UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

PATRÍCIA FÉLIX ÁVILA

UTILIZATION OF LIGNOCELLULOSIC AGROINDUSTRIAL WASTES FOR ENZYMATIC PRODUCTION OF OLIGOSACCHARIDES AIMING PREBIOTIC AGENT FOR HUMAN HEALTH

APROVEITAMENTO DE RESÍDUOS AGROINDUSTRIAIS LIGNOCELULÓSICOS PARA PRODUÇÃO ENZIMÁTICA DE OLIGOSSACARÍDEOS VISANDO A OBTENÇÃO DE AGENTE PREBIÓTICO PARA A SAÚDE HUMANA

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas, como parte dos requisitos exigidos para o título de Doutora em Engenharia de Alimentos.

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Orientação: Profa. Dra. Rosana Goldbeck

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RESUMO

Os materiais lignocelulósicos representam uma fonte de matéria prima sub-explorada em processos biotecnológicos, visto que um grande excedente de resíduos a partir do processamento industrial de matérias primas agrícolas são gerados anualmente. Desta forma, a produção de xilo-oligossacarídeos (XOS) a partir da hidrólise enzimática da xilana presente na hemicelulose proveniente destes resíduos, bem como de celo-oligossacarídeos (COS) a partir da celulose apresentam uma alternativa promissora e sustentável para valorização dos mesmos. Este estudo teve como objetivo produzir oligossacarídeos (XOS e COS) a partir da hidrólise enzimática de resíduos agroindustriais lignocelulósicos (palha de cana-de-açúcar e casca de café) como fonte de arabinoxilana e celulose, de modo a avaliar o seu potencial prebiótico. Diferentes estratégias foram utilizadas para o aprimoramento do processo alcalino integrado para fracionamento do arabinoxilano e celulose partindo dos materiais lignocelulósicos previamente desliginificados, os quais proporcionaram rendimentos e eficiência de extração do arabinoxilano em torno de 40,35% e 82,32%, respectivamente. Em uma segunda etapa, delineamentos compostos centrais rotacionais (DCCR) foram empregados para desenvolver misturas de enzimas para a produção de XOS e COS a partir dos arabinoxilanos extraídos e dos resíduos ricos em celulose obtidos, respectivamente. Misturas enzimáticas eficientes foram desenvolvidas com endoxilanase (GH11), α-l-arabinofuranosidase (GH51), e feruloyl esterase (CE1), proporcionando altas concentrações de XOS alcançando valores de até 511,5 $(mg).(g_{substrato})^{-1}$. Bem como usando endo-1,4-β-D-glucanase (GH12), exo-1,4-β-D-glucanase (GH5) e feruloyl esterase (CE1) para a produção de COS atingindo valores maximizados em torno de 63,56 $(mg).(g_{substrato})^{-1}$. Como segunda estratégia para a aumentar produção de COS e investigar os possíveis efeitos de inibição pela celobiose, foi realizado um estudo empregando endo-1,4-β-D-glucanase (GH12) previamente adsorvida aos substratos em múltiplos estágios de hidrólise de modo a controlar os processos de inibição enzimática onde foi possível alcançar valores em torno 85.43 $(mg).(g_{substrato})^{-1}$. Como última etapa do processo foi realizado um fracionamento dos oligosscarídeos produzidos utilizando membranas de nanofiltração em série de modo a avaliar o potencial prebiótico das frações obtidas. As etapas *in vitro* demonstraram que os oligossacarídeos produzidos exibiram considerável resistência à hidrólise pelas enzimas do sistema gastro-instestinal. Bem como algumas culturas probióticas de *Lactobacillus* e *Bifidobacterium* apresentaram notável crescimento nos meios contendo XOS e COS. Os microorganismos consumiram preferencialmente xilobiose (X2) e xilotriose (X3) e em relação aos meios contendo COS consumiram majoritariamente as frações celobiose (C2) e celotriose

(C3) Assim, os resultados apresentados mostraram que ambas as biomassas utilizadas foram fontes potenciais para produção de oligossacarídeos prebióticos e que seu fracionamento em frações de menor peso molecular contribuiu significativamente para o aumento do potencial prebiotico tanto dos XOS e COS produzidos a partir de ambas as biomassas.

Palavras-chave: Palha da cana-de-açúcar; Casca de café; Oligossacarídeos; Hidrólise Enzimática; Prebióticos.

ABSTRACT

Lignocellulosic materials represent a source of raw material that is under-explored in biotechnological processes, since a large surplus of waste from the industrial processing of agricultural raw materials is generated annually. Thus, the production of xylooligosaccharides (XOS) from the enzymatic hydrolysis of xylan present in hemicellulose from these residues, as well as celloligosaccharides (COS) from cellulose present an intelligent and sustainable alternative for their valorization. This study aimed to produce oligosaccharides (XOS and COS) from enzymatic hydrolysis of lignocellulosic agroindustrial residues (sugarcane straw and coffee husk) as a source of arabinoxylan and cellulose, in order to evaluate their prebiotic potential in human health. Different strategies were used to improve the integrated alkaline process for fractionation of arabinoxylan and cellulose from the prior delignified lignocellulosic materials which provided yields and efficiency of arabinoxylan extraction around 40.35% and 82.32%, respectively with insignificant values of lignin content. In a second step, central rotating composite rotating designs (CCRD) were used to develop mixtures of enzymes for the production of XOS and COS from the extracted arabinoxylans and the cellulose-rich products obtained, respectively. Efficient enzyme mixtures were developed with endoxylanase (GH11), α-l-arabinofuranosidase (GH51), and feruloyl esterase (CE1), providing high concentrations of XOS reaching values of up to 511.5 (mg).(gsubstrate)$^{-1}$. As well as using endo-1,4-β-D-glucanase (GH12), exo-1,4-β-D-glucanase (GH5) and feruloyl esterase (CE1) for the production of COS reaching values maximized around 63.56 (mg).(g substrate)$^{-1}$. As a second strategy to increase the COS production and and investigate the possible effects of inhibiting by cellobiose, a study employing adsorbed endo-1,4-β-D-glucanase (GH12) to substrates in multiple stages of enzymatic hydrolysis was performed, where it was possible to reach values around 85.43 (mg). (g substrate) $^{-1}$. As the last step of the process, a fractionation process of the oligosaccharides was carried out using nanofiltration membranes in series in order to assess the prebiotic potential of the previous fractions. In vitro steps showed that oligosaccharides developed exhibited resistance to hydrolysis by enzymes of the gastro-intestinal system. As well as that probiotic cultures of *Lactobacillus* and B*ifidobacterium* stream growth on media containing XOS and COS as the sole carbon source. The microorganisms consumed preferentially xylobiose (X2) and xylotriosis (X3) and in relation to the media containing COS they consumed mostly as fractions cellobiose (C2) and cellotriosis (C3). Thus, the results found that both biomasses use are potential sources for the production of prebiotic oligosaccharides

and that their fractionation into fractions of lower molecular weight contributes to increase the prebiotic potential of both XOS and COS as from both biomass.
Key-words: Sugarcane straw; Coffee husk; Oligosaccharides; Enzymatic hydrolysis; Prebiotics.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA - Análise de Variância

EG - Endoglucanase

BG – β-glicosidase

CBH - Celobiohidrolase

CMC - Carboximetilcelulose

CMCase – Atividade de endoglucanase sobre o CMC

DCCR - Delineamento composto central rotacional

DNS - Ácido 3,5-dinitrosalicílico

LCM – Materiais Lignocelulósicos (*Lignocelulosic Materials*)

FPase - Atividade de celulases total sobre o papel de filtro (*Fiter paper activity*)

FPU - Unidade de papel de filtro (*Filter paper unit*)

HPLC – Cromatografia líquida de alta eficiência *(High Performance Liquid Chromatography)*

CH – Casca de café (*Coffee husk*)

SS – Palha da cana de açúcar (*Sugarcane straw)*

CHX- Xilana da palha da casca de café (*Coffee Husk Xylan*)

SSX- Xilana da palha da cana-de-açúcar *(Sugarcane Straw Xylan)*

CHC- Celulose da palha da casca de café (*Coffee Husk Cellulose*)

SSC- Celulose da palha da cana-de-açúcar *(Sugarcane Straw Cellulose)*

NREL- National Renewable Energy Laboratory

OS - Oligosaccharides

PAD- Detecção Amperométrica Pulsada (*Pulsed Amperometric Detection*)

XOS – Xilo-oligossacarídeos

X2- Xilobiose

X3- Xilotriose

X4- Xilotetraose

X5- Xilopentaose

X6- Xilohexaose

COS – Celo-oligossacarídeos

C2- Celobiose

C3- Celotriose

C4- Celotetraose

C5- Celopentaose

C6- Celohexaose

NF – Nanofiltração (*Nanofiltration)*

DP – Grau de polimerização (*Degree of polymerization*)

SSHX – Hidrolisado da palha da cana-de-açúcar rico em xilo-oligosscarídeos (*Sugarcane straw xylooligosacchahrides hydrolysate*)

SSHC- Hidrolisado da palha da cana-de-açúcar rico em celo-oligossacarídeos (*Sugarcane straw cello-oligosaccharides hydrolysate*)

SDPX- Xilo-oligossacarídeos de curto grau de polimerização (*Short degree of poymerization xylo-oligosaccharides).*

SDPC- Celo-oligossacarídeos de curto grau de polimerização (*Short degree of polymerization cello-oligosaccharides).*

HDPX- Xilo-oligossacarídeos de alto grau de polimerização (*Short degree of polymerization xylooligosaccharides).*

HDPC- Celo-oligossacarídeos de alto grau de polimerização (*Short degree of polymerization cello-oligosaccharides).*

DF –Diafiltração (*Diafiltration*)

SCFA – Ácidos graxos de cadeia curta (*Short chain fat acids*)

SUMÁRIO

Capítulo 1

Introdução Geral e Objetivos

1.1 INTRODUÇÃO GERAL

Grandes excedentes de resíduos do processamento de produtos agrícolas são gerados todos os anos. Contudo, tais resíduos podem vir a ser fontes potenciais para produção de muitos compostos de interesse industrial, tais como compostos antioxidantes e com potencial bioativo (BHATIA et al., 2020). A biomassa lignocelulósica é um recurso abundante e renovável composta majoritariamente por celulose e hemicelulose. A celulose é um polímero constituído por moléculas de glicose unidas por ligações glicosídicas β(1–4) de onde é possível produzir glicose e celo-oligosscarídeos. As hemiceluloses ou polioses são polímeros ramificados de caráter bastante heterogêneo, os quais são constituídos por uma gama de polissacarídeos de baixo peso molecular. Dentre eles os mais abundantes são os xilanos, de onde é possível produzir xilo-oligossacarídeos (XOS), que por sua vez são compostos por cadeias de resíduos de xilose unidos por ligações β-(1,4) podendo ou não apresentar diferentes ramificações em sua cadeia, dando à cada tipo uma característica específica (CANO et al., 2020).

A hidrólise enzimática de materiais lignocelulósicos é considerado um método eficiente para a produção de açúcares provenientes da hemicelulose e celulose por ser um processo que pode ser facilmente controlado, além de ser seletivo e exigir condições amenas de pH e temperatura. O que consequentemente implica na minimização da geração de compostos que podem ser prejudiciais em etapas subsequentes de fermentação ou purificação (CANO et al., 2020). As enzimas celulolíticas são capazes de atuar na hidrólise do polímero de celulose através da ação sinérgistica dos seus três principais grupos de enzimas: endoglucanases (EG) as quais clivam aleatoriamente ligações β-1,4 glicosídicas nas regiões amorfas da celulose gerando novos terminais redutores e não-redutores, favorecendo principalmente a produção de celo-oligossacarídeos. As celobiohidrolases (CBH) são enzimas processivas e liberam celobiose a partir de terminais redutores e não redutores de fragmentos de celulose produzidos por endoglucanases, e por fim as β-glicosidases (BG) hidrolisam celobiose em glicose (BARBOSA; SILVELLO; GOLDBECK, 2020). Já a degradação total das frações de hemicelulose requerem uma gama de enzimas que agem cooperativamente para convertê-la em unidades de xilose e podem ser divididas em dois grandes grupos: o primeiro é composto pelas enzimas que clivam a cadeia de xilano principal, o qual engloba as endo-β-xilanases (EC 3.2.1.8) e β-xilosidades (EC 3.2.1.37). Outro grupo é composto por enzimas denominadas como acessórias, as quais desemepenham a função de promover o acesso das endo-β-xilanases e β-xilosidades durante a hidrólise. São elas: α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase, acetil xilano esterases (EC 3.1.1.6) ácido para-coumárico esterases,

soleninas e feruloil esterases (EC 3.1.1.73) (JUTURU; WU, 2013). Devido a heterogeneidade do polímero de hemicelulose o perfil dos XOS é dependente não apenas da enzima, mas também da fonte de xilano empregada (AKPINAR; ERDOGAN; BONSTANCI, 2009).

Os carboidratos não digeríveis são constituintes da fibra alimentar e são parcialmente digeridos por seres humanos, no qual as porções não digeríveis servem como nutrientes para as bactérias que fazem parte da flora natural tais como a *Bifidobactérias* e *Lactobacilos* (ZHONG et al., 2020). Esses oligossacarídeos complexos são denominados como prebióticos quando os mesmos atuam como ingredientes nutricionais que afetam beneficamente o hospedeiro, estimulando seletivamente o crescimento e a atividade de uma ou mais bactérias intestinais benéficas, melhorando a saúde do hospedeiro (SINGH; BANERJEE; ARORA, 2015). Muitos benefícios à saúde já foram relatados, dentre os quais o aumento da biodisponibilidade de minerais, a redução do nível de colesterol sérico, manutenção da saúde gastrointestinal, prevenção do risco de câncer de colón, efeito benéfico na diabetes mellitus tipo 2, efeitos anti-carninogenicos e toxicologicos em células humanas e efeito benéfico sobre a diabetes mellitus tipo 2 (MANNING; GIBSON, 2004; ANDO et al., 2004). Alguns pesquisadores apontam ainda os oligossacarídeos não digeríveis como suplementos alimentares, podendo ser adicionados em diversos produtos na indústria de alimentos como em produtos derivados do leite e em alimentos simbióticos (SEBASTIÁN et al., 2019; BALTHAZAR et al., 2017; FONTELES; RODRIGUES, 2018).

Nesse sentido esse trabalho propõe o estudo de um processo integrado para fracionamento da xilana e celulose de resíduos agroindustriais de modo a estudar diferentes estratégias de produção enzimática dos oligossacarídeos provenientes dessas frações com o principal propósito de investigar o seu potencial prebiótico.Este presente trabalho encontra-se dividido em nove capítulos, sendo que neste primeiro capítulo apresenta a introdução e os objetivos propostos nesta tese. No Capítulo 2 é apresentado uma revisão bibliográfica relacionada com a biomassa lignocelulósica e seus principais constituintes, abordando principalmente as biomassas da cana-de-açúcar e da casca de café; as estratégias de fracionamento da biomassa lignocelulósica nas frações hemicelulose e celulose; atuação de diferentes enzimas na hidrólise enzimática; os desafios na elaboração de misturas enzimáticas para a produção de XOS a partir desses constituintes e as potenciais aplicações desses oligossacarídeos na indústria alimentícia e farmacêutica. No capítulo 3 ainda é apresentado um estudo de revisão de rotas de produção e potenciais aplicações emeregentes dos celo-oligosscarídeos, o qual é enfatizado neste capítulo por ainda ser muito pouco abordado na

literatura. No Capítulo 4 são introduzidos os resultados obtidos na primeira vertente de estudo da tese a qual se concentrou no estudo de xilo-oligosscarídeos. Dessa forma neste capítulo é apresentado o estudo do aprimoramento dos processos de extração alcalina do arabinoxilano partindo da holocelulose da palha da cana-de-açúcar e casca de café para a otimização das concentrações de diferentes enzimas para formulação de misturas enzimáticas para a produção XOS. No capítulo 5 as misturas enzimáticas otimizadas no capítulo anterior são empregadas para estudo do potencial prebiótico e antioxidante dos XOS produzidos a partir das frações de arabinoxilano das biomassas estudadas. Já no capítulo 6 são introduzidos resultados que representam a segunda vertente de estudo desta presente tese, a qual se concentrou no estudo de celo-oligossacarídeos. Neste capítulo é apresentado o o estudo da recuperação da celulose nos processos alcalinos discutidos no Capítulo 4. Como também são apresentados estudos de otimização das concentrações de enzimas para formulação de misturas enzimáticas para a produção de COS a partir dos sólidos ricos em celulose recuperados. Em seguida no Capítulo 7 são apresentadas outras estratégias de produção enzimática de COS, como a hidrólise enzimática em múltiplos estágios visando aumentar os rendimentos obtidos anteriormente, de modo a também investigar possíveis processos de inibição enzimática e avaliar seu potencial prebiotico. No capítulo 8 o estudo desta presente tese é concluído com o estudo do fracionamento de ambos oligosscarídeos produzidos (XOS e COS) utilizando membranas em serie de nanofiltração de modo a avaliar o impacto da apliacação das diferentes frações no crsscimento de cepas probióticas. Por fim no capítulo 9 são apresentados as discussões e conclusões gerais em relação aos resultados apresentados ao logo dos capítulos 4, 5, 6, 7 e 8 desta trabalho.

1.2 OBJETIVOS

1.2.1 Geral

Produzir xilo-oligossacarídeos (XOS) e celo-oligossacarídeos (COS) através da hidrólise enzimática a partir das frações xilana e celulose, respectivamente de diferentes resíduos agroindustriais lignocelulósicos visando à obtenção de agentes com potencial prebiótico.

1.2.2 Específicos

- Caracterizar quimicamente as biomassas estudadas (palha de cana-de-açúcar, casca de café);
- Deslignificar as biomassas da palha da cana-de-açúcar e casca de café de modo a obter a fração holocelulose usando clorito de sódio em meio ácido.

