged between 27.8% and 32.3%, with the highest value at 30°C. These results showed that a considerable delignification occurs under mild conditions, without any correlation with temperature within the temperature range studied. A similar trend was found for time (data not shown).

The use of very mild delignification conditions is an important trait of the presented results compared to the literature. In fact much higher severity conditions are usually applied either in direct organosolv delignification (Toledano et al. 2013b) or the sequential process of autohydrolysis and organosolv of eucalypt wood (Romani et al. 2011). Furthermore, the utilisation of this two-step mild process also allowed obtaining higher purity lignin in the soluble form, *i.e*. free of sugars. This is particularly relevant for the samples extracted at 30ºC for which, as expected, no sugars were detected by HPLC analysis in the delignification liquors. This is an advantage for the subsequent purification stage and strengthens the proposed strategy.

Glucan and residual xylan mostly remained in the solid phase after delignification demonstrating the selectivity of organosolv process for lignin, without affecting the polysaccharides in the pretreated sample. The recovery of glucan was very high, in most cases higher than 85%, enabling to obtain a solid enriched in glucan, and residual xylan was actually not removed at all (data not shown).

The organosolv liquors were analysed for their total phenolics content and profile. Fig. 2 shows the concentrations of phenolic compounds obtained for the delignification conditions studied. The concentration of total phenolics was practically not affected by temperature, although there was a slight increase, in particular for 130°C, with 6.0 g/L of total phenolic compounds. At 70°C and 110°C the concentration of total phenolics was also high, corresponding to 5.1 and 5.2 g/L, respectively.

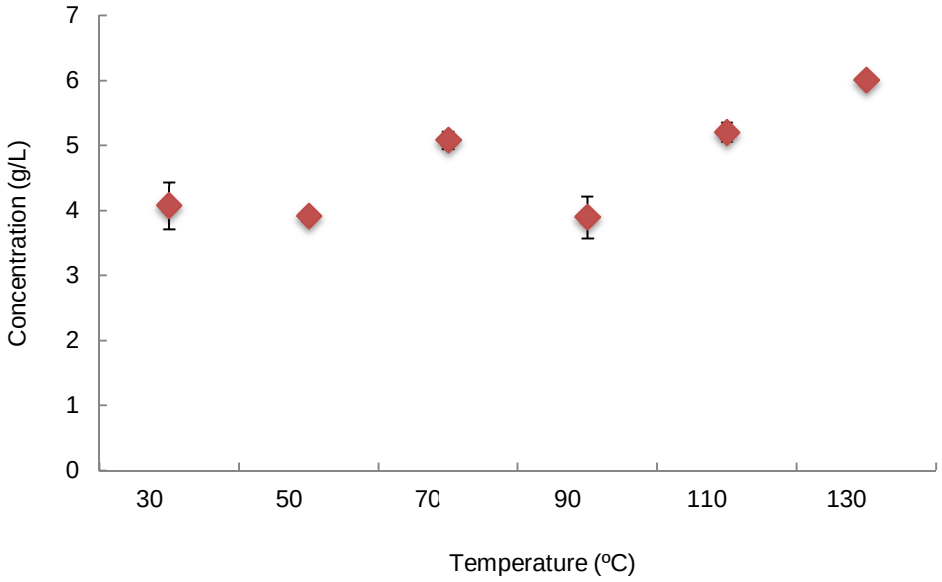

Fig 2 - Total phenolic compounds concentration (GAE equivalents) obtained for the organosolv conditions tested.

Soluble lignin characterisation by CZE

The phenolic profile of all the liquors obtained by the organosolv delignification was determined by capillary zone electrophoresis (CZE). As an example, Fig 3 shows the electropherogram for the sample obtained after delignification at 30°C.