- Fracionar as frações arabinoxilana e celulósica da holocelulose extraída das biomassas (palha de cana-de-açúcar e casca de café e casca) para a produção de XOS e COS respectivamente;
- Determinar concentrações ótimas de enzimas comerciais para formulação de misturas enzimáticas ideais para hidrólise enzimática da arabinoxilana ou celulose obtidas das biomassas estudadas para obtenção de XOS e COS de cadeia curta (grau de polimerização 2-6) respectivamente;
- Avaliar a digestão *in vitro* dos oligossacarídeos (XOS e COS) obtidos pela ação da enzima amilase presente na saliva humana, bem como pela ação do suco gástrico artificial e das enzimas pancreáticas;
- Avaliar o crescimento *in vitro* de bactérias probióticas do gênero *Bifidobacterium* e *Lactobacillus* em meios contendo oligos (XOS ou COS) produzidos como única fonte de carbono, de modo a compara-los com meios contendo glicose;
- Fracionar os oligosscarídeos produzidos em frações de baixo peso molecular utilizando membranas de nanofiltração em séries.
- Realizar ensaios de fermentação com cepas probióticas do gênero *Bifidobacterium* e *Lactobacillus* de modo a avaliar o seu cresimento nas respectivas frações obtidas de XOS e COS;
- Quantificar o teor de lactato e ácidos graxos de cadeia curta (ácido acético, ácido lático, ácido propiônico e ácido butírico) após os ensaios de fermentação com XOS ou COS e suas respectivas frações obtidas;

Referências

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Capítulo 2

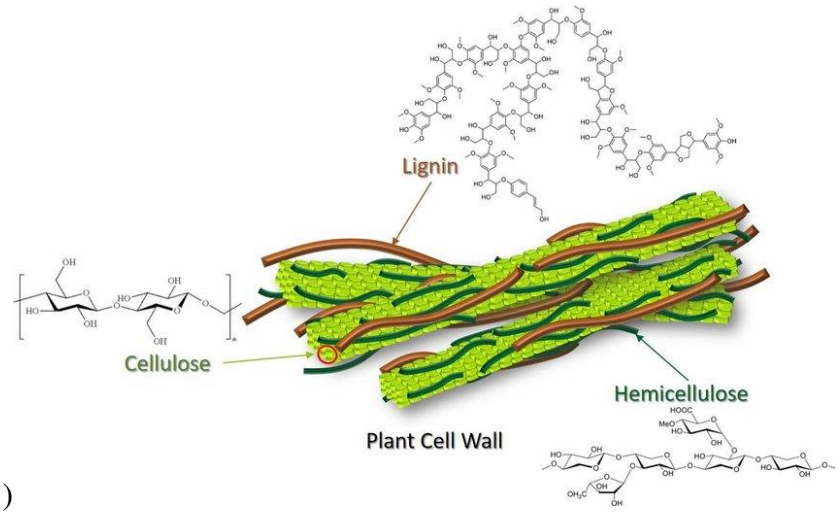
Revisão Bibliográfica

2. REVISÃO BIBLIOGRÁFICA

2.1 Biomassa Lignocelulósica

A biomassa lignocelulósica é um dos recursos renováveis mais abundantes no meio ambiente e por meio de diferentes processos biotecnológicos é um forte aliado a produção de biocombustíveis, biomoléculas e produtos de alto valor agregado (BHATIA et al., 2020). Dessa forma nos últimos anos vários estudos tem se direcionado no aproveitamento de resíduos agroindustriais tais como polpa e cascas de café, resíduos de frutas, farelo de soja, bagaço e palha de cana-de-açúcar, entre outros. (CANO et al., 2020).

A biomassa lignocelulósica é constituída mjoritoriamente por celulose, hemicelulose e lignina (BALAT, 2011). Outros constituintes como pectina, proteínas, cera, extrativos e cinzas podem estar presentes, no entanto em frações minoritárias. As quantidades de cada um dos constituintes presentes podem variar de acordo coma espécie e idade da planta. A celulose forma microfibrilas que são mantidas unidas a hemicelulose e lignina através de combinações de ligações covalentes e ligações de hidrogênio formando a matriz lignocelulósica (Figura 2.1). (ZHANG et al., 2012).



2011)

Figura 2.1. Esturutura da matriz lignocelulósica (Adaptado de ZHANG et al., 2012).

2.1.1. Composição química

2.1.1.1 Celulose

A celulose é o polisscarídeo majoritário em grande parte dos materiais lignocelulósicos, o qual é constituído por uma cadeia linear por moléculas de glicose unidas

por ligações glicosídicas β(1–4), com grau de polimerização varindo entre 10.000 a 15.000 unidades monoméricas (Figura 2.2) (BHATIA et al., 2020).

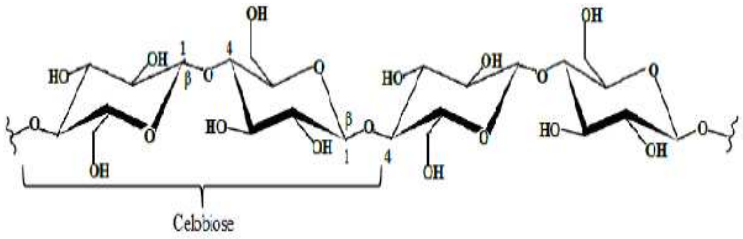


Figura 2.2. Estrutura molecular e polimerização da cadeia de celulose (Adaptado de FENGEL; WENGER,1984).

A linearidade das cadeias de celulose favorece a união de cadeias por meio de ligações de hidrogênio de forma que feixes de moléculas de celulose se agreguem na forma de microfibrilas na qual regiões altamente ordenadas denominadas como cristalinas se alternam com regiões menos ordenadas denominadas como amorfas (Figura 2.3) (DUFRENSE, 2012). Essas ligações dificultam a livre rotação dos anéis em torno das ligações glicosídicas, resultando no enrijecimento da cadeia. Dessa forma a estrutura altamente fibrosa promove alta resistência à tração e insolubidade na maioria dos solventes. (BHATIA et al., 2020).

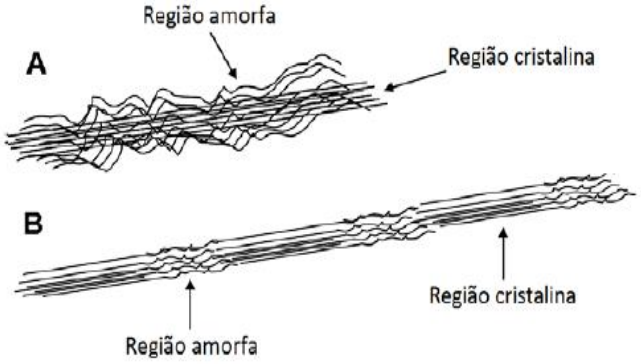


Figura 2.3. Regioes amorfo e cristalina na cadeia de celulose (Adaptado de ANDERSEN, 2007).

2.1.1.2 Hemicelulose

A hemicelulose é um heteropolímero composto por monossacarídeos, como pentose, hexoses, açúcares acetilados e ácidos urônicos. Os vários tipos podem variar abrangentemente em diferentes espécies de plantas (SAHA, 2003). A sua principal cadeia é constituída de um homopolímero de xilana ou um heteropolímero de glucomananas, o qual é formado por

unidades de arabinose, galactose e ácido 4-O-metilglucorônico que se ligam uns aos outros essencialmente por ligações glicosídicas β-(1→4), sendo também possível encontrar ligações glicosídicas β-(1→3), β-(1→6), α-(1→2), α-(1→3) e α-(1→6) (LIMAYEM; RICKE, 2012).

Ao contrário da celulose, os vários tipos de hemiceluloses não são cristalinas, e apresentam cadeias mais curtas e ramificadas (CUNHA; GANDINI, 2010). Na parede celular dos vegetais, as hemiceluloses estão associadas a vários outros constituintes, como celulose, proteínas, pectina e lignina (SUN et al., 2000). Na superfície das microfibrilas de celulose estão fortemente aderidas por meio de ligações de hidrogênio. Há relatos que essa adesão evitaria a formação de fibrilas maiores de celulose por agregação lateral (MUSSATTO, TEIXEIRA, 2010). Por meio de ligações do tipo éter e ligações do tipo éster entre as unidades acetil e ácidos hidroxicinâmicos principalmente em ácidos ferúlico e p-coumárico, também se encotram aderidas a lignina, o que dificulta significativamente a extração da hemicelulose da matriz lignocelulósica da parede celular dos vegetais (XU et al., 2006).

Nos resíduos agroindustriais, como a da cana-de-açúcar o tipo de hemicelulose predomeninante é a arabinoxilana, constituída por uma estrutura molecular proveniente de resíduos de xilose ligado por ligações β-(1→4). De forma que ligados a estes resíduos, nas posições C-2 e/ou C-3, estão resíduos de L-arabinose e ácido glucurônico. (PENG et al., 2009). Além disso, os resíduos de xilose podem apresentar grupos *O*-acetil substituindo alguns dos grupos hidroxil.Na biomassa da cana-de-açúcar, o tipo de hemicelulose descrita como predominante é o 4-*O*-metil-glucuronoarabinoxilana (Figura 2.4) (PENG et al., 2009; BIAN et al., 2012). No entanto o percentual e estrutura química da hemicelulose é bastante heterogêneo entre diferentes biomassas e diferentes frações de uma única biomassa (caule, folhas, raízes, casca) (PENG et al., 2012).

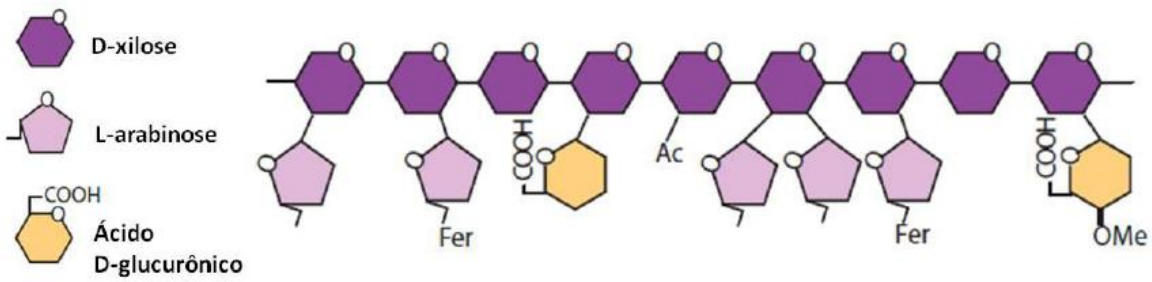


Figura 2.4. Estrutura esquemática da molécula de glucuronoarabinoxilana, onde "Fer" representa a esterificação com ácido ferúlico. (Adaptado de DA SILVA, 2013).

2.1.1.3 Lignina

A lignina é o terceiro polímero presente mais abundante na parece celular dos vegetais, conferindo a mesma rigidez e impermeabilidade ao ataque microbiano. Esse heteropolimero é constituído por unidades formadoras de fenil propano (p-cumarílico, álcool coniferílico e álcool sinapílico) unidos por diferentes ligações (Figura 2.5) (AGBOR et al., 2011).

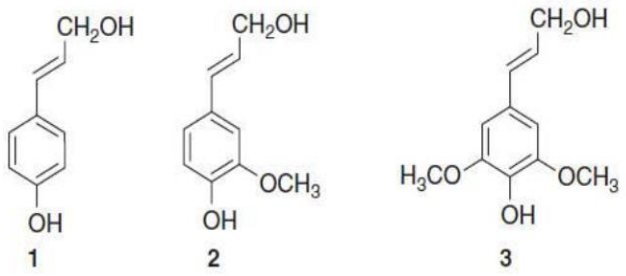


Figura 2.5. Unidades formadoras de fenilpropano, (1) álcool *p*-coumarílico, (2) álcool coniferílico e (3) álcool sinapílico (Adaptado de GELLERSTEDT; HENRIKSSON, 2008).

A presença e estrutura desses compostos se diferem dependendo do tipo e especie de plantas. Na biomassa da cana-de-açúcar os três compostos estão presentes. Em coníferas a lignina é composta quase que exclusivamente pelo álcool coniferílico, apresentando pequenas quantidades de álcool *p*-coumarílico. Já em árvores folhosas (*hardwoods*), estão presentes os alcoóis coniferílico e sinapílico (SARKANEN, 1971; FENGEL; WEGENER, 1984; SJÖSTRÖM, 1993). Esse polímero é geralmente associado com um agente de 'Cola' o que promove a união dos diferentes componentes da biomassa lignocelulósica, tornando-a insolúvel em água. Devido a sua intensa associação com microfibrilas de celulose, é apontada como um dos principais fatores limitantes ao ataque enzimático da celulose (ZHU et al., 2008). Dessa forma nos últimos anos muitos estudos tem mostrado que sua remoção da biomassa lignocelulósica tem sido crucial para se obter rendimentos elevados de hidrólise enzimática (AGBOR et al., 2011).

2.1.2 Biomassa da Cana-de-açúcar

A cana-de-açúcar foi introduzida no período colonial e desde então se transformou em uma das principais culturas da economia brasileira. No Brasil cerca de 600 milhões de toneladas de subprodutos agroindustriais são produzidos anualmente, e os provenientes da cana-de-açúcar estão entre os mais representativos (BHATIA et al., 2020).

Os subprodutos fibrosos que podem ser gerados após a colheita da cana-de-açúcar são denominados bagaço e palha. O bagaço da cana-de-açúcar (fração fibrosa da cana-de-açúcar) é o resíduo abundante produzido globalmente fração obtida pode ter considerável aumento com a colheita das folhas e pontas da cana-de-açúcar. Já a palha corresponde às porções foliares e às ponteiras da cana, que, geralmente, são colhidas simultaneamente com os colmos, no entanto deixados no próprio lugar de plantio de modo a devolver ao solo parte dos nutrientes minerais imobilizados durante o crescimento (ALMEIDA, 2008). A utilização da palha da cana-de-açúcar tem o benefício adicional de não competir como fonte de alimento e ter um conteúdo energético semelhante ao bagaço por unidade de peso, mas é freqüentemente queimada para facilitar a colheita dos colmos. Com as melhorias na tecnologia de colheita e cogeração da cana-de-açúcar, o bagaço e a palha da cana tornaram-se importantes fontes de bioenergia e para produção de diversos biprodutos por demandarem um baixo investimento financeiro para sua aquisição, o que é altamente vantajoso em processos de biorrefinaria(CANILHA et al., 2010; WOLF, 2011).

2.1.3. Biomassa da Casca de Café

A casca de café é oriunda do processamento do café cereja por via seca ou úmida, o qual passa por etapas de secagem e beneficiamento, com posterior retirada da casca. O método de processamento por via seca, é o mais utilizado no Brasil e consiste em uma fase de secagem do fruto seguido pelo descascamento mecânico. Já no método por via úmida o é incluído uma primeira etapa de despolpamento (SANTOS et al., 2007). No Brasil, cerca de 75% do café são processados por "via seca", gerando em média cerca de uma tonelada de casca para cada tonelada de grãos produzidos (SILVA, 2012). O Seu rendimento pode atingir em torno de 50% do peso colhido e sua produção a qual é concentrada nos meses de julho a dezembro pode atingir um máximo de até 75% nos quatro primeiros meses de processamento (MACHADO et al., 2020; VEGRO, CARVALHO, 1994). Segundo Zoca (2012), o tratamento e destinação desses resíduos podem elevar diretamente o custo de produção. Atualmente, a utilização destes resíduo vem sendo cada vez mais frequentes por apresentarem substâncias de alto valor agregado e por seu reaproveitamento também minimizar o impacto ambiental. Normalmente a casca é empregada como fertilizante, por serem fontes de potássio, como também na alimentação de ruminantes. Essa matéria vegetal também pode ser utilizada como combustível com alto poder calorífico de 3500 kcal/kg, na produção de gás metano e vinagre, na composição de baterias elétricas, podendo ser transformadas em carvão como forma de concentrar energia (VEGRO; CARVALHO, 1994). A casca de café também é rica em celulose e hemicelulose e

portanto são usadas como substrato para o crescimento de cogumelos e para processos de fermentação para produção de enzimas, ácidos orgânicos e bioetanol (PANDEY et al., 2000). Dessa forma a casca de café representa um dos subprodutos do café com o maior número de aplicações.

2.2. Pré-tratamento

O pré-tratamento de materiais lignocelulósicos é uma etapa crucial, que visa a desorganização da estrutura recalcitrante da parede celular lignocelulósica, possibilitando maior acessibilidade das enzimas aos polissacarídeos durante a hidrólise enzimática (Figura 2.6) (SHILL et al. , 2011). Portanto, o pré-tratamento é essencial para a remoção da lignina, redução da cristalinidade da celulose e aumento da porosidade do material.

Biomassas lignocelulósicas requerem diferentes pré-tratamentos com base em sua composição para melhor disponibilizar e/ou fracionar seus polissacarídeos presentes. Uma classificação generalizada de métodos de pré-tratamento os agrupa em; pré-tratamento físico, químico, biológico como também pré-tratamentos múltiplos ou combinados. Em métodos de pré-tratamento combinados, parâmetros físicos como temperatura, pressão ou uma etapa biológica são combinados com tratamentos químicos e são denominados métodos de pré-tratamento físico-químico ou bioquímico. Estratégias de pré-tratamento combinados são geralmente mais eficazes para aumentar a digestibilidade e fracionamento da biomassa (BRANDT et al., 2013).

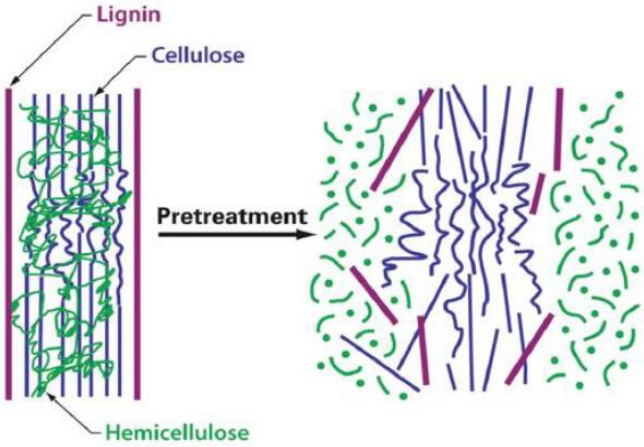


Figura 2.6. Ação dos pré-tratamentos na desconstrução dos componentes da parede celular vegetal (Adaptado de Brandt et al., 2013).

A susceptibilidade aos processos enzimáticos estão intrinsicamente relacionados ao índice de cristalinidade, uma vez que quando há um decréscimo do índice de cristalinidade da celulose a susceptibilidade das celulases aumenta consideravelmente. Dessa forma é reportado que a celulose pode ser eficientemente sacarificada quando apresenta grau de cristalinidade inferior a 1,0 (BHATIA et al., 2020). De forma bastante abrangente além da redução da cristalinidade da celulose e aumento da porosidade do material é desejável que o pré-tratamento também apresente características operacionais e efeitos como a preservação da parte celulósica e hemicelulose, minimização da geração de compostos inibidores para etapas de fermentação, menor demanda de energia, baixo custo e reciclagem de produtos químicos. A etapa de pré-tratamento deve ser escolhida com base no componente que se deseja isolar (FOSTON; RAGAUSKAS, 2012). Dessa forma existem diversos tipos de pré-tratamento que podem ser utilizados para aumentar a susceptibilidade da associação celulose-lignina e assim melhorar a eficiência da hidrólise enzimática. Os processos físicos, em geral, são empregados para reduzir o tamanho das partículas, o grau de cristalinidade e polimerização, bem como aumentar a fração solúvel. No entanto os processos químicos têm apresentado uma maior aplicação, já que os pré-tratamentos físicos são relativamente ineficientes em relação ao aumento da digestibilidade da biomassa (BHATIA et al., 2020). Há relatos que os pré-tratamentos que apresentam temperaturas e tempos de incubação mais elevados são mais eficientes, indicando que pode haver uma temperatura efetiva de transição de lignina vítrea que deve ser excedida para melhor solubilização da lignina (BRANDT et al., 2013).

Devido a extensa variedade de materiais lignocelulósicos que diferem nas suas propriedades químicas e físicas, há uma interdependência entre o pré-tratamento e o tipo de substrato (FOSTON; RAGAUSKAS, 2012). Assim é evidente que diferentes pré-tratamentos terão impactos diversos nas propriedades físico-químicas da biomassa pré-tratada, melhorando a conversão e consequentemente a produção de oligossacarídeos ou açúcares fermentescíveis que serão liberados durante a etapa de hidrólise enzimática (CANO et al., 2020).

2.2.1. Deslignificação com Clorito de Sódio

O processo de remoção de lignina, também conhecido por deslignificação pode ser realizado através de métodos oxidativos obtidos por uma variedade de métodos. A polpação Kraft é industrialmente o mais utilizado para a remoção de lignina da madeira; entretanto, não é o mais utilizado em escala laboratorial para isolar a holocelulose devido a considerável degradação de polissacarídeos, extração de hemiceluloses e remoção incompleta de lignina. Dessa forma o método mais empregado e melhor estabelecido para a remoção de lignina da

biomassa é a deslignificação com clorito de sódio em meio ácido. Este método efetivamente branqueia e dessa forma solubiliza a lignina em temperaturas moderadas. Além disso é seletivo na remoção de lignina com solubilização de apenas traços das frações glucana e xilana (AHLGREN E GORING, 1971). De acordo com Gierer (1986), os dois cenários mais prováveis para a degradação da celulose durante a deslignificação com ácido-clorito são a clivagem ácida das ligações glicosídicas e/ou degradação oxidativa dos polissacarídeos. A hidrólise ácida é um mecanismo bem estabelecido para a redução do peso molecular da celulose, mesmo sob condições ligeiramente ácidas. A oxidação da celulose com clorito é inespecífica e a degradação ocorre mais rapidamente perto de um pH neutro No entanto a degradação oxidativa é limitada em condições ácidas, onde o ácido acético é geralmente adicionado no processo para redução do pH.

2.2.2. Extração alcalina

Devido a estrutura bastante complexa dos materiais lignocelulósicos a extração seletiva da hemicelulose e celulose são importantes para a realização de etapas que as utilizem como substrato para processos biotecnológicos com a finalidade de obter produtos de maior valor agregado (CANO et al., 2020). Considerando a produção de xilo-oligossacarídeos (XOS) e celo-oligosscarídeos (COS), a hemicelulose e celulose presente nos materiais lignocelulósicos podem ser isoladas por meio de extração alcalina. Para extração alcalina, produtos químicos como hidróxido de cálcio, hidróxido de sódio, hidróxido de potássio ou hidróxido de amônio sãonormalmente usado (BEHERA et al., 2014). No decorrer do processo de extração, as ligações éster entre a hemicelulose e a lignina são hidrolisadas, liberando assim a hemicelulose e celulose da matriz lignocelulósica, permitindo sua extração e recuperação, respectivamente (EBRINGEROVÁ, HEINZE, 2000). Assim o pré-tratamento alcalino pode remover diferentes grupos provenientes da hemicelulose como acetil e ácidos urônicos; o que pode consequentemente alterar a estrutura da hemicelulose extraída em relação a estrutura nativa. Além disso em comparação com outros métodos de pré-tratamento, a extração alcalina requer menor grau de severidade em relação condições operacionais (temperaturas e pressões mais baixas). Existem várias razões que influenciam a escolha de um método de pré-tratamento para biomassa; nomeadamente, evitando a redução do tamanho da biomassa, preservando a fração da celulose e hemicelulose, evitando a formação de frações provenientes de produtos de degradação, bem como baixo consumo de energia e eficácia de custos. Dessa forma cada método de extração tem suas vantagens e desvantagens, embora seja um método que forneça

um alto rendimento de hemicelulose solubilizada e uma pureza considerável (SUN, TOMKINSON, 2002).

2.4. Hidrólise Enzimática

Embora a catálise química e física sejam capazes de hidrolisar biomassa lignocelulósica em produtos de interesse, as reações enzimáticas apresentam algumas vantagens tais como requerer temperaturas moderadas, possuirem estereoespecificidade e eliminarem o uso de produtos químicos e reagentes tóxicos, os quais dificultam o processo de purificação e são de díficil reciclo. O uso de enzimas é ecologicamente correto e bem estabelecido industrialmente, aplicado na fabricação de biocombustíveis, resinas, carnes processadas, queijos, sucos, óleos, bebidas alcoólicas etc. Atualmente, as técnicas de purificação e a engenharia genética tem ampliado significativamente o desempenho dos processos enzimáticos (LOPES et al., 2018). A heterogeneidade e complexidade da estrutura lignocelulósica da biomassa resulta em uma multiplicidade de enzimas que diferem em suas propriedades estruturais e físico-químicas, modo de ação e especificidade para o substrato (OBENG et al., 2017).

2.4.1. Celulases

As principais enzimas necessárias para a hidrólise da celulose microcristalina, polissacarídeo majoritário na parede celular, são as celobiohidrolases, endoglucanases, exoglucanases e β-glucosidases, as quais trabalham cooperativamente para reforçar a hidrólise das microfibrilas complexas de celulose (OBENG et al., 2017). Individualmente nenhuma enzima do complexo é capaz de hidrolisar a celulose de forma eficiente, assim é necessária a ação sinérgica desse complexo (Figura 2.12) (BARBOSA; SILVELLO; GOLDBECK, 2020).

Endo-1,4-β-D-glucanases (EC 3.2.1.4) são enzimas que hidrolisam aleatoriamente as ligações internas das fibras de celulose presentes na parte amorfa da celulose, liberando celo-oligossacarídeos e celobiose, a qual é hidrolisada em glicose por a ação da β−glucosidase ou celobiases (EC 3.2.1.21) (POLIZELI et al., 2017).

As β−glicosidases possuem uma estrutura rígida com o sítio ativo dentro de uma grande cavidade, correspondente a uma bolsa de sítio formada por quatro alças hidrofílicas com diferentes conformações para aumentar a ligação de carboidratos, o que favorece a entrada da celobiose. Além disso, essas enzimas são essenciais para aliviar a inibição de feedback dos produtos provenientes das glucanases. No entanto, as β-glicosidases também sofrem inibição

de seus produtos e, portanto, a busca por β-glicosidases tolerantes à glicose tem sido bastante estudada (OBENG et al., 2017).

As exo-1,4-β-D-glucanases. têm grande finidade para os sítios cristalinos ao longo das cadeias de celulose externas. Os loops formam um túnel em torno do sítio catalítico para onde o substrato é direcionado. Eles incluem 1,4-β-D-glucano glucohidrolases (EC 3.2.1.74), que liberam glicose de 1,4-β-D-glucanos e hidrolisam lentamente celobiose, e 1,4-β-D-glucano celobiohidrolases (CBH), que liberam celobiose de 1,4-β-glucanos das extremidades redutoras (CBH I) ou não redutoras (CBHII) (OBENG et al., 2017; POLIZELI et al., 2017).

A celobiose desidrogenase (EC 1.1.99.18), capaz de oxidar a celobiose, pertence à família das atividades auxiliares (AA) e possui uma atividade auxiliar sinérgica, ativando celulases hidrolíticas por meio do alívio da inibição enzimática pelo produto celobiose, de forma semelhante às β-glicosidases (OBENG et al., 2017).

2.4.2 Hemicelulases

Como já descrito anteriormente, a hemicelulose possui uma composição mais abrangente que a celulose e, por isso, necessita de um maior número de enzimas para ser hidrolisada de forma eficiente. A xilana é um polímero de xilose, mas que na natureza, geralmente está associada com outros açúcares formando glucuronoxilanas, glucuronoarabinoxilanas, glucomananas, arabinogalactanas, e galactoglucomananas (FENG et al., 2010).

As endo-1,4-β-xilanases (EC 3.2.1.8) são enzimas que atuam na cadeia principal da xilana clivando as ligações glicosídicas entre as unidades de xilose na, liberando xilo-oligossacarídeos. Apenas duas famílias pertencem a este grupo de enzimas: GH10 e GH11. Enquanto a família GH10 são capazes de hidrolisar cadeias de xilana mais próximas dos pontos de ramificação, liberando cadeias mais curtas de xilo-oligossacarídeos, a família GH11 é capaz de clivar em regiões mais distantes das cadeias laterais. Assim, a distribuição dos resíduos ramificados na estrutura da xilana interfere significativamente na ação dessas enzimas. As β-xilosidases (EC 3.2.1.37) hidrolisam pequenos xilo-oligossacarídeos de suas extremidades não redutoras em xilose. Eles geralmente não são capazes de hidrolisar a xilana, mas podem clivar substratos artificiais como o p-nitrofenil-β-d-xilopiranosídeo (POLIZELI et al., 2005).

2.4.3. Misturas Enzimáticas e Sinergismo

A biomassa lignocelulósica é um substrato extremamente complexo, que requer várias enzimas para se degradar. Dessa forma é esperado que haja sinergismo entre essas enzimas na

natureza, produzindo rendimentos muito maiores quando atuando de forma cooperativa. Além disso, a cooperação mais efetiva reduz as cargas enzimáticas e, consequentemente, os custos de produção. Grandes esforços têm sido dedicados ao aprimoramento de coquetéis enzimáticos para substratos específicos (LOPES et al., 2018).

Celulossomas são um meio natural de aproveitar a natureza sinérgica de certas enzimas hidrolíticas para degradar a lignocelulose. Um celulossoma é um complexo enzimático exocelular encontrado na superfície celular do crescimento do meio extracelular de alguns fungos anaeróbicos (por exemplo, *Neocallimastix frontalis*) e bactérias (por exemplo, *Clostridium thermocellum*) onde várias enzimas sacarolíticas são ancoradas a proteínas de suporte (SCHUSTER;CHINN, 2013). Desde sua descoberta, muitos pesquisadores usaram uma variedade de preparações para demonstrar a sinergia entre várias enzimas lignocelulósicas: mais recentemente, Goldbeck et al. (2014) investigaram que misturas enzimáticas aumentam a despolimerização da hemicelulose, e Ávila et al. (2020) relataram a sinergia entre endoxilanase e arabinofuranosidase, usada para otimizar a produção de xilo-oligossacarídeos. Em outra abordagem, Furtado et al. (2013) projetaram uma enzima bifuncional que mostra sinergismo entre β-glucanase (EC 3.2.1.73) e a lacase oxidase dependente de cobre (EC 1.10.3.2) que catalisa a oxidação de compostos aromáticos, aumentando a redução da produção de açúcares. Inspiradas na natureza, proteínas quiméricas artificiais, que são domínios de duas ou mais enzimas expressas em uma única sequência peptídica, foram projetadas para reduzir os custos de produção e melhorar o desempenho enzimático (MARTINS, DINAMARCO, GOLDBECK, 2020).

2.5. Oligossacarídeos prebióticos

Oligossacarídeos podem ser definidos como carboidratos de ocorrência natural que consistem em 2–10 unidades monossacarídicas, sendo estas lineares ou ramificadas, conectados por ligações α- e / ou β-glicosídicas. Essas moléculas podem ser compostas por diferentes monossacarídeos e as classes principais são compostas por unidades de frutose, galactose, glicose e xilose. Sua produção em grande escala se dá por meio da hidrólise de polissacarídeos, como xilana, celulose, quitosana, pectina e inulina, e mecanismos de transglicosilação quando a sacarose e a lactose são os substratos (IBRAHIN, 2018).

Um prebiótico foi definido pela primeira vez como um "ingrediente alimentar não digerível que afeta beneficamente o hospedeiro ao estimular seletivamente o crescimento e/ ou a atividade de uma ou de um número limitado de bactérias no cólon e, assim, melhora a saúde do hospedeiro" (GIBSON; ROBERFROID, 1995). No entanto muitos oligossacarídeos e

polissacarídeos alimentares (incluindo fibra dietética) foram declarados como atividade prebiótica, porém nem todos os carboidratos dietéticos são prebióticos. Para ser classificado como prebiótico o oligossacarídeo deve seguir alguns critérios, como, apresentar resistência à acidez gástrica e à ação de algumas enzimas no intestino, ser fermentado seletivamente pela microflora intestinal, e estimular o crescimento ou atividade de bactérias probióticas ligadas à saúde intestinal (GIBSON, 2004).

Os oligosscarídeos prebióticos estimulam o crescimento de bactérias benéficas na microbiota intestinal, mostrando um efeito benéfico no controle do peso corporal, melhorando os níveis de glicose e lipídios no sangue e reduzindo os sintomas do câncer de cólon (MOLDES et al., 2017). Dessa forma, os prebióticos atuam como substratos para microrganismos probióticos que produzem metabólitos importantes, que estão associados à maioria dos efeitos de promoção da saúde humana (IBRAHIM, 2018). Os prebióticos também são citados entre as dez principais tendências no setor de alimentos funcionais (PATEL, GOYAL, 2011). Entre os principais representantes desse grupo estão os fruto-oligossacarídeos (FOS), os xilo-oligossacarídeos (XOS) e os galacto-oligossacarídeos (GOS), cujas propriedades funcionais e aplicações já são conhecidas, o que ainda não é verdade para os celo-oligossacarídeos (COS), mostrando assim um caminho promissor para pesquisas futuras (IBRAHIN, 2018).

As fontes lignocelulosicas são consideradas matérias-primas atraentes para a produção de oligossacarídeos prebióticos. Três classes principais de polímeros constituem a biomassa lignocelulósica: celulose, hemicelulose e lignina, que podem ser clivadas por hidrólise enzimática ou autocatálise, liberando uma ampla gama de compostos com propriedades diferentes, dentre eles se destacam os oligossacarídeos provenientes da xilana no que se refere aos xilo-oligossacarídeos (XOS), e os provenientes da celulose no caso dos celo-oligossacarídeos (COS), os quais serão abordados a seguir no item 2.5.1 e no Capítulo 3, respectivamente.

2.5.1 Xilo-oligossacarídeos

Xilooligossacarídeos (XOS) são oligômeros constituídos por unidades de xilose ligadas por meio de ligações β-1,4 glicosídicas, os quais podem ser produzidos a partir da xilana de resíduos lignocelulósicos, que podem conter em média 25–50% em massa seca de xilana (SAMANTA et al., 2015). De acordo com a fonte de xilana para a produção XOS, sua estrutura se diferente em grau de polimerização e quantidade de unidades monoméricas liberadas (Figura 2.7). A xilana é geralmente encontrada em combinação com grupos laterais, como resíduos de

arabinofuranosil e grupos acetil, que podem levar à formação de XOS ramificado com diversas propriedades biológicas (AACHARY, PRAPULLA, 2011). Além disso os XOS apresentam maior atividade bifidogênica em comparação com outros prebióticos, e muitos estudos relataram seu potencial para inibir a carcinogênese (VAZQUES, 2000) e suas propriedades antiinflamatórias e antioxidantes (MARQUES et al., 2021). A atividade antioxidante do XOS está associada à presença de grupos acetil e uronila e também derivados do ácido hidroxicinâmico ligado a ésteres como resíduos ácido caféico, ferúlico, singárico e cumárico e ácido metil glucurônico presente nas cadeias laterais de xilana (LEDDOMADDO et al., 2021). Como ingrediente alimentar, os XOS são estáveis em uma ampla faixa de temperaturas (até 100 ºC) e pH (de 2,5 a 8), tem odor e sabor aceitáveis e doçura relativa. Eles são comumente usados como um agente gelificante para modificar a viscosidade e estabilizar a espuma (VAZQUES, 2000)

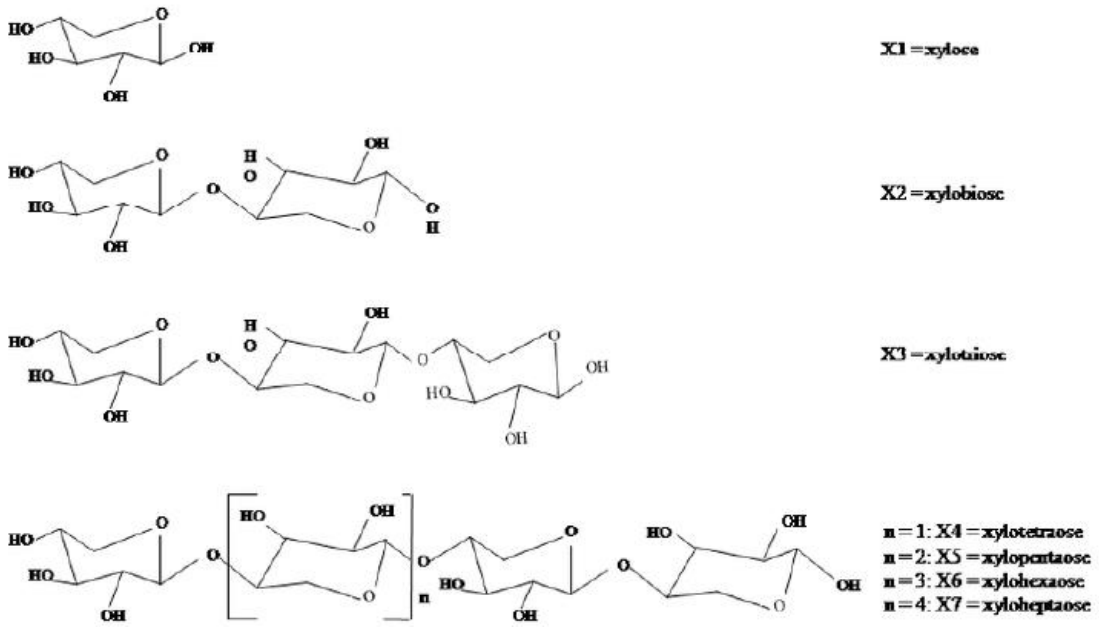


Figura 2.7. Estrutura molecular e polimerização das cadeias de xilo-oligossacarídeos (Adaptado de BRIENZO et al., 2016).