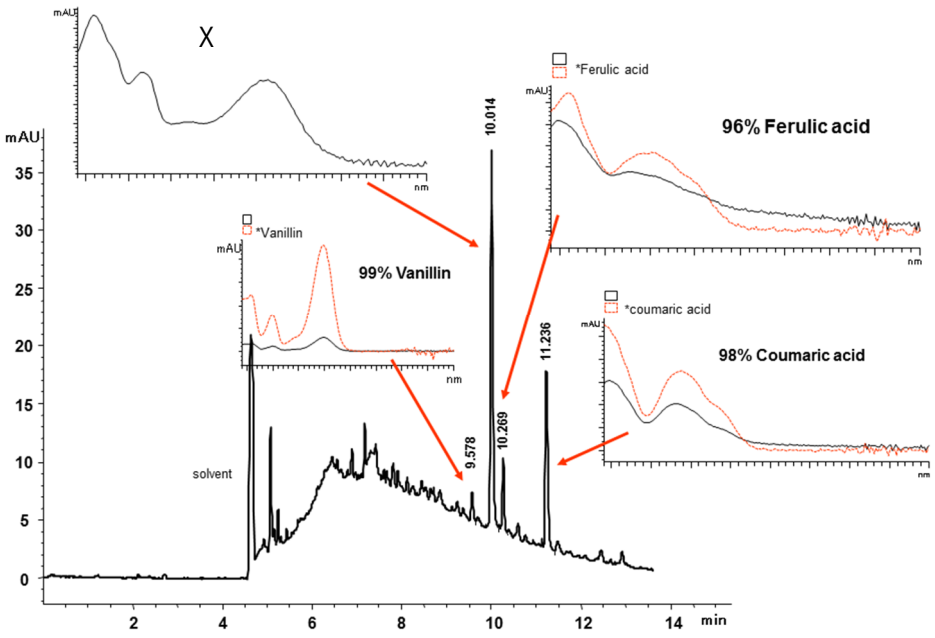

Fig 3 - Electropherogram (200 nm) showing the phenolic profile for the sample obtained after organosolv delignification at 30°C. Matching % were obtained by comparison with authentic standards run at the same conditions as sample.

Identification of compounds was done by comparison of spectra and migration times with the ones of authentic standards. Some phenolic compounds present in the complex sample could be identified with a good matching, namely vanillin, and ferulic and coumaric acids. These compounds were also identified in rice straw by other authors (Buranov and Mazza 2008) and are well known for their bioactivities, particularly as antioxidants (Boeriu et al. 2004). These samples have therefore potential as a source of added-value compounds.

The spectrum obtained for the compound present in the highest amount (X) shows a characteristic flavonoid profile although the identification with a good matching was not possible. Previous studies suggest that this compound could be tricine, a flavone with biological activities, such as anti-oxidant and anti-carcinogenic, normally found in rice bran (Karimi et al. 2014).

Membrane separation of phenolic compounds

Membrane technology was used to study the separation of phenolic compounds from the liquor mixture resulting from the delignification process. The sample selected for the nanofiltration trials was the sample treated at 30°C (OLRS30), which corresponded to the best delignification efficiency. The three important phenolic compounds (coumaric and ferulic acids, and vanillin) identified by *CZE* with a matching higher than 95% (Fig. 3) were chosen as the target compounds for the separation process and the nanofiltration membrane used was chosen accordingly with their molecular weight (152.15 Da, 164.16 Da and 194.18 Da for vanillin, coumaric acid and ferulic acid, respectively). Therefore, a membrane with a cut-off of 280 Da and resistant to organic solvents was used to separate the compounds with smaller molecular mass from the compounds with higher molecular mass present in the liquor. The separation depends not only on the molecular weight but also on the compounds interaction with the membrane surface, including chemical interactions due to the configurations of the molecules and the electrical loads.

Different transmembrane pressures (15-40 bar) were tested, in order to identify the best conditions for the separation of the target compounds and draw some conclusions about their rejection. The membrane presented a permeability of 0.14 L/(m^2.h.bar) for the delignification sample.

The phenolic profile of the permeate sample obtained at 15 bar (Fig. 4) is quite different from the one obtained for the liquor after organosolv delignification at 30° C (Fig. 3), due to the fact that many compounds were retained by the membrane, resulting in a less complex profile and well-defined peaks, i.e. a more purified sample.

Membrane rejection also enabled the separation and identification of other phenolic compounds in the permeate such as syringaldehyde and hydroxybenzaldehyde that had not been identified previously in the liquor before membrane processing due to the spectrum complexity and overlapping peaks. The percentage of matching in the permeate sample (data not shown) is lower than the matching obtained for the same compounds in the delignified samples at 30°C (Fig. 3) which could be explained by the fact that the permeated samples are very diluted. Nevertheless, migration times are the same.