Entre as propriedades biológicas estudadas estão a digestibilidade pelas enzimas digestivas, propriedades anticariogênicas e não-cariogênicas, ação bacteriostática, seletividade a proliferação de bifidobactérias e lactobacillus (NAKAKUKI, 2002). Atualmente há um crescente interesse pela busca e desenvolvimento de novos prebióticos (de FIGUEIREDO de et al., 2017). A utilização de XOS já é uma realidade em alguns países, como no Japão, por

exemplo, essa substância já é comumente empregada em vários produtos alimentícios para humanos e animais (GIBSON; ROBERFROID, 1995). Alguns benefícios para a saúde de quem realiza a suplementação alimentar estão sendo citados como: melhora constante das funções intestinais, a absorção de minerais, proporcionando efeitos benéficos sobre o sistema imunológico e cardiovascular, promovendo melhor desempenho da atividades alérgicas e anti-inflamatórias (de FIGUEIREDO et al., 2017).

2.5.1.1. Produção de Xilo-oligossacarídeos

Os XOS podem ser obtidos basicamente por processos hidrolíticos: ácido, basico e enzimático (VAQUEZ et al., 2000). Assim podem ser produzidos por auto-hidrólise, ou através da hidrolise enzimática da xilana a partir de materiais lignocelulósicos (AKPINAR, ERDOGAN, BONSTANCI, 2009). Para a produção de XOS a partir de materiais lignocelulósicos, muitas vezes a fração xilana é previamente separada do complexo lignocelulósico por meio de tratamentos alcalinos conforme abordado no item 2.2.2. No entanto, alguns autores relataram a hidrólise enzimática direta da biomassa, que é uma tarefa árdua, uma vez que o complexo xilana-lignina é altamente resistente ao ataque enzimático.

Na hidrólise ácida, as ligações glicosídicas β-1,4 são clivadas e os polissacarídeos são fracionados em oligossacarídeos ou monossacarídeos. A concentração do reagente ácido deve ser baixa o suficiente para evitar a hidrólise da hemicelulose em monômeros, como a xilose. De forma a também minimizar a degradação dos monossacarídeos liberados em produtos como furfural ou hidroximetil furfural. A quebra da cadeia de xilana através da hidrólise ácida pode ocorrer através de ataques aleatórios, reduzindo o grau de polimerização e liberando oligômeros ou através da despolimerização, a qual depende do tamanho da cadeia e dos monossacarídeos que são liberados. Para maximizar a produção do XOS, esses dois tipos de reação precisam ser controlados, e o ataque aleatório deve ocorrer com mais frequência (AKPINAR, ERDOGAN; BOSTANCI,2009; BRIENZO et al., 2016,).

No processo de auto-hidrólise a xilana proveniente do material lignocelulósico é despolimerizada pelos os íons hidronium provenientes da auto-ionização da água durante o processo de aquecimento de forma a gerar xilo-oligossacarídeos e xilose. (GARROTE et al., 2002). Neste pré-tratamento hidrotérmico, a concentração de XOS dependerá do equilíbrio entre a decomposição da xilana em XOS e sua posterior decomposição em xilose. No entanto há uma considerável produção de compostos indesejáveis, como xilose e produtos da degradação de açúcares e lignina.

A hidrólise enzimática da hemicelulose é um processo que envolve um complexo sólido-líquido de uma reação catalítica de duas fases, onde as enzimas são adsorvidas da fase líquida para o substrato sólido. As enzimas adsorvidas na fase sólida catalisam o substrato insolúvel simultaneamente com as enzimas dissolvidas que catalisam o substrato solúvel na fase líquida. As condições da hidrólise enzimática da hemicelulose em xilo-oligossacarídeos devem ser otimizadas de modo a se obter uma forma mais econômica e eficiente de bioprocessamento desses oligomeros. Alguns aspectos microbiológicos e bioquímicos da hidrólise enzimática pelas endoxilanases podem influenciar a produção de xilo-oligossacarídeos, e por essa razão deve-se atingir a concentração ideal de enzima e substrato, reação tempo e temperatura para a otimização deste bioprocesso. Além disso, a ausência ou a presença de pequenas quantidades de β-xilosidases são importantes para se obter uma maior concentração de XOS e pouca conversão destes em xilose (POLETTO et al., 2020)

A enzima endo-β-1,4-xilanase atua na estrutura da xilana e gera XOS com baixo grau de polimerização. Para hidrólise completa da hemicelulose, enzimas acessórias que atuam nas ramiacaçoes da cadeia de xilano são necessários e a sua presença ou ausência pode gerar XOS com ou sem ramificação. Xilo-oligossacarídeos ramificados podem ser produzidos por hidrólise enzimática sem a presença de enzimas auxiliares e tal ramificação pode influenciar a taxa de hidrólise da xilana, que depende fortemente da quantidade dessas enzimas (CANO et al., 2020). As endoxilanases das famílias GH10 e GH11 diferem-se em especificidade para o substrato. Aqueles da família GH10 são menos específicos para a xilana como o substrato, e são capazes de hidrolisar formas substituintes da cadeia de xilano que produzindo oligossacarídeos contendo substituintes no terminal não redutor da molécula. Endoxilanases da Família GH11, conhecida como verdaeiras xilanases, é restrita à hidrólise da cadeia principal de xilana dessa froma atuam nas regiões não ramificadas do polissacarídeo. Assim, as endoxilanases da família GH10 produzem XOS com um grau inferior de polimerização do que os da família GH11 (BRIENZO et al., 2016).

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Capítulo 3

Cello-oligosaccharides production from lignocellulosic biomass and their emerging prebiotic applications

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Cello-oligosaccharides production from lignocellulosic biomass and their emerging prebiotic applications.

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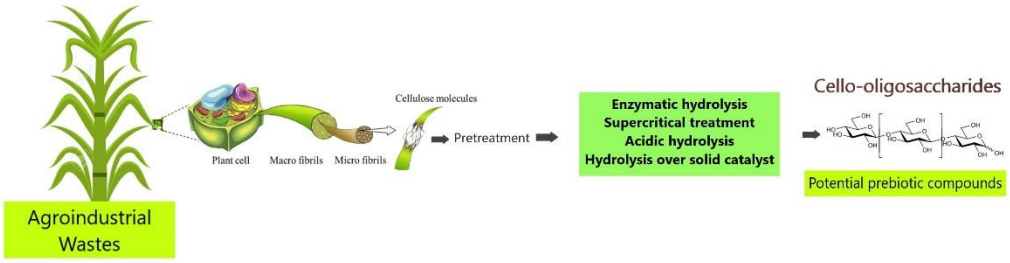
Abstract

Cello-oligosaccharides (COS) are linear oligosaccharides composed of β-1,4-linked glucopyranose units. They comprise a group of important new oligosaccharides of significant interest and potential applications in the pharmaceutical, food, chemical, and feed industries, currently emerging as potential prebiotic compounds. COS from lignocellulosic biomass, specifically the agro-industrial residues and by-products of the forestry industry, constitute a new attractive process that imposes the sustainable use of biomass resources. Two main strategies have been used for the production of COS: acid-based and enzyme-based cellulose-based hydrolysis. The latter has been considered more attractive due to the use of milder reaction conditions and less production of monomers. This review summarizes that although COS is emerging as a potential prebiotic with also other potential applications, there is a lack of information regarding the large-scale production, which could be associated with the recalcitrant nature of cellulose compared to other polysaccharides, which hinders the hydrolysis of its dense network.

Key-words: Lignocellulosic biomass, Cellulose, Oligosaccharides, Prebiotics;

Graphical abstract



3.1. Introduction

Lignocellulosic biomass has been proven increasingly necessary given the negative impacts of the indiscriminate use of non-renewable sources for biofuels production. But besides the obtention of green fuels, lignocellulosic biomass – such as agricultural residues (e.g., sugarcane straw, corn cobs, and coffee husk) – earned attention due to its abundance and potential to produce high value-added compounds, not competing with food production (Sakamoto et al. 2012). The three main classes of polysaccharides that constitute the lignocellulosic biomass – cellulose, hemicellulose, and lignin – can be cleaved by enzymatic hydrolysis or autocatalysis, releasing a wide range of compounds with different properties, such as glucose, xylose, furfural, xylo-oligosaccharides, and cello-oligosaccharides. The cellulosic portion, albeit the most abundant (representing 30% to 50% of dry biomass, depending on the source), presents high crystallinity and recalcitrance against enzymatic hydrolysis (Champreda et al. 2019). Therefore, this fraction represents one of the most challenging research fields to reduce costs associated with the production of fermentable sugars (glucose) and cello-oligosaccharides. Also, the conversion of lignocellulose into cello-oligosaccharides, which is an intermediate bio-product generated during hydrolysis to obtain glucose, seems to be a promising novel substrate for ethanol fermentation, reducing risks of contamination and glucose inhibition (Barbosa et al. 2020a; Chu et al. 2014).

Cello-oligosaccharides (COS) are oligomers of β-1,4-linked D-glucose units. The disaccharide cellobiose and shorter COS, ranging from 3 (cellotriose) to 6 (cellohexaose) glucose units, are water soluble; longer COS tend to form insoluble cellulose material (Fujii et al. 2016; Karnaouri et al. 2019b; Sanz et al. 2005). The soluble COS are not digested by humans, and a number of studies support their prebiotic properties, stimulating *in vitro* growth of several *Lactobacillus* and *Bifidobacterium* strains (Fujii et al. 2016; Karnaouri et al. 2019b; Sanz et al. 2005). The increasing pieces of evidence about the dynamic composition of the gut microbiota

and its effects on human health raises the search for new prebiotic ingredients, especially those from agro-industrial residues (Tuohy et al. 2005; Zhong et al. 2020). However, their applicability is limited due to low availability and elevated cost production suggested by recalcitrant nature of cellulose compared to other polysaccharides, which hinders the hydrolysis of its dense network (Tolonen et al. 2015; Zhong et al. 2020; Cano et al. 2020). Additionally, there is a lack of information regarding their *in vivo* prebiotic effects and large-scale production (Barbosa et al 2020a; Zhong et al. 2020). Thereunto, this review explains the possible production process of these compounds and covers their emerging potential applications, emphasizing their evident prebiotic potential.

3.2. Lignocellulosic biomass: abundant cellulose source for cello-oligosaccharides

World development over the years has undergone several transformations to meet global dynamics, which promoted biorefineries based on lignocellulosic biomass, an important asset as a renewable base material (Champreda et al. 2019;Bhatia et al. 2019). Under the sustainability regime, lignocellulose biorefineries provide renewable fuels, chemicals, and specific products, such as oligosaccharides (Bhatia et al. 2019; Cano et al. 2020, Mano et al. 2018).

Cellulose is a natural source for obtaining cell-oligosaccharides and has applications in any other industrial sectors such as the paper textile fibers, and biofuels production (Bhatia et al. 2019). It is a linear polysaccharide that consists of D-anhydroglucopyranose units (AGUs) linked by carbons with β-1,4 bond. This polysaccharide exists as a polymer with a degree of polymerization (DP) of up to 10,000 AGUs (Klemm et al. 2005). The cellulose chain can be presented under different domains. In the crystalline domain, the cellulose chains are parallel and form leaves that are stacked on top of each other in an orderly manner, being interrupted by less ordered domains (amorphous region). In this way, a rigid network of intra- and inter-chain hydrogen bonds is formed in the cellulose sheets, while these sheets are joined by Van der Waals bonds, the main factor for cellulose recalcitrance (Bergenstråhle et al. 2010; Tolonen et al . 2015). The different cellulose domains and their molecular structure are illustrated in Figure 3.1.

The lignocellulosic materials, specifically from agricultural forest residues and agro-industrial by-products, are rich in cellulose and their respective utilization represents an attractive process that imposes the sustainable use of biomass resources (Karnaouri et al. 2019a, 2019b). However, the use of the cellulose fraction of these materials is still limited due to the inherent complexity and strong cross-links between cellulose, hemicellulose, and lignin in the

lignocellulosic matrix, which makes it a very complex structure hindering the accessibility of cellulolytic enzymes and affecting the conversion of cellulose into its constituents (Barbosa et al. 2020c; Bhatia et al. 2019; Karnaouri et al. 2019a, 2019b). Thus, the choice of the most favorable pretreatment for a given raw material is essential to reduce the recalcitrance of biomass, and consequently increase the accessibility of cellulose for the production of COS via enzymatic hydrolysis (Barbosa et al. 2020a; Barbosa et al. 2020b). There are limited reports that include pretreated lignocellulosic materials source as cellulose fraction to COS production, such as sugarcane straw (Barbosa et al. 2020a, 2020b), birch and spruce (Karnaouri et al. 2018, 2019b), wheat straw, maize bran (Birhade et al. 2017), and corncob (Chu et al. 2014).

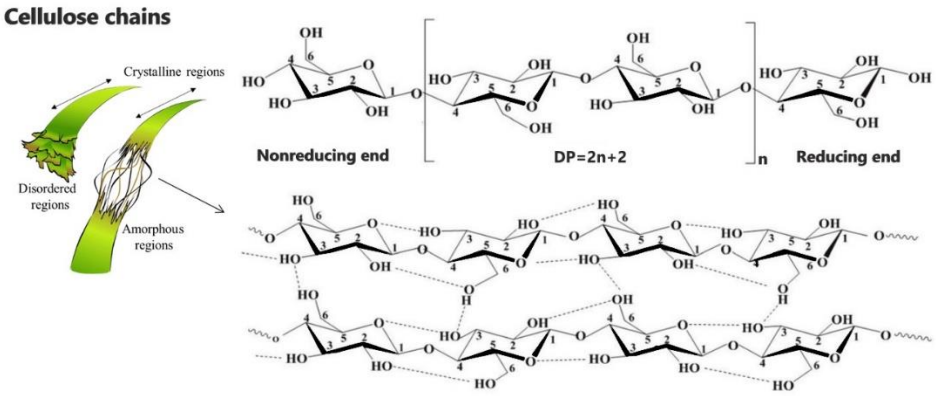


Figure 3.1. Cellulose domains and molecular structure.

3.3. Concepts and Classifications

Cello-oligosaccharides (COS) or cello-oligomers comprise a new class of non-digestible oligosaccharides (NDOs) and have been studied by researchers in recent years, who are looking for optimum conditions for their obtaining from lignocellulosic biomass, in addition to evaluations of their properties and potential industrial applications. NDOs are defined as non-starchy oligomers, which can be derived from fractions of plant matrices, such as pectin, hemicellulose and cellulose, of low degree of polymerization (DP), normally containing 3-10 units of sugars, such as fructose, galactose, mannose, arabinose, xylose and / or glucose (Karnaouri et al. 2019b). Among the main representatives of this group are the fructo-oligosaccharides (FOS), the xylo-oligosaccharides (XOS) and the galacto-oligosaccharides (GOS), as shown in Figure 3.2, whose functional properties and applications are already well-known, a fact that is yet not true for COS, thus showing a promising path for future research (Mano et al. 2018).

From a biochemical point of view, cello-oligosaccharides are defined as important biomolecules constituted of glucose units interconnected through β-1,4 type bonds. They are differentiated according to their DP and can be classified and / or characterized as: cellobiose (2 glucose monomers), cellotriose (3 glucose monomers), cellotetraose (4 glucose monomers), cellopentose (5 glucose monomers) and cellohexose (6 glucose monomers) (Barbosa et al. 2020b).

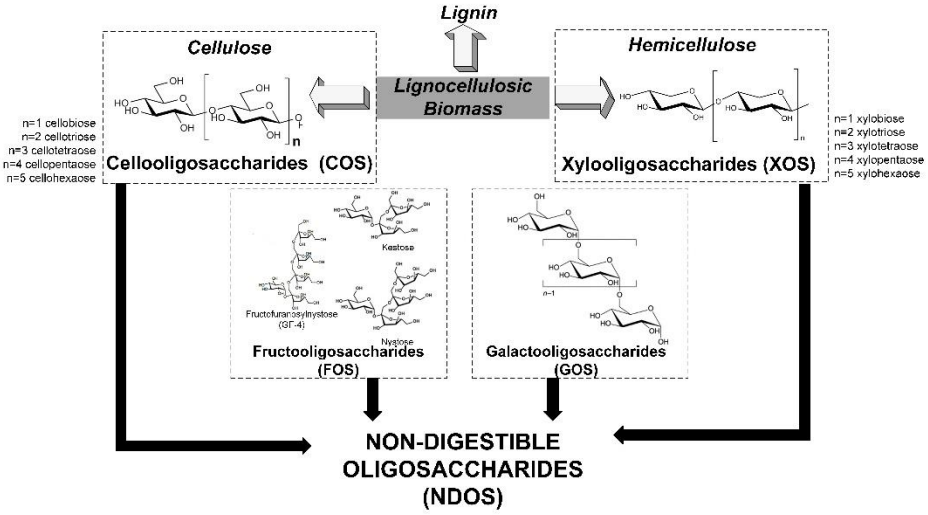


Figure 3.2. Cello-oligosaccharides and the main representatives of non-digestible oligosaccharides.

3.4. Production of cello-oligosaccharides

The plant cell wall is a tangle of constituents encompassing crystalline cellulose microfibrils surrounded by hemicellulose and lignin. Thus, it is necessary to loosen this structure for later hydrolysis of the cellulose source into high added-value constituents, such as cello-oligosaccharides (COS). These cellulose oligosaccharides are considered important emerging products for different applications, mainly in the field of animal feed and the food sector (Song et al. 2013; Otsuka et al. 2004; Uyeno et al. 2013). However, limited information is still available for large-scale production of cello-oligosaccharides (Karnaouri et al. 2019b; Zhou et al. 2020). In general, methods based on physical-chemical and enzymatic process are used for the production of COS, which are discussed in the following topics.

3.5. Physical-chemical methods

Physical-chemical or simple hydrolytic treatments, with or without the addition of small amounts of acid catalysts, are the highly effective treatments for hydrolysis of hemicellulose and partial hydrolysis of cellulose, producing xylo-oligomers and cello-

oligomers along with their sugar monomers. However, the right set of conditions (temperature, residence time, acid load, biomass loading) are crucial to obtain satisfactory yields of oligosaccharides from biomass feedstock (Awasthi et al. 2019). The combined severity factor implied during biomass treatment directly affects the oligosaccharides recovery. If the combined severity factor is increased beyond a certain point, then the released monomers and oligosaccharides are dehydrated into furfural and 5-hydroxymethyl furfural followed by levulinic acid production (Cano et al. 2020; Cho et al. 2020).

3.5.1. Supercritical treatment

The discovery of the crystalline cellulose conversion into water-soluble species via supercritical water treatment occurred in the early 1990s (Adschiri et al. 1993). This process (Figure 3.3) has the advantage of dissolving the hydrolyzed cellulose not as a monomer but rather as oligomers or short polymers, whose DP depends on the treatment temperature (Yu and Wu 2010). Recently, Tolonen et al. (2015) reported a reaction liquid content of 6.1% monomeric sugar and 42.0% cello-oligosaccharides via physical hydrolysis by supercritical water. According to the authors, when the temperature reaches the critical point (374 °C and 22.1 MPa), the internal energy of the system increases almost stepwise, and the physical properties of water are drastically changed. These factors were attributed to a stepwise acceleration of cellulose dissolution. Overall, the rate of cellulose dissolution increases faster than the corresponding rate of degradation of sugar, which enables the high recovery yields of the dissolved compounds.

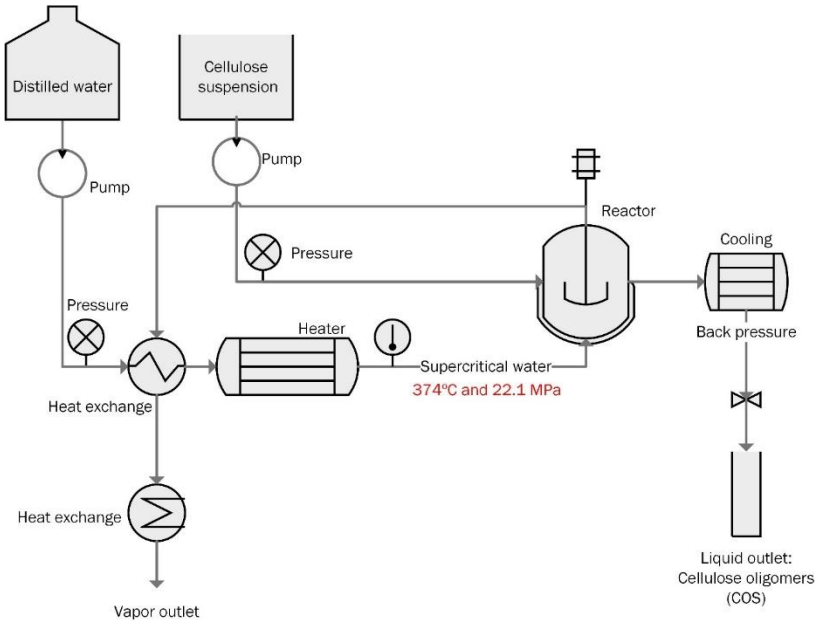


Figure 3.3. Schematic process of supercritical water treatment for cellulose dissolution.

3.5.2. Acidic hydrolysis

The acid hydrolysis has been the main treatment before enzymatic hydrolysis of the lignocellulosic biomass. Exclusively chemical degradation in acidic conditions has been, in general, conducted in order to obtain, among others, glucose, xylose, furan derivatives or levulinic acid (Cano et al. 2020). Cellulose can be very hard to hydrolyze due to its rigid crystalline structure that difficult the acid to penetrate the dense network (Cano et al. 2020; Bergenstråhle et al. 2010; Tolonen et al. 2015). In this case, hydrolysis is driven by concentrated or supported catalyst acids (Figure 3.4). However, there are few reports of acidic hydrolysis performed in a controlled process leading to COS production; some of them are covered below.

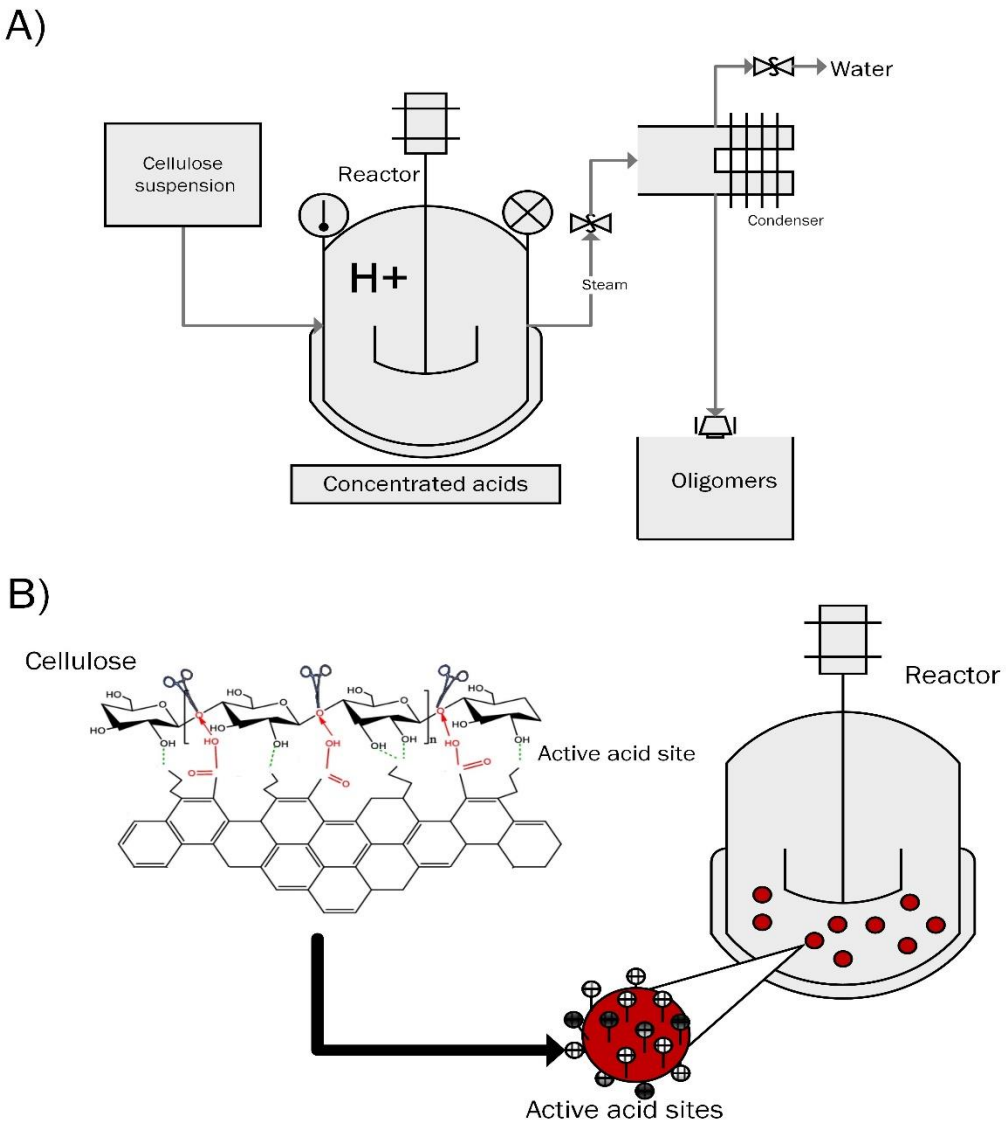


Figure 3.4 Schematic acidic hydrolysis using a solid support catalyst (A) and without solid support catalyst (B).

Bouchard et al. (2016) evaluated different conditions to improve the preparing of cellulose nanocrystals (CNC) from bleached kraft pulp to obtain cello-oligosaccharides by acid hydrolysis. When carried out at 45°C for 25 min, hydrolysis in sulfuric acid 64% led to COS with DP 7-20. In another study, COS with DP 3-11 were produced by treating microcrystalline cellulose at room temperature (22°C) using a 4:1 mixture of concentrated hydrochloric acid and concentrated sulfuric acid for 4-6 h, and soluble oligosaccharides were precipitated with acetone and purified by ion exchange column chromatography (Zhang and Lynd, 2003). Isogai et al. (2008) study with cellulose from different sources (cotton linters, fibrous microcrystalline cellulose and its derivatives after alkaline treatment) using 1M HCl at 105°C for 3 h showed a bimodal elution pattern consisting of a major component of high molecular weight of DP 35–101 and a low molecular weight component of DP 18–24 (Isogai et al. 2018).

3.5.3. Acidic hydrolysis over carbon support catalyst

Carbon materials containing oxygenated functional groups are potential heterogeneous catalysts for the synthesis of cello-oligosaccharides from cellulose (Chen, et al. 2019). These functional groups are stable under the ball-milling and hydrothermal conditions required for cellulose hydrolysis. During the reaction, cellulose adsorbs on the carbon surface by CH–π and hydrophobic interactions, and then the β-1,4-glycosidic bonds are hydrolyzed by the acidic functional group present on the catalyst surface and can be readily used in the food and agricultural industries (Kobayashi et 2014, Yabushita et al., 2014). A solid catalyst was recently prepared by oxidation of activated carbon under high temperature air (425°C) for 10 h. Cellulose was hydrolyzed in the presence of this microporous catalyst in a semi-flow reactor by mix-milling during 1 h at 180°C. These semi-flow conditions provided 71% of cello-oligosaccharides (Chen et al., 2019). The authors suggested that the adsorption of cellulose on the catalyst's surface prevents further degradation.

Cello-oligosaccharides can also be obtained through chemical homogeneous catalysts, such as 3.8% HF/SbF5 (Martin-Mingot et al. 2012,) and 85% H_3PO_4 (Liebert et al. 2008), which could dissolve and hydrolyze cellulose, resulting in high DP COS. However, there are some limitations to the application of these methods in the food and agricultural industries, due to difficulty in separating the catalyst, as well as its toxicity (Chen et al. 2019).

3.5.4. Enzymatic Process

In comparison to chemical-physical methods, the enzymatic hydrolysis is a more attractive one, due to the relatively mild reaction conditions and fewer by-products (Bhatia et

al. 2019). The knowledge about the huge amounts of lignocellulosic materials produced as residues in agriculture opened a new variety of raw material that could be subjected to enzyme saccharification (Cano et al. 2020).

Cellulases are a group of enzymes that affect their lytic activity over cellulose, having a synergistic effect amidst all different enzyme activities (Barbosa et al. 2020c; Vermaas et al. 2015; Selig et al. 2015). This group is composed of three kinds of group enzymes, namely exoglucanases, endoglucanes and β-glucosidases.(Barbosa et al. 2020c; Soccol et al.2010). As illustrated in Figure 3.5, endoglucanases have affinity for amorphous cellulose regions and promote a random attack on internal β -glycosidic bonds, releasing oligomers of different lengths, such as cellobiose. Exoglucanases are processive enzymes with affinity for crystalline cellulose. This group can act on the reducing ends of the molecule (CBH I, exo-1,4-b-cellobiosidase) or on non-reducing ends (CBH II, exo-1,4-b-d-glucanase), releasing cellobiose. Finally, β-glucosidases hydrolyze disaccharide cellobiose or COS to two glucose molecules (Barbosa et al. 2020c; Soccol et al.2010; Kuhad et al. 2011; Zabed et al. 2017).

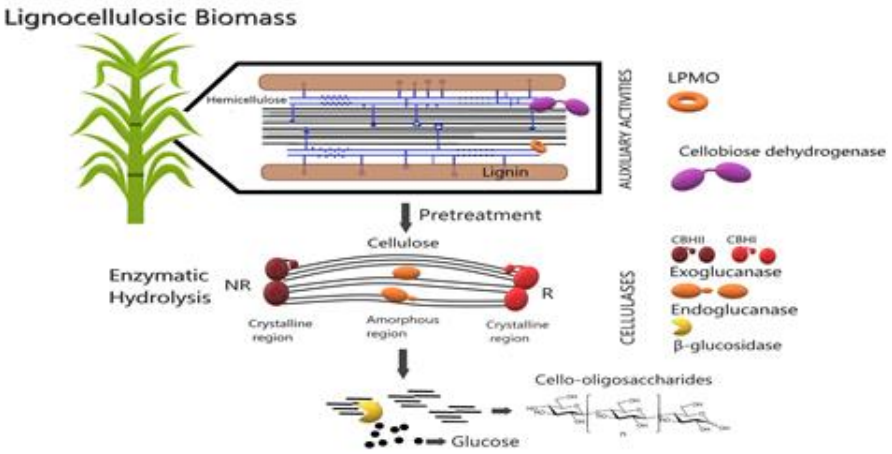


Figure 3.5. Schematic action of cellulases and accessory enzymes in lignocellulosic biomass to glucose and cello-oligosaccharides releasing.

Several factors can affect the enzymatic hydrolysis conversion yield and are related both to enzymes and biomass properties resulted from the pretreatments (Cano et al. 2020, Bhatia et al. 2019, Barbosa et al. 2020b). Regarding the enzymes, parameters such as loading, synergy, temperature, pH, and agitation must be considered (Bhatia et al. 2019; Cano et al. 2020; Barbosa et al. 2020a; Barbosa et al. 2020b). Thus, different strategies for enzyme-mediated production of cello-oligosaccharides have been proposed. These include, among others, the design of tailor-made enzymatic cocktails that offer a controlled polysaccharide cleavage breaking and produce less monomers (Karnaouri et al. 2018), the modification of

reaction conditions (e.g., buffer exchange to abolish the end-product inhibition of enzymatic activity) (Vanderghem et al. 2010) or the fine-tuning of the performance of commercially available enzyme cocktails (e.g., addition of-glucosidase inhibitor (Tsuji et al. 2013).

Cello-oligosaccharides are intermediates of the cellulose hydrolysis process into glucose; on the other hand, during COS production, the monosaccharides (glucose) are the by-products. Researchers pointed out that the existence of monosaccharides would bring negative consequences to the application of cello-oligosaccharides in the food industry, such as risks of dental caries (Clemens et al. 2016). Also, β-glucosidase impedes COS accumulation since this kind of enzyme hydrolyses the glycosidic bond within cellobiose, then yielding a high amount of glucose. Therefore, removing some enzymes, or reducing their activities in the cocktail, enables the release of oligosaccharides with a higher DP. That is the reason why some procedures are focused on the adsorption of these enzymes on their natural substrate (Chu et al. 2014). The multi-stage enzymatic hydrolysis of cellulose consists of stages, with the removal of the products after each one (Chu et al. 2014). This process has been reported as a potential strategy to enhances hydrolysis efficiency due to removing product inhibition (Chu et al. 2014; Yang et al. 2010; Yu et al. 2012).

Chu et al. (2014) demonstrated that the selective removing of β-glucosidase activity in a corncob residue doubles the cello-oligosaccharides production in the subsequent enzymatic hydrolysis (from 0.15 g/g to 0.25 g/g, corresponding to a final concentration of 7.6 g/L and 12.6 g/L, respectively) and the selectivity (from 30 to 60%). Birhade et al. (2017), on the other hand, discovered that these differential adsorption processes are deeply influenced by the accessibility of cellulose. The adsorption applying wheat straw treated with ammonia maximized the production of a mixture of cello-oligosaccharides DP > 6 (0.11 g/g), DP 2–6 (0.10 g/g), and monosaccharides (0.11 g/g).

Another effective strategy increased the purity of resultant cello-oligosaccharide through the simultaneous production of high value-added products of sugar monoester. This process converted the excessive glucose in the cello-oligosaccharides solution into glucose mono-decanoate, which is a well-known biodegradable non-ionic surfactant. A paper-filter was the model used to investigate the feasibility of the process, which increased the purity of the cello-oligosaccharides from 33.3% to 74.3% and simultaneously produced glucose mono-decanoate with 92.3% purity. The verification of the process with different lignocellulosic materials (switchgrass, cornstalk, and reed) also indicated a good performance (Zhou et al. 2020).

The cellobiohydrolase (CBH) belonging to the glycosyl hydrolase family 7 (CBH7) and the endoglucanase of the family 5 (EG5) have been considered two enzymes of pivotal importance for the production of COS, especially cellobiose from lignocellulosic materials (Karnaouri et al. 2018). However, other enzymes acting on the amorphous areas, such as endoglucanases of the family 7 (EG7) and cellobiohydrolase of family 6 (CBH6,) are needed as the reaction proceeds (Karanaouri et al. 2019b, 2018). In addition, individual cellulolytic activity of endoglucanases has been related to rapid and efficient liquefaction of cellulose-rich lignocellulosic materials under high dry matter loadings (19% (w/w)), which is of out most importance in order to achieve high COS yields (Karnaouri et al. 2014). When CBHs act in concert with the endoglucanases hydrolysis yields increase drastically due to the endo–exo synergy between these two classes of the enzymes (Karanouri et al. 2019b, Barbosa et al. 2020c).

Lately, new approaches for COS production optimization have been using regular endoglucanases, processive endoglucanases, and auxiliary enzymes, such as lytic polysaccharide monooxygenases (LMPOs) (Barbosa et al. 2020a; Karnaouri et al. 2018; Karnaouri et al. 2019a). LPMOs act in strong synergism with endoglucanases both for the release of neutral and oxidized sugars (Fushinobu, 2014; Barbosa et al. 2020c). Moreover, some studies support the supposition that LPMOs create new chain breaks in crystalline substrates, reducing the biomass crystallinity (Barbosa et al. 2020b; Hemsworth et al. 2015; Vermaas et al. 2015; Selig et al. 2015).

In a recent study, organosolv-pretreated forest residues (birch and spruce) were tested in the presence of processive endoglucanases of bacterial and fungal origin, belonging to GH9, GH6, and GH48 families and LPMO auxiliary family 9 for their ability to release COS. The optimal enzyme combinations using birch and spruce led to 22.3 wt% and 19.1 wt% cellulose conversion into cellobiose, respectively (Karnaouri et al. 2019b). Barbosa et al. (2020a) also optimized the COS production, reaching approximately 60 mg/g of pretreated sugarcane straw using a combination of heterologous expressed processive endoglucanases (10 U/g), LPMO (2 mg/g), and cellobiose dehydrogenase (1 mg/g).

The advent of new enzymes, such as LPMOs, raised novel essential components of cellulolytic cocktails due to the ability of these enzymes in facilitating the activity of hydrolases by creating new terminals to the cellulose chains, promoting the defibrillation of the fibers, and improving the overall degradation process (Karnaouri et al. 2019b; Barbosa et al. 2020b; Hemsworth et al. 2015; Vermaas et al. 2015; Selig et al. 2015). In depth study about hydrolytic

processes with classical hydrolytic enzymes and acids created new opportunities to obtain bioactive oligomers, even from recalcitrant cellulose. However, the enzyme separation procedure is complicated and expensive, hampering its application on a large scale (Zhou et al. 2020).