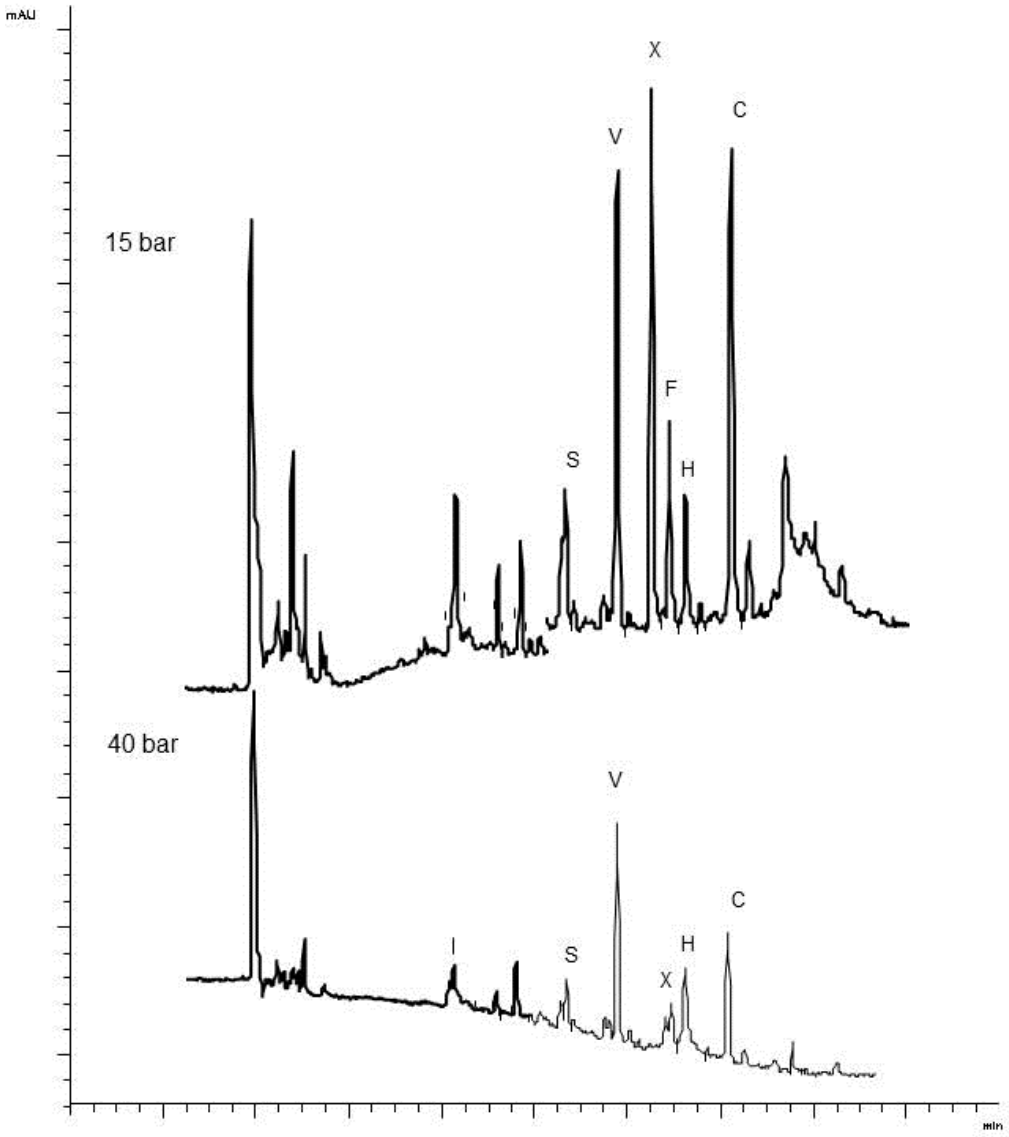

Fig 4 - Electropherogram (200 nm) showing the phenolic profile for the sample obtained after membrane separation at 15 and 40 bar.

S - syringaldehyde, V - vanillin, X - unidentified compound, H - hydroxybenzaldehyde and C -coumaric acid. Matching % were obtained by comparison with authentic standards run at the same conditions as sample.

As an example, and considering that these are the identified phenolic compounds present at higher concentrations in the initial sample, Fig 5 shows the percentage of ferulic and coumaric acid rejections by the membrane at different pressures. At higher transmembrane pressure there is increased membrane retention of the target compounds, resulting from membrane compactation leading to increased retention. For lower pressures there is a greater relaxation of the membrane, allowing higher molecular weight compound throughputs.

The rejection for the membrane was always lower for coumaric acid when compared to ferulic due to its lower molecular weight. At a transmembrane pressure of 40 bar the membrane revealed 100%

rejection for ferulic acid and 97% to coumaric acid (Fig.5), demonstrating that transmembrane pressure of 40 bar is not suitable for the separation of these compounds. The best separation was achieved for the lowest pressure (15 bar), where it is possible an effective separation (Fig. 4).

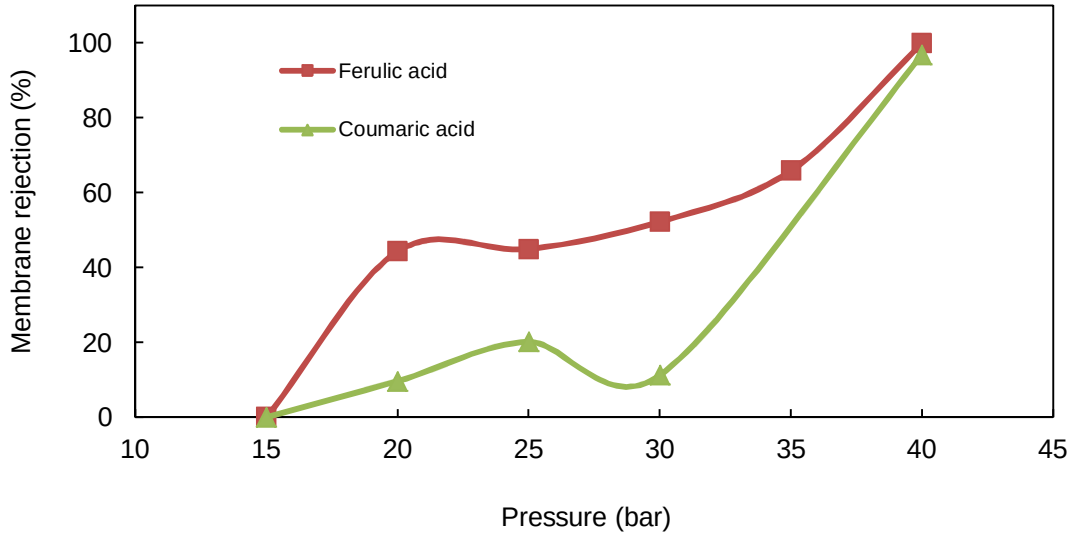

Fig 5 - Membrane rejection (%) at different pressures for ferulic and coumaric acids.

The use of nanofiltration allowed the separation of the target phenolic compounds from the liquor. It was also shown that it was possible to make their fractionation by selecting the pressure in the nanofiltration process. Koncsag and Kirwan (2012) showed also the possibility of separation of phenolic compounds from lignin by the membrane rejection of high molecular weight compounds.
Liquors resulting from delignification are complex samples for which there are still few studies. The results obtained here seem promising for the purification/separation of phenolic compounds in these types of samples.

Soluble lignin characterisation

The soluble lignin samples isolated after organosolv delignification OLRS30 and OLRS110 have low molecular weight and low dispersity (Table 5). They are small oligomers with a degree of polymerisation lower than 12. The increase of process temperature from 30ºC to 110ºC reduced slightly (by approximately 8 %) the molecular weight of the lignin-derived products due to enhanced depolymerisation. This is in agreement with the slightly higher phenolic content obtained during processing at temperatures above 100ºC, as described in section 3.2. These lignin fractions have lower molecular weight and lower heterogeneity than commercial lignins like organosolv hardwood lignin (Alcell, entry 3 in Table 5), soda lignin from a mixture of wheat straw and Sarkanda grass (P1000, entry 4 in Table 5) and soda wheat straw lignin (entry 5 in Table 5).

Table 5 - Molecular masses and FTI-IR analysis of lignins after organosolv delignification at 30°C and 110ºC.