3.6. Properties and Applications

COS have some particular physical-chemical, rheological and functional properties, becoming important compounds with elevated potential applications in the most varied industrial segments, such as in the food, medicines, cosmetics, and chemical industries, as well as in the production of animal feed, bioenergy, among others. COS have excellent oil retention capacities, form highly fluid powders, have good resistance to compression and malleability, have considerable resistance to heat, in addition to remaining practically intact at acidic pH. All of these characteristics enable the use of these potential prebiotics to improve dispersions and rheological properties of the materials and / or formulations to which they can be applied (Kluge et al. 2019).

In general, no studies were found in the literature that report specific characteristics and applications for each type of celloligosaccharide in isolation, which highlights and praises possible fields of study to be evaluated by researchers in the future. The properties and applications of COS are discussed here in general, in its integral form, as a mixture of oligomers of 2 to 6 glucose units.

One of the most important properties of cello-oligosaccharides is their prebiotic functional activity, thus stimulating the growth of beneficial probiotic cultures, such as bacteria of the genus *Bifidobacterium sp*. and *Lactobacillus sp*. (Jiao et al. 2014). COS are able to resist the initial stages of the digestion process in the stomach, and to reach the large intestine almost intact, thus serving as a substrate for these beneficial microorganisms present in the intestinal microbiota. The growth of these beneficial bacteria promotes the production of short-chain fatty acids (SCFA), such as acetate, propionate and butyrate, bringing several benefits to the host (Mano et al. 2018).

SCFA act in the body by reducing luminal pH, inhibiting the development of enteropathogens. Acetate and butyrate act synergistically as a primary substrate for the growth of the colon epithelium, inducing the production of immunomodulatory cytokines, in addition to promoting the excretion of ammonia and amine compounds. All of these effects play an important role in strengthening the immune system, regulating and stimulating the absorption of mineral salts, regulating lipid metabolism, inhibiting the development of cancer cells, mainly

in the colon and stomach, preventing cardiovascular diseases, and preventing other metabolic syndromes (Jiao et al. 2014; Mano et al. 2018).

All these mechanisms triggered by their prebiotic effect favor cello-oligosaccharides, considering that, like other prebiotic substances, COS have considerable potential to be incorporated into food formulations, such as cereal bars, bakery products, sweets, ice creams, dairy desserts, juices, nectars, sauces, symbiotic foods, etc. They can also be incorporated into dietary supplements or even into medications that assist in the replacement of the intestinal microbiota (de Freitas et al. 2019; Scolforo and Madeira 2013).

In addition to these applications, COS can be incorporated into feed formulations, in the view of the multiple benefits they promote for animal health and development. During the evaluation of the effect of cello-oligosaccharide supplementation on the intestinal microflora of weaned pigs, it was observed that the increased consumption of these prebiotics promoted a significant growth of *Lactobacillus sp.*, in addition to bringing improvements to the mucosa architecture and nutrient transport in the small intestine of piglets (Jiao et al. 2014). Studies have also shown that the use of COS as a supplement in diets for calves helps the healthy development of these animals, mainly by promoting microbial fermentation and subsequent short-chain fatty acids that favors their intestinal and general (Cangiano et al. 2020).

COS present an important role in inhibiting pathogenic microorganisms, in addition to other properties that make them biomolecules of great interest and the target of research in the medical, pharmaceutical, and cosmetic fields. They have stood out as an important bacteriostatic agent, and can therefore compose the formulation of antibiotics or other medications, since they can efficiently inhibit the growth of *Helicobacter pylori*, a bacterium that can resist stomach acid pH mainly because it causes serious gastroenteritis (Kluge et al. 2019). In addition, there is the inhibition of other pathogens, such as bacteria of the genus *Clostridium sp*.,that can cause damage to the gastrointestinal system and to other organs (Uyeno et al. 2013). By oral ingestion, cello-oligomers are also responsible for lowering total cholesterol and the concentration of neutral fat in the liver, preventing or aiding in the cure of diseases and, consequently, promoting a better lifestyle (Kluge et al. 2019). Due to their considerable moisturizing effect and improvement of the skin barrier, mainly acting in the inhibition of microorganisms that cause dermatitis, such as *Staphylococcus sp.*, *Pseudomonas sp*., COS are also improving the efficiency of creams and cosmetics. Combined with fatty acids, they can also be used as new bio-based amphiphilic compounds (Billès et al. 2016). Hydroxyl

groups in cello-oligomers can be derivatized by a variety of methods, thereby adjusting their properties and opening other fields of application (Kluge et al. 2019).

Cello-oligosaccharides have also been proposed by researchers in recent years as new substrates for fermentation and ethanol production, given the potential advantages over glucose (conventional process), including a reduced risk of contamination of the process, due to the use of engineered and selective yeast strains for the consumption of these prebiotics and ethanol production and for consuming the substrate faster, resulting in shorter total fermentation times, in addition to the effects of inhibiting the process limited by the high concentration of glucose, which is avoided when using these oligosaccharides (Barbosa et al. 2020a; Liang et al. 2013).

3.7. Conclusion

COS have a considerable potential to be utilized in a number of useful applications and the most relevant is their emerging prebiotic functional activity, although so little has been studied. An evident reason for the lack of extensive research on COS is the limited availability and high price, which is in turn caused by the recalcitrant nature of cellulose compared to other polysaccharides. Thus, the advent of new enzymes, such as LPMOs, and the more in-depth study of hydrolytic processes with classical hydrolytic enzymes and with supported acids enhance the action over the more recalcitrant cellulose, which needs harsh conditions to be hydrolyzed, creating new opportunities to COS production. Therefore, the use of enzymes reduces the risk of sugar dehydration to undesirable compounds, since fine-tuning of acidity and temperature is needed to obtain satisfactory results in acid-based process.

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Capítulo 4

Enzymatic production of xylooligosaccharides from alkali-solubilized arabinoxylan from sugarcane straw and coffee husk

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Enzymatic production of xylooligosaccharides from alkali-solubilized arabinoxylan from sugarcane straw and coffee husk

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Abstract

Enzymatic hydrolysis of lignocellulosic biomass has opened new perspectives for the production of biofuels and functional food ingredients, such as prebiotic xylooligosaccharides (XOS). This study aimed to improve the arabinoxylan extraction process from sugarcane straw (SS) and coffee husk (CH) to produce short-chain XOS by enzymatic hydrolysis using ideal enzyme mixture formulations. The improvement of arabinoxylan extraction process provided the highest extraction yield about 40.35% and arabinoxylan extraction efficiency around 82.32% with insignificant lignin content values. In a second step, a central composite rotatable design was used to develop an enzyme mixture for XOS production from SS and CH arabinoxylan. Three commercial enzymes were tested, endoxylanase (GH11), α-L-arabinofuranosidase (GH51), and feruloyl esterase (CE1). An efficient enzyme mixture was developed, affording high XOS concentrations from SS arabinoxylan around 10.23 g L^{-1} and CH arabinoxylan 8.45 g L^{-1}.

Keywords: Lignocellulosic biomass, Holocellulose, Enzymes, Optimization

4.1 Introduction

Sugarcane straw (SW) and coffee husk (CH) are some of the most widely distributed and abundant agricultural wastes in Brazil [1]. The lignocellulosic nature of plant wastes allows them to be fractionated into cellulose, hemicellulose, and lignin and then used as renewable sources for the production of value-added products [2]. The xylan-rich hemicellulose fraction of plant wastes is a potential starting material for the production of xylose-based compounds [3].

Xylooligosaccharides (XOS) are sugar oligomers produced by the hydrolysis of xylan, a homopolysaccharide backbone of xylose units [4]. XOS can be used as sweeteners, stabilizers, emulsifiers, and functional ingredients in a variety of food products [8]. One of the most important applications of XOS is in functional foods owing to their prebiotic effects. In the gastrointestinal environment, XOS, especially short-chain XOS, are metabolized by bifidobacteria, stimulating their growth and that of *lactobacillus* in detriment to the growth of harmful bacteria. XOS also increase the levels of short-chain fatty acids in the gastrointestinal tract and exert gastric ulcer protective effects [7, 9, 10].

Production of XOS from xylan-rich lignocellulosic materials (LCM) generally includes chemical methods, enzymatic methods, and a combination of these methods [11]. The production of XOS with chemical methods can be accomplished by steam, diluted solutions of mineral acids, or alkaline solutions. Extraction of xylan with steam or acid produces large amounts of monosaccharides and their dehydration product [12]. Steam or hydrolytic degradation of xylan, known as autohydrolysis, involves the deacetylation of xylans to produce acetic acid, which hydrolyzes the hemicellulose [13]. This method eliminates the use of corrosive chemicals for the extraction of xylan. However, it requires special equipment that can be operated at high temperatures resulting in hydrolysates containing a large variety of undesirable components, such as xylose and lignin fractions, demanding a time-consuming purification process [11,13]. In contrast to autohydrolysis and chemical treatment methods, enzymatic hydrolysis avoids production of undesirable by-products or high amounts of monosaccharides, or require high-pressure or high-temperature equipment [14].The industrial XOS production can be performed by enzymatic hydrolysis of xylan present in the LCM, such as corn cobs, rice husks, barley straw, tobacco stalk, cotton stalk, sunflower stalk, wheat straw and sugarcane biomass [11]. Alternatively, XOS can be produced in two stages that consist of the xylan extraction first, which may be an alkaline treatment. In a second stage, an enzymatic hydrolysis of xylan can be performed with a consequent reduction in the purification step requirement [12-14].

XOS can be produced using endoxylanases, which hydrolyze the β-1,4-glycosidic linkages in xylan. The enzyme complex should have high endo-1,4-β-xylanase activity and low exoxylanase or β-xylosidase activity to avoid generating high amounts of free xylose, which acts as a reaction inhibitor [7]. However the composition of hemicellulose can vary significantly among plant species and even within the separate components of a single plant (leaves, stem and roots). Thus a diverse combination of enzymes is required for complete hemicellulose

hydrolysis: endo and exoxylanases initiate the breakdown of cross-linked hemicelluloses generating XOS of varying lengths; arabinofuranosidase hydrolyses arabinose units into both furanose and pyranose forms; glucoronisidase hydrolyses methyl glucuronic acid substitutes; while acetylxylan esterase and ferulic acid esterase hydrolyze acetyl groups substitutes and ferulic acid, respectively [15].

There are numerous studies on enzymatic hydrolysis of LCM using enzyme mixtures. However, there are few studies employing sugarcane straw or coffee husk as the substrate, not even using the isolated holocellulose fraction to XOS production. However the better understanding of which enzymes, and their proportions, is essential for hemicellulose degradation, and could lead to rational design of more efficient, and consequently less expensive enzyme mixtures [16]. In a study of Wong and Maringer [17] significant synergism was observed between xylanases during the hydrolysis of acetylated pine hemicellulose. Ávila et al. [18] verified synergistic associations between commercial cocktails including and endoglucanase and arabinofurnosidase to produce XOS employed a substrate mixture containing sugarcane straw and bagasse. Goldbeck et al. [16] also developed an efficient enzyme mixture with synergistic effect employing two hemicellulases, the endo-1,4-xylanases (GH11) from *Penicillium funiculosum* (XynC11/CAC15487) and the feruloyl esterase (CE1) from *Clostridium thermocellum* (CtFAE/ATCC27405), which effectively broke-down hemicellulose from pretreated sugarcane bagasse (up to 65%) along with the production of XOS.

The breakage of chemical bonds in xylan is relatively difficult and costly, limiting XOS production [19]. Therefore, methods to increase xylan extraction efficiency and reduce enzymatic hydrolysis costs are important for industrial applications. This present work proposes to improvement of hemicellulose alkaline extraction and optimize enzymes mixtures aimed to produce linear short-chain XOS by enzymatic hydrolysis of xylan extracted from isolated holocellulose of different lignocellulosic biomass wastes (SS and CH), which are by-products of the agroindustry with considerable hemicellulose source.

4. 2 Materials and Methods

4.2.1 Raw materials

SS was kindly supplied by the Brazilian Bioethanol Science and Technology Laboratory (CTBE/CNPEM, Campinas, SP, Brazil). CH was donated by Coxupé Ltd.

(Guaxupé, MG, Brazil). Moisture content was quantified according to AOAC [20]. SS and CH contained 75% and 57% moisture, respectively.

Materials were washed with water at room temperature to remove excess soil [1], oven-dried at 80 °C to 10% moisture [21], ground in a cutting mill, and sieved to obtain particles of 0.3–0.7 mm it diameter for use in the experiments[18].

4.2.2 Chemical composition analysis

The chemical composition analysis were perform fallowing the NREL protocols with minor adaptations. Cellulose, hemicellulose, and lignin contents were determined according to the NREL/TP-510-42619 procedure [22]. Representative samples of 300 mg were collected from each biomass and hydrolyzed by treatment with 72% H_2SO_4 at 30 °C for 60 min and with 4% H_2SO_4 at 121 °C for an additional 60 min. Samples were quenched in ice and filtered. Cellulose and hemicellulose contents were determined by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) using a Dionex DX-500 system (Sunnyvale, CA, USA) and a CarboPac PA1 column (4 × 250 mm, Dionex, USA. Ash content was determined by burning 1 g of sample in a muffle furnace for 6 h at 600 °C, according to the NREL/TP-510-42622 method [23]. Extractives were determined by Soxhlet extraction with ethanol and water, according to NREL/TP-510-42619 [24]. Three independent replicates were performed for each assay, and results are presented as mean and standard deviation.

4.2.3 Holocellulose and arabinoxylan fraction

The arabinoxylan extraction method of Zilliox and Debeire [25] was used as a reference in this study to extract arabinoxylan from holocellulose fraction of sugarcane straw and coffee husk. Ground SS and CH were pre-incubated in 400 mL of deionized water at 70 °C for 16 h. The holocellulose fraction was obtained according to Wise et al. [26] procedure. The reaction was carried out at 80°C under moderate agitation in 250 mL Erlenmeyer flasks containing 2 g of biomass and 120 mL of a 7.5% acetic acid solution. Sodium chlorite (20 mg) was added to the mixture 4 times in 1 h intervals, totaling 4 h of process. The delignified product was filtered, washed with distilled water several times, and oven-dried at 60 °C for 48 h. This additional step was included in the traditional extraction procedure to increase biomass delignification and improve the efficiency of enzymatic hydrolysis.

Holocellulose fraction (8% solids loading) was mixed with 250 mL of 24% (w/v) KOH solution containing 1% (w/v) $NaBH_2$ and incubated for 3 h at 35 °C (Treatment 1). The liquid

fraction was filtered with gauze until no solids were observed in the liquid phase. Arabinoxylans were precipitated from the filtrate by the addition of a solution of 60% (v/v) ethanol and 6.7% (v/v) acetic acid. The precipitate was separated by centrifugation at 4000 × *g* for 15 min, washed 5 times with equal volumes of acetic acid and an ethanol solution (50% ethanol and 0.5% EDTA in deionized water), and dried at 60 °C to constant weight [6, 19, 27]. A flow diagram of the procedure is presented in Figure. 4.1.

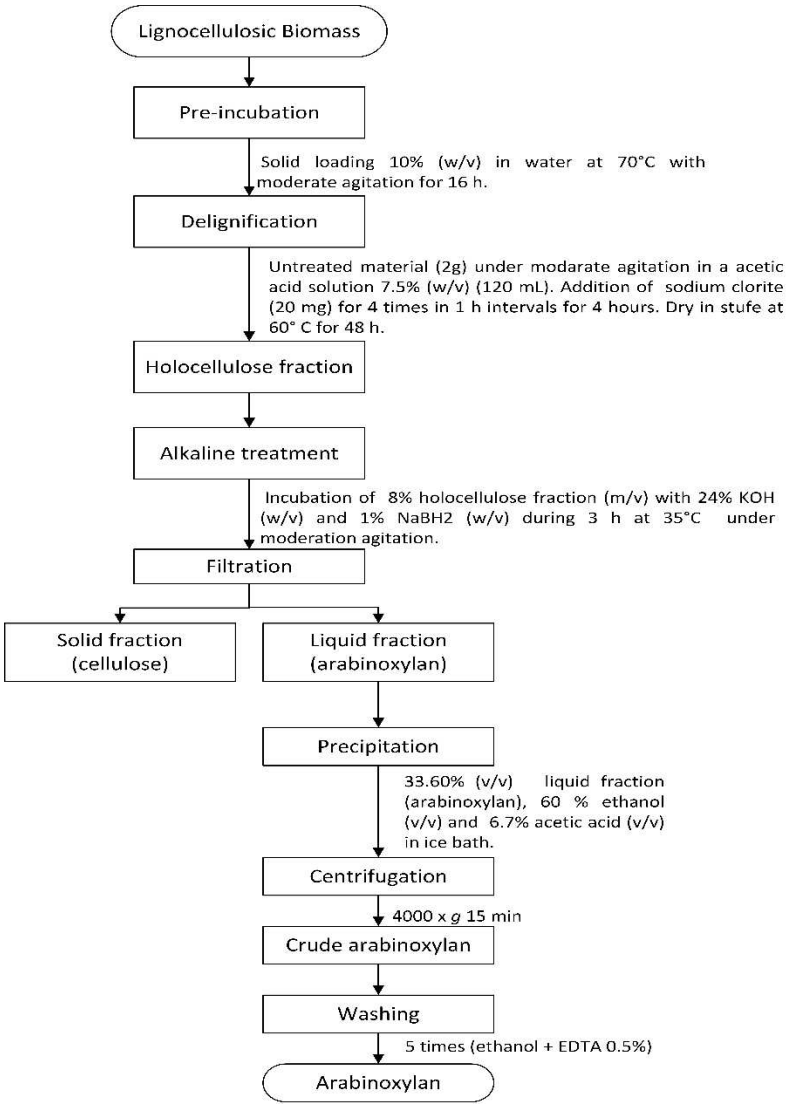

Figure 4.1. Schematic diagram of arabinoxylan extraction from sugarcane straw and coffee husk.

Solids loading, incubation time, KOH concentration, temperature, hydrolysis time, ethanol/acetic acid ratio, and number of washings were varied to study their effects on xylan yield and extraction efficiency. In all cases, xylan precipitate were washed until neutral. The treatment conditions are presented in Table 4.1.

Table 4.1. Treatment and extraction conditions for separation of arabinoxylans from isolated holocellulose of sugarcane straw and coffee husk.

Treatment	Solids loading (%)	Pre-incubation time (h)	KOH (% w/v)	Temp. (°C)	Reaction time (h)	Ethanol/acetic acid ratio (v/v)	Number of washings
Treatment 1	8	16	24	35	3	60:6.7	5
Treatment 2	8	10	24	35	6	63:6.3	4
Treatment 3	8	10	14	70	3	60:6.7	4
Treatment 4	8	10	8	80	3	60:6.7	4
Treatment 5	8	10	4.5	121	0.5	60:6.7	4
Treatment 6	15	0	4.5	121	0.5	60:6.7	4

4.2.4 Extraction yield and xylan extraction efficiency

Extraction yield (Y) and arabinoxylan extraction efficiency (E) were calculated using the following equations [19]:

$$Y = \left(\frac{W_e}{W_h}\right) \times 100 \quad (1)$$

where W_e is the dry weight of extracted material (g) and W_h is the dry weight of isolated holocellulose fraction (g).

$$E = \left(\frac{X_e}{X_h}\right) \times 100 \quad (2)$$

where X_e is the arabinoxylan content in the precipitate material (mg g^{-1}) and X_h is the arabinoxylan content in the isolated holocellulose fraction (mg g^{-1}).

4.2.5 Commercial enzymes and determination of protein concentration

The following commercial enzymes were used: endo-1,4-xylanase from *Aspergillus niger* (GH11), α-L-arabinofuranosidase from *Aspergillus niger* (GH51), and feruloyl esterase from *Clostridium thermocellum* (CE1). Enzymes were purchased from Megazyme (Bray, Ireland). Protein concentration was determined according to Lowry [28].

4. 2.6 Enzyme activity assays

α-L-Arabinofuranosidase activity was analyzed according to da Silva et al. [29]. Briefly, 100 µL of 10 mM *p*-nitrophenyl-α-L-arabinofuranoside, 200 µL of 0.5 M sodium acetate buffer pH 5.0, 650 µL of Milli-Q water, and 50 µL of appropriately diluted enzyme solution were mixed and incubated at 45 °C for 10 min. The reaction was stopped by adding 500 µL of 1 M Na_2CO_3. *p*-Nitrophenol was quantified spectrophotometrically at 420 nm. One

unit of α-L-arabinofuranosidase was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute.

Endo-1,4-xylanase activity was assayed using soluble birchwood xylan (Sigma–Aldrich, San Luis, MO, USA) as substrate. Appropriately diluted enzyme solutions (0.1 mL) and 500 μL of 2% birchwood xylan were mixed with 50 mM sodium acetate buffer pH 5.0 to a total volume of 1 mL. The reaction was incubated at 45 °C for 30 min [29], and reducing sugars were analyzed by the dinitrosalicylic acid method [30]. One unit of xylanase was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per minute.

Feruloyl esterase activity was determined using naphthyl acetate as substrate, according to the method of Koseki et al. [31]. One unit of feruloyl esterase was defined as the amount of enzyme that released 1 μmol of α-naphthol per minute.

4.2.8 Monosaccharides and XOS quantification

Xylose, arabinose and XOS were quantified by HPLC-PAD using a Dionex DX-500 system (Sunnyvale, CA, USA), a CarboPac PA1 column (4 × 250 mm), a CarboPac PA1 guard column (4 × 50 mm), and an electrochemical detector. Separation was achieved using a linear gradient of A (Milli-Q water), B (250 mM NaOH), C (100 mM NaOH and 1000 mM NaOAc), and D (Milli-Q water). Integrated peak areas were normalized relative to those of commercial standards (Megazyme, Bray, Ireland) [18].

4.2.9. Statistical analysis

All experiments were performed in triplicate, and results are presented as mean and standard deviation. Means were compared by Tukey's test ($p < 0.05$) using Statistica v. 8.0 (Statsoft, Inc., Tulsa, OK, USA). The results of experimental design were analyzed at 90% confidence level using Protimiza Experimental Design online software.

4.3 Results and Discussion

4.3.1. Arabinoxylan extraction

4.3.1.2. Chemical composition

As an initial step, we compared different process conditions for solubilization/extraction of hemicellulose from SS and CH. Although there are several reports of the solubilization of hemicellulose in the literature [6, 19, 25, 27], adequate process

conditions for industrial application have not yet been determined. The major challenges include reducing reagent consumption and process time.

The proximate composition of the agroindustrial wastes used in this study is presented in Table 4.2. Our results were in agreement with literature data showing that SS is composed of 24–29% total hemicellulose, 19–25% total lignin, 38–50% cellulose, and 1–7% ash [21, 33, 34]. The proximate composition of CH was similar to that reported by de Carvalho Oliveira et al. [35]. CH composition is greatly influenced by crop variety, cultivation conditions, and processing methods [36], which explains the differences found between this and previous studies [34, 37]. The wide availability of SS and CH, added to their high hemicellulose content, make them interesting substrate materials for industrial-scale production of XOS [19].

Table 4.2. Proximate composition (% w/w, dry basis) of untreated sugarcane straw and coffee husk biomass.

Components	**Sugarcane straw**	**Coffee husk**
Cellulose	39.40 ± 0.23	27.78 ± 0.78
Hemicellulose	29.86 ± 0.55	24.67 ± 0.61
*Xylan	22.65 ± 0.65	20.32 ± 0.54
*Arabinose groups	5.86 ± 0.85	3.67 ± 0.71
Total Lignin	21.83 ± 0.91	35.58 ± 1.55
Ash	4.05 ± 0.12	0.98 ± 0.56
Extractives	6.67 ± 0.56	10.78 ± 0.78
Total	98.81 ± 2.37	99.79 ± 4.28

*Xylan and arabinose groups corresponds fractions of hemicellulose source, thus they were not included in the total mass balance.

Table 4.3 shows the chemical composition of isolated holocellulose from sugarcane straw and coffee husk and their respective arabinoxylan extracts from different alkaline treatments. Extracts were composed of heterogeneous polymers of pentose (arabinoxylan) and hexose sugars (glucan/cellulose). The extracted material after alkaline treatments from sugarcane straw holocellulose presented higher range values of arabinoxylan concentration (purity) (78.67- 84.71%) than CH (74.62-78.55%) and consequently a lower glucan content after alkaline treatments (Table 4.3). This result is in agreement with the higher initial hemicellulose content found in SS biomass (Table 4.2).

Except for treatment 2, all treatments yielded products with similar arabinoxylan concentrations ($p < 0.05$) (Table 4.3). The difference in arabinoxylan content between treatment 2 and other treatments may be due to the higher concentration of ethanol (63%) and lower concentration of acetic acid (6.3%) in the reaction medium, as shown in Table 4.1. These findings indicate that higher arabinoxylan extraction can be achieved using higher proportions of ethanol and lower proportions of acetic acid. The isoelectric point of xylan was reached when using an alcohol/acid ratio of 63:6.3, contributing to the efficiency of the process. The concentration of arabinoxylans in the precipitate extracted from both isolated holocellulose sources were higher than that reported in the literature for other substrates because arabinoxylans were extracted from holocellulose rather than from raw lignocellulosic biomass [6, 19]. Fractionation holocellulose reduced the lignin content by 87.35 and 89.37% for SS and CH biomass, respectively. This step was crucial for obtaining high-purity arabinoxylan extracts. Several studies have shown that delignification is necessary for an effective hemicellulose conversion after enzymatic hydrolysis [19, 38].

Table 4.3. Chemical composition of isolated holocellulose fraction from sugarcane straw and coffee husk and their respective arabinoxylan rich precipitate obtained after alkaline treatments.

Treatments	**Sugarcane straw**		
	Glucan (%)	Lignin (%)	Arabinoxylan (%)
Holocellulose	51.78 ± 0.78a	2.73 ± 0.58a	41.52 ± 1.36a
Treatment 1	4.12 ± 0.72b	0.77 ± 0.16b	80.90 ± 0.93b
Treatment 2	2.14 ± 0.44c	0.45 ± 0.07c	84.71 ± 0.96c
Treatment 3	4.81 ± 0.62b	0.72 ± 0.13b	80.40 ± 1.27b
Treatment 4	4.42 ± 0.63b	0.75 ± 0.12b	79.94 ± 1.24b
Treatment 5	4.54 ± 0.51b	0.71 ±0.17b	78.67 ± 1.35b
Treatment 6	5.10 ± 0.72b	0.74 ± 0.78b	79.70 ± 1.33b
Treatments	**Coffee husk**		
	Glucan (%)	Lignin (%)	Arabinoxylan (%)
Holocellulose	56.40 ± 0.23a	3.78 ± 0.75a	35.97 ± 0.87a
Treatment 1	7.12 ± 0.87b	0.32 ± 0.15b	75.78 ± 0.94b
Treatment 2	4.19 ±0.78c	0.26 ± 0.05c	78.55 ± 1.20c
Treatment 3	6.81 ± 0.62b	0.42 ± 0.14b	75.31 ± 1.03b
Treatment 4	7.42 ± 0.63b	0.56 ± 0.13b	74.62 ± 1.13b
Treatment 5	8.54 ± 1.21b	0.53 ± 0.12b	74.65 ± 0.89b
Treatment 6	9.40 ± 0.72b	0.61 ± 0.22b	75.17 ± 1.15b

Means followed by the same letter in a specific column of each material (Sugarcane straw and Coffee husk) do not differ at p<0.05 by Tukey's test.

4.3.1.3. Extraction yield and arabinoxylan extraction efficiency

Treatment conditions influence extraction yield and efficiency. Milder extraction conditions can afford comparable results to severe conditions with reduced formation of enzyme inhibitors [19]. Extraction yield and arabinoxylan extraction efficiency were evaluated for all treatments. Results are presented in Table 4.4.

The extraction yield obtained from both holocellulose materials using treatment 3 did not differ from that obtained using the Zilliox and Debeire [25] method conditions (Treatment 1). However the highest arabinoxylan extraction efficiency was obtained with treatment 2 for both holocellulose materials (Table 3.4). The use of a longer extraction time (6 vs. 3 h) and a higher ethanol proportion (63 vs. 60%) improved extraction efficiency by 5 percentage points in comparison to the treatment 1. The short pre-incubation time used in treatment 2 (10 h) contributed to reducing the total reaction time by 3 h, which is especially interesting in an industrial setting. Although the two holocellulose sources have present a similar tendency under alkaline treatments, the arabinoxylan present in the SS holocellulose was more easily precipitate than arabinoxylan present in the CH holocellulose, achieving thus higher arabinoxylan extraction efficiency (Ex) results (Table 4.4).

Table 4.4. Extraction yield (Y) and arabinoxylan extraction efficiency (E) of sugarcane straw and coffee husk isolated holocellulose.

Treatments	Sugarcane straw		Coffee husk	
	***Y* (%)**	***E* (%)**	***Y* (%)**	***E* (%)**
Treatment 1	39.78 ± 1.56ab	77.53 ± 1.65b	30.78 ± 0.83ab	64.84 ± 0.82b
Treatment 2	40.35 ± 1.85a	82.32 ± 1.76a	31.76 ± 1.02a	69.35 ± 0.68a
Treatment 3	39.35 ± 1.96ab	76.19 ± 1.75c	27.64 ± 0.87c	57.86 ± 0.62e
Treatment 4	38.15 ± 1.1b	73.35 ± 1.62d	28.99 ± 0.89c	60.13 ± 0.46d
Treatment 5	32.80 ± 1.87c	62.14 ± 1.62e	30.34 ± 0.78b	62.96 ± 0.57c
Treatment 6	28.80 ± 1.56d	55.28 ± 1.71f	24.18 ± 0.82d	50.53 ± 0.67f

Means followed by the same letter in a column do not differ at $p < 0.05$ by Tukey's test.

Solids loading was increased to 15% in treatment 6, but this did not result in improved extraction yield or efficiency. Extracts obtained with treatment 6 had high viscosity (data not shown), which probably hampered the catalytic activity of KOH [19, 39]. The absence of pre-incubation step could also has contributed for this result, although in previous works de Figueiredo et al.[19] had reported satisfactory yield values using a lower solid loading (8%) without a prior incubation step. As a result, treatment 6 afforded the lowest extraction yields

and arabinoxylan extraction efficiencies (Table 4.4). High amounts of salt residues were obtained after precipitation in treatment 6; consequently, large amounts of ethanol were used during the washing step. de Figueiredo et al. [19] experienced similar difficulties in the hydrolysis of sugarcane bagasse. In treatments 5 and 6, the amount of KOH used per gram of extracted xylan was 80% lower than that used in the treatment 1. de Figueiredo et al. [19] reduced all chemicals used for enzymatic hydrolysis of sugarcane bagasse by 75% compared to the method of Zilliox and Debeire [25] without affecting the results. However, the authors did not perform holocellulose extraction prior to hydrolysis. de Figueiredo et al. [19] were able to reduce the reaction time by 27 times by excluding the pre-incubation step and washing products only 4 times. The reaction time of treatments 3 and 4 was 15 min shorter than that of the reference method, as 4 washings were carried out instead of 5, but temperatures were higher (70 and 80 °C, respectively), increasing the amount of electric energy used in the process.

In previous work our research group performed the arabinoxylan extraction from sugarcane straw biomass using Zilliox and Debeire method [25] without prior delignification procedure. Martins et al. in press [40] reported arabinoxylan efficiency around 70%, which were significantly lower those obtained from holocellulose fraction in this work (82.32 ± 1.76) (Table 3.4).The extraction yield and arabinoxylan extraction efficiency were also higher than those reported by Carvalho et al. [18] and de Figueiredo et al. [19] for sugarcane bagasse. This demonstrates that the obtaining of holocellulose process prior to extraction results in a substantial improvement in arabinoxylan yield. As shown in Figure. 1 the untreated material was delignified generation the holocellulose that corresponds to the α-cellulose + hemicellulose fraction of lignocellulosic materials [26]. Then the holocellulose material was submitted to alkaline treatments providing a solid cellulose fraction and arabinoxylan-rich liquid fraction. Fractions can be used to obtain not only prebiotic oligosaccharides but also fermentable sugars for biofuel and xylitol production. Agroindustrial wastes are a low-cost source of cellulose and hemicellulose that may reduce the production costs of important biotechnological products.

The findings show that treatments 2 gave the best results for both substrates, namely higher arabinoxylan concentration (Table 4.3). Treatment 2 was carried out under moderate conditions, resulting in higher extraction yield and efficiency; therefore, it was selected to extract xylan for subsequent optimization experiments.

4.3.2. Enzymatic hydrolysis

4.3.2.1. Optimization of enzyme mixtures

The conversion of xylan from lignocellulosic biomass to liquid fuels and other chemicals can be achieved using multienzymatic systems. In this step, we assessed the combinatorial effects of enzymes on XOS production using CCRD. Three independent variables were analyzed: endoxylanase, α-L-arabinofuranosidase, and feruloyl esterase concentrations. The response variable was XOS concentration.

CCRD results showed that the highest XOS release from arabinoxylan from sugarcane straw (SSX) (10.28 g L^{-1}) was achieved in run 8 (Table 4.5). XOS concentration was similar to that obtained in run 10 (10.25 g L^{-1}), which α-L-arabinofuranosidase and feruloyl esterase were used at 2.65 and 1.63 mg g^{-1} (level 0). This result indicates that α-L-arabinofuranosidase and feruloyl esterase can be used at lower concentrations without affecting the response variable, which translates into a reduction in production costs. The highest XOS concentration from extracted arabinoxylan from coffee husk (CHX) was obtained in run 6 (8.29 g L^{-1}). The results of runs 8 and 10 for CHX show that α-L-arabinofuranosidase and feruloyl esterase concentrations can be reduced by about 17% and 60%, respectively, without causing considerable changes to XOS yield. XOS production from CHX required lower concentrations of endoxylanase than that from SSX, as substrates differed in arabinoxylan composition (Table 4.3). Center point runs (15, 16, and 17) showed similar results, indicating good reproducibility.

Table 4.5. Central composite rotatable design matrix and experimental results of xylooligosaccharides (XOS) production from sugarcane straw arabinoxylan (SSX) and coffee husk arabinoxylan (CHX) using enzyme mixture.

Run	Enzymes (mg protein. g-1 substrate)			SSX		CHX	
	GH11	GH51	CE1	XOS (g L^{-1})	XOS yield (%)	XOs (g L^{-1})	XOS yield (%)
1	2.0 (−1)	1.50 (−1)	0.75 (−1)	3.86	19.30	2.86	14.30
2	4.80 (+1)	1.50 (−1)	0.75 (−1)	7.05	35.25	8.05	40.25
3	2.0 (−1)	3.80 (+1)	0.75 (−1)	5.66	28.30	2.81	14.05
4	5.50 (+1)	3.80 (+1)	0.75 (−1)	9.19	45.95	8.19	40.95
5	2.0 (−1)	1.50 (−1)	2.50 (+1)	4.08	20.40	2.89	14.45
6	5.50 (+1)	1.50 (−1)	2.50 (+1)	8.29	41.45	8.23	41.15
7	2.0 (−1)	3.80 (+1)	2.50 (+1)	5.44	27.20	2.87	14.35
8	5.50 (+1)	3.80 (+1)	2.50 (+1)	10.28	51.40	8.15	40.75
9	0.80 (−1.68)	2.65 (0)	1.63 (0)	2.56	12.80	1.15	5.75
10	6.68 (+1.68)	2.65 (0)	1.63 (0)	10.25	51.25	8.2	41.00
11	3.75 (0)	0.70 (−1.68)	1.63 (0)	4.71	23.55	5.71	28.55
12	3.75 (0)	4.58 (1.68)	1.63 (0)	8.87	44.35	6.81	34.05
13	3.75 (0)	2.65 (0)	0.14 (−1.68)	7.09	35.45	5.69	28.45

14	3.75 (0)	2.65 (0)	3.09 (+1.68)	8.79	43.95	6.83	34.15
15	3.75 (0)	2.65 (0)	1.63 (0)	8.85	44.25	6.85	34.25
16	3.75 (0)	2.65 (0)	1.63 (0)	8.91	44.55	6.91	34.55
17	3.75 (0)	2.65 (0)	1.63 (0)	8.81	44.05	6.81	34.05

GH11, endo-1,4-xylanase; GH51, α-l-arabinofuranosidase; CE1, feruloyl esterase.

The results corroborate those obtained by Brenelli [41], who observed synergistic effects between α-L-arabinofuranosidase and endo-1,4-xylanase on rye arabinoxylan and sugarcane bagasse. According to the authors, XOS branched with arabinose bagasse were preferentially hydrolyzed by enzymes, as observed in this study for SSX. Thus, synergism between enzymes is correlated to their mode of action on XOS branched with arabinose residues. Goldbeck et al. [15], in studying the mode of action of six hemicellulolytic enzymes on sugarcane bagasse and wheat arabinoxylan, observed a synergistic effect between endo-1,4-xylanase and feruloyl esterase on hemicellulose conversion.