Entry	Sample	Molecular weight			FT-IR analysis		
		Mn (g mol^{-1})	Mw (g mol^{-1})	PD	I_{1326}	I_{1265}	S/G
1	OLRS30	606	2011	3.3	0.18	0.23	0.77
2	OLRS110	567	1839	3.2	0.22	0.28	0.78
3	Alcell	675	3180	4.7			
4	P1000	743	4495	6.1			
5	Soda wheat straw	834	5671	6.8			

The FT-IR spectra of the two lignins are almost identical and show all the features characteristic of a GS type lignin (Fig. 6). The following peaks were present: 835±7 cm^{-1} (γ=CH aromatic ring, guaiacyl-syringyl), 1030 ±3 cm^{-1} (δ_{C-H} guaiacyl aromatic ring and δ_{C-OH} primary alcohol), 1115 ±6 cm^{-1} (δ_{C-O} stretching syringyl aromatic ring, C-H in-plane deformation of the syringyl unit), 1220 ±5 cm^{-1} ($\nu_{s\ C-O}$ syringyl ring), 1265 ±4 cm^{-1} (ν_s guaiacyl ring, $\nu_{asC-O-C}$), 1330 ±2 cm^{-1} (ν_s syringyl ring), 1510 ±8 cm^{-1} ($\nu_{C=C}$ aromatic ring , guaiacyl- syringyl), 1605 ±7 cm^{-1} ($\nu_{C=C}$ aromatic ring), 2850±1 (ν_{sCH2}, guaiacyl-syringyl), 2920±5 (ν_{asCH2}, guaiacyl- syringyl). In the carbonyl/carboxyl region, weak bands are found at 1658 cm^{-1} and 1701 cm^{-1}, specific for the conjugated and non-conjugated C=O, respectively.

The intensities of the peaks at 1326 cm^{-1} (syryngyl unit, S) and 1265 cm^{-1} (guaiacyl unit, G) were used to determine the ratio S/G, which is an indication of the reactivity of lignin. The two lignin samples obtained by organosolv delignification have similar S/G ratio (entry 1 and 2 in Table 5), which is comparable with the reported S/G ratio of 0.8 determined by ^{31}P-NMR analysis for wheat straw lignin extracted by the soda process (Boeriu et al., 2013).

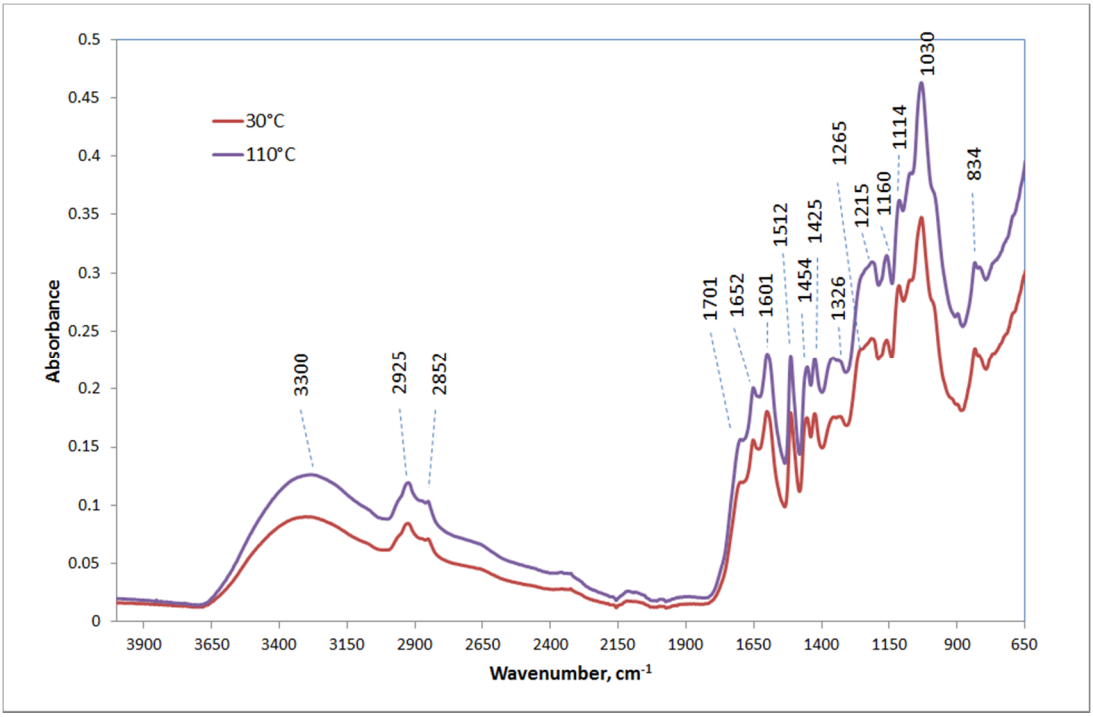

Fig.6 - FT-IR spectra of organosolv delignification samples OLRS30 (30ºC,1 h) and OLRS110 (110ºC, 1 h)

Given the chemical composition of the obtained lignin-derived compounds, e.g. their S and G units and the relatively high S content which give them a higher stability, envisaged applications should be based on their antioxidant properties as well as surfactant properties.

Conclusions

The combined use of hydrothermal (autohydrolysis) followed by an organosolv process under mild conditions is an effective strategy to separate and recover hemicelluloses and lignin-derived compounds from rice straw. The solubilised lignin is free from polysaccharide-derived compounds and sulphur-free, presenting a low molecular weight and low polydispersity. Membrane filtration could be applied to separate the monomeric phenolic compounds from the delignification liquor. Lignin-derived products seem attractive added value compounds for their antioxidant and surfactant properties.

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Capítulo VII. Conclusões e perspetivas

Este capítulo apresenta o resumo das principais conclusões retiradas do trabalho de investigação desenvolvido.

A ideia subjacente a esta tese foi desenvolver uma abordagem diferente para o processo integrado de valorização de materiais lenhocelulósicos no âmbito de uma biorrefinaria, usando como materiais modelo as palhas de arroz e de milho, dois dos subprodutos agrícolas com maior relevância nacional e internacional, e para os quais ainda não há processos de valorização industrial concretos. Os processos de valorização das biomassas industriais focam-se principalmente na da fração celulósica, enquanto este estudo incidiu essencialmente na valorização das frações de hemiceluloses e lenhina.

Tendo em conta este objetivo, foi delineado um processo de aproveitamento de hemiceluloses e lenhina a partir destas matérias-primas. O processo proposto para esta valorização consistiu nos seguintes estágios: hidrólise seletiva das hemiceluloses, seguida de solubilização seletiva da lenhina e identificação de possíveis aplicações para os compostos obtidos.

A solubilização seletiva das hemiceluloses das palhas de arroz e milho foi feita por um processo de auto-hidrólise que permitiu obter xilo-oligossacáridos (XOS) como produtos principais, substituídos principalmente com arabinose e alguns grupos acetilo.

Este pré-tratamento de auto-hidrólise mostrou-se altamente seletivo para a fração hemicelulósica. Foram identificadas as condições operacionais que conduziram ao rendimento em XOS mais elevado e concluiu-se que a extensão da despolimerização das xilanas dependia da severidade do processo. Obtiveram-se rendimentos em XOS elevados, em condições relativamente suaves, que conduziram à formação em concentrações muito baixas de possíveis inibidores e produtos de degradação, tais como HMF, furfural, ácido acético e compostos fenólicos.

Os hidrolisados ricos em XOS foram fracionados por cromatografia de filtração em gel (GFC) e obtiveram-se três categorias principais de produtos: polissacáridos pequenos, oligossacáridos médios, e monossacáridos e subprodutos (ácido acético, HMF, furfural). Os estudos de purificação dos hidrolisados por fracionamento GFC permitiram a separação das frações de XOS com maior interesse do ponto de vista funcional, com potencial efeito prebiótico.

O processo de purificação dos hidrolisados permitiu a obtenção de uma fração de XOS com elevada pureza. Os testes de fermentação *in vitro* utilizando inóculos fecais confirmaram o aumento da população de bifidobactérias e a produção de ácidos gordos de cadeia curta, comprovando o efeito bifidogénico dos XOS testados. Este resultado permite afirmar que os XOS produzidos têm potencial de inserção no mercado, nomeadamente ao nível da industrial alimentar (quer humana, quer animal), constituindo-se assim como um produto de valor acrescentado.

Os hidrolisados ricos em XOS e o enriquecimento em glucanas e em lenhina na fase sólida fazem das palhas de arroz e de milho matérias-primas adequadas para serem usadas no âmbito de uma biorrefinaria e o tratamento hidrotérmico um método favorável ao primeiro passo do processamento.

A celulose e lenhina não foram solubilizadas pelo tratamento auto-hidrólise, e o resíduo sólido rico em celulose e lenhina mostrou um aumento substancial da digestibilidade enzimática da celulose em relação aos materiais não tratados. Este resultado permite perspetivar a utilização destes sólidos para outras valorizações no âmbito da biorrefinaria, nomeadamente para a produção de compostos

de grande volume, mas de menor valor acrescentado, tais como sejam, etanol ou o ácido láctico, como tradicionalmente proposto na literatura.

A extração da lenhina foi estudada utilizando o processo organosolv com etanol em condições suaves e sem catalisadores, e focou-se na otimização das condições operacionais de deslenhificação, tendo por finalidade aumentar os rendimentos em lenhina solubilizada. A deslenhificação do sólido resultante da auto-hidrólise com uma mistura etanol/água foi estudada para várias temperaturas, tempos e concentrações de etanol.

Os resultados obtidos mostraram que ocorreu uma deslenhificação considerável em condições operacionais suaves, e sem correlação entre o rendimento em lenhina e o tempo/temperatura utilizados. O processo organosolv nas condições utilizadas mostrou elevada seletividade para a lenhina, sem afetar os polissacáridos presentes nos sólidos pré-tratados. Este processo também permitiu a obtenção de lenhina de pureza elevada, ou seja, livre de açúcares. Nos licores resultantes da deslenhificação não se encontraram açúcares, o que se traduz numa vantagem para a fase de purificação subsequente.

Os melhores resultados, do ponto de vista da composição dos licores ricos em lenhina, foram obtidos a temperatura baixa (30°C) o que constitui uma vantagem relativamente a outros processos descritos na literatura. Acresce que o etanol utilizado para a deslenhificação é barato, fácil de recuperar e é ele próprio um produto das biorrefinarias. Também o facto de não serem utilizados outros produtos químicos para além da água e etanol, proporciona a obtenção de lenhinas de elevada qualidade.

Os principais compostos presentes nos licores resultantes da deslenhificação são o ácido ferúlico, cumárico e a vanilina e um composto maioritário possivelmente um flavonoide. A utilização de membranas e de nanofiltração permitiu a purificação e separação de vários compostos fenólicos derivados da lenhina, entre eles os ácidos ferúlico e cumárico, a vanilina, o siringaldeído e o hidroxibenzaldeído. Esta técnica também permitiu a separação do composto maioritário dos compostos anteriores.

O conjunto sequencial dos processos utilizados, auto-hidrólise, deslenhificação organosolv e separação por membranas permitiu a obtenção de licores ricos em compostos fenólicos, todos eles compostos de alto valor acrescentado.

A estratégia proposta foi assim estudada ao nível experimental, que permitiu obter dados para a realização de uma análise económica preliminar, sabendo desde já que a produção dos compostos de alto valor acrescentado aumenta a viabilidade económica da proposta.

Para continuação dos trabalhos efetuados neste âmbito, propõem-se as seguintes linhas de investigação:

- Valorização da fração de celulose, para completar o processo de utilização integral do recurso por uma biorrefinaria de materiais lenhocelulósicos, com base na biomassa de palhas de arroz e milho. A fração de celulose poderá ser utilizada para a produção de bioetanol, produção ou de fibras celulósicas. Também a nanocelulose tem um mercado crescente em setores como o têxtil,

alimentos, cosméticos, produtos farmacêuticos e médicos, e como tal, seria uma aplicação de estudo interessante.

- Estudo de outros processos de valorização de lenhina, como a pirólise ou gaseificação ou a formulação de resinas.

- Estudo do aumento de escala dos dois processos estudados, considerando estudos energéticos e incluindo uma análise de viabilidade económica.

- Análise laboratorial sobre a possibilidade de inclusão de uma operação inicial de remoção de extrativos, determinando rendimentos e composição dos extrativos solubilizados, assim como o modo como esta operação afetaria as subsequentes operações de auto-hidrólise e de deslenhificação.

- Aplicação da estratégia de valorização a outros resíduos biomássicos.


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