Exploratory studies on XOS production are necessary because these compounds have excellent prebiotic properties and are one of the few nutraceutical products that can be obtained from cheap and abundant lignocellulosic biomass [15]. XOS have important immunomodulatory, anti-inflammatory, and antioxidant properties and have been used for the treatment of various diseases.

Compounds obtained from chemical or autohydrolysis processes generally have a high degree of polymerization, different from XOS produced by enzymatic processes. Furthermore, enzymatic hydrolysis has high specificity and minimal production of undesirable by-products [42]. Surek and Buyukkileci [43] reported XOS yield around 62% by autohydrolisis of halzenut husk obtained at 190 °C and 30 min of holding time. However xylose, acetic acid and furfural concentrations increased considerably with treatment severity. In contrast Parajó et al. [11] reported XOS yields relatively lower provided by autohydrolysis process using different lignocellulosic materials such as barley husks (27.1%) and corncobs (24.8%), rice husks (18.0%) and Eucalyptus wood (15.4%).

Previous studies have successfully produced XOS by enzymatic hydrolysis of xylan extracted from lignocellulosic biomass. Brienzo et al. [44] obtained XOS concentrations of 0.43–6.4 mg mL^{-1} and maximum conversion of 37% using pretreated sugarcane bagasse using substrate concentration range of 0.5–3.5% (w/v), and Samanta et al. [45] produced 2.8 mg mL^{-1} XOS and XOS yield of 11% using *Sehima nervosum* grass employing the same substrate loading of this work. These results are considerably lower than those achieved in the current

study, suggesting that arabinoxylan extraction from holocellulose allied to the employment of ideal enzymes mixture can improve XOS yield.

Regression models were generated on the basis of CCRD results, and coefficients were evaluated at $p < 0.1$ [46]. Second-order models for XOS production by enzymatic hydrolysis of SSX and CHX are shown in the falowing equations:

$$XOS_{(SSX)\,(g.L-1)} = 8.87 + 2.10x_1 - 0.91x_1^2 + 1.04x_2 - 0.78x_2^2 + 0.38x_3 - 0.37x_3^2 + 0.29x1x_3$$

$$XOS_{(CHX)\,(g.L-1)}) = 6.87 + 2.42x_1 - 0.81x_1^2 - 0.25x_2^2 - 0.25x_3^2$$

where x_1, x_2, and x_3 correspond to coded levels of endoxylanase, α-L-arabinofuranosidase, and feruloyl esterase concentrations, respectively.

Regression models were subjected to analysis of variance (Supplementray material, Tables S4.1 and S4.2). For both substrates, the F value was much higher than the F critical value (approximately 39 times higher for SSX and 45 times higher for CHX). The high coefficient of determination ($R^2 = 97\%$) indicated a good fit of models to experimental data. In addition it is important to note that the lack of fit parameter was not significant in the level of significance studied ($p < 0.1$) for XOS from SSX, since the calculated F critical value for lack of fit were lower than the F value. However it was not observed to XOS from CHX, according to Rodrigues and Iemma [46] it occurs when the lack of adjustment is low, but the pure error tends to zero, indicating therefore a good reproducibility of experiments.

Figure 4.2 shows the contour curve plots of XOS production by enzymatic hydrolysis of SSX and CHX. Experimental design is a valuable tool for optimizing enzyme mixtures for biomass hydrolysis [46]. Contour curves plot generated from CCRD data predicted that XOS release from SSX is highest (10.00 g L^{-1}) when the following enzyme protein concentrations are used (per gram of substrate): 0.63 mg of feruloyl esterase, 5 mg of endo-1,4-xylanase, and 2.70 mg of α-L-arabinofuranosidase (Fig 2 IA and IB). Optimum XOS release from CHX (8.00 g L^{-1}) requires the use of 0.63 mg of feruloyl esterase, 6 mg of endo-1,4-xylanase, and 0.80 mg of α-L-arabinofuranosidase (Figure 4.2 IIA and IIB).

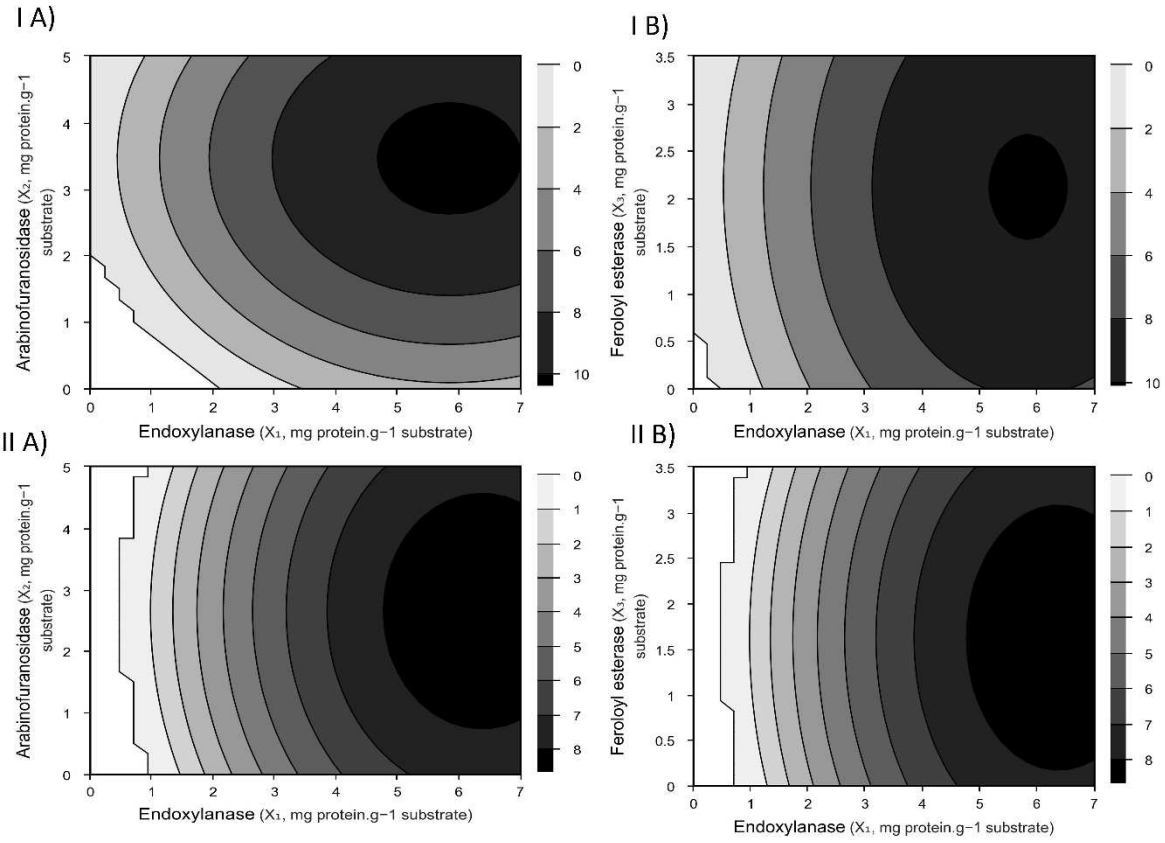

Figure 4.2. Contour curves plots of central composite rotatable design results illustrating the optimum concentrations of endo-1,4-xylanase and α-L-arabinofuranosidase (A) and endo-1,4-xylanase and feruloyl esterase (B) for the release of XOS from sugarcane straw arabinoxylan (I) and coffee husk arabinoxylan (II).

The models were validated experimentally in triplicate. Validation runs afforded XOS concentrations of 10.25 and 8.45 g L^{-1}, XOS yields of 51.25 % and 42.25 %, and production rates of 4.27 and 3.52 mg g^{-1} h^{-1} for SSX and CHX, respectively. Predicted results were similar to experimental results, demonstrating that optimization was successful. In addition base on monosaccharides released the XOS rich hydrolysate purity was estimate around 94% and 95% for SS and CH biomass, respectively, which could also implies in a possible simplification and cost reduction in the purification stage.

In order to validate the synergistic effect of the enzymes on XOS production, experiments were carried out with the enzymes in isolated way and the possible enzyme combinations in concentrations optimized in the experimental design. According to Figure 3.3, combining GH11 and CE1 resulted in a release of X2 as major product from both arabinoxylan substrates. As well as the substitution of CE1 for GH51 increased arabinose (Ara) and

xylotriose (X3) concentration products. The present increase on arabinose concentration is probably by addition the of GH51, which act on arabinoxylan chain removing arabinosyl substituents consequently contributing to XOS of linear chain releasing [18].

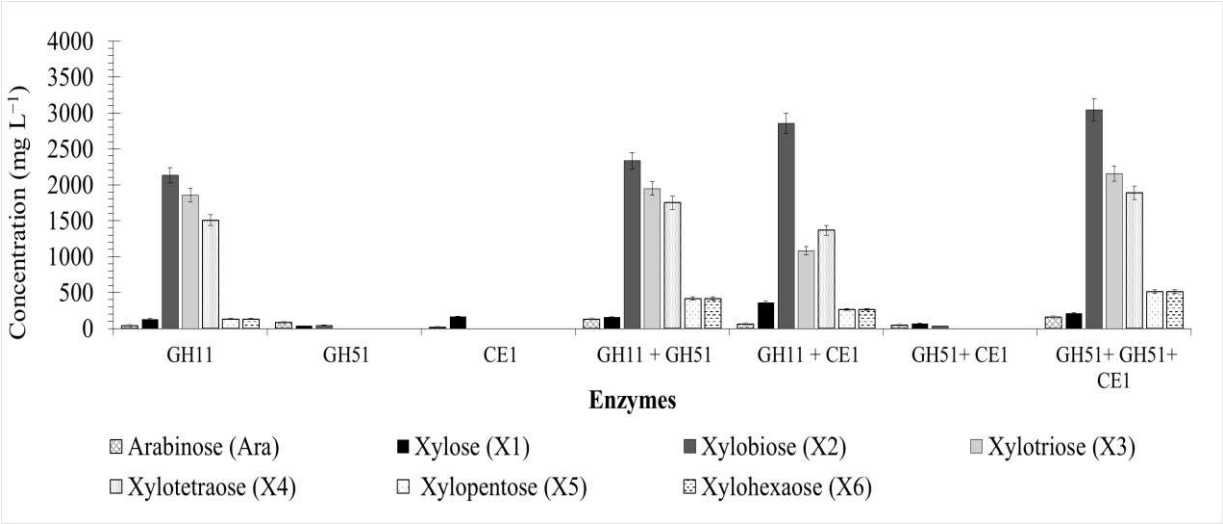

Figure 4.3. HPLC–PAD analysis of arabinose, xylose and XOS profile released from sugaracane straw arabinoxylan (A) and coffee husk abainoxylan (B) after 48 h of enzymtic hydrolisis with enzymes in isolated way and possible combinations enzymes in ideal concentrations optimized in experimental design. GH11 correspond to endo-1,4-xylanase from *Aspergillus niger*. GH51, α-L-arabinofuranosidase from *Aspergillus niger* and CE1, feruloyl esterase from *Clostridium thermocellum*.

It is important to observe that when the combination (GH51+GH11+CE1) was employed a considerable increase in the total XOS production was observed (Figure 4.3). These results are in accordance with the pareto chart (Figure 4.4), which shows that the three enzymes have presented significant effect on X2 and X3 releasing (p <0.1), which were the major products of hydrolysis, demonstrating thus a potential synergistic effect to produce X2 and X3. In addition these XOS polymerization degree (DP) are the greatest interesting to food and pharmaceutical industry due to reports of their prebiotic potential [47]. Although GH51 and CE1 have present significant effect on the enzyme mixture formulation (Figure 3.4), when they were employed in isolated way they have not presented capacity of XOS releasing (Figure 4.3).

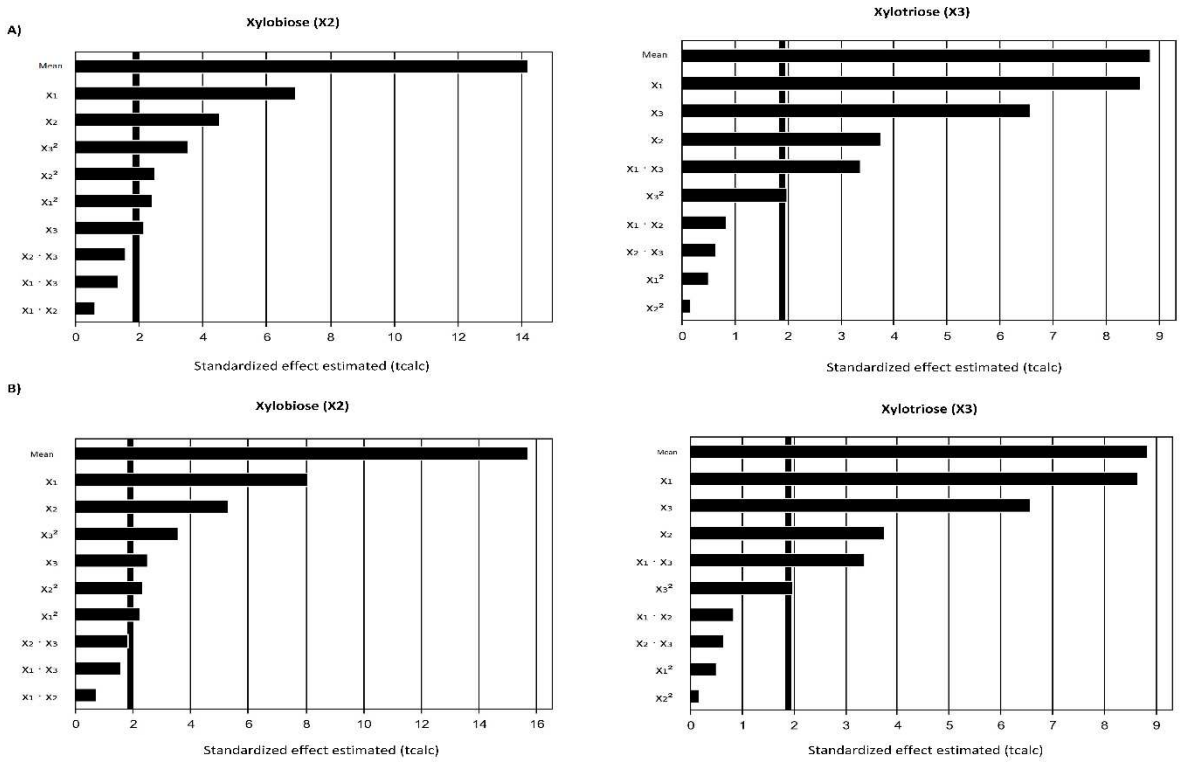

Figure 4.4. Pareto chart of standardized effects ($p < 0.1$) on Xylobiose (X2) and Xylotriose (X3) production from sugarcane straw arabinoxylan (SSX) (A) and coffee husk arabinoxylan (CHX) (B). Where x1=endo-1,4-xylanase (GH11), x2=α-L-arabinofuranosidase (GH51) and x3= feroloyl esterase (CE1).

As previously explained, the enzyme protein concentration in the combinatory assays were confirmed to be suitable for these experimental purposes. Thus this comprehensive study demonstrate that the mode of operation ratio of enzymes in cocktails can lead to positive results for rational designing of enzymatic mixtures with better efficiencies which are also cost effective in the process.

4.3.2.2. Kinetics of XOS release

Hydrolysis assays of 12, 24, and 48 h were performed using the optimized enzyme mixture for each xylan source to assess XOS profile over time. A previous study using xylanases from *A. niger* to produce XOS from different agricultural wastes showed that the degree of polymerization of XOS depends not only on the enzyme but also on the time of reaction [47].

Figure 4.5 shows that the enzyme mixtures released mainly short-chain XOS (xylobiose, xylotriose, and xylotetraose) and smaller concentrations of xylose, xylopentaose, and xylohexaose from both substrates.

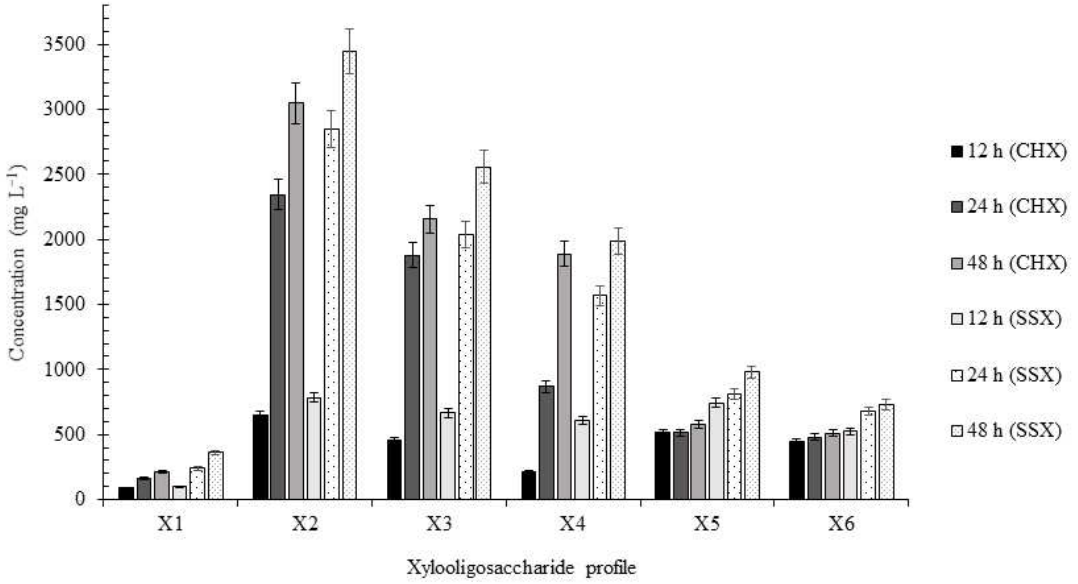

Figure 4.5. Xylooligosaccharide profile obtained after 12, 24, and 48 h of enzymatic hydrolysis of sugarcane straw arabinoxylan (SSX) and coffee husk arabinoxylan (CHX). X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose.

A similar carbohydrate profile was observed by Manisseri and Gudipati [48] in the hydrolysis of wheat bran by purified xylanases and by Goldbeck et al. [15, 16] in the hydrolysis of sugarcane bagasse by purified hemicelluloses. XOS with a low degree of polymerization (2 to 4) are preferred for food-related applications [9], as they are more easily metabolized by probiotic bacteria and have increased prebiotic activity [9, 49]. Experiments in humans showed that XOS intake has a beneficial effect on intestinal flora and that xylobiose is not excreted in feces or urine up to 24 h after ingestion. XOS are not hydrolyzed by saliva, pancreatin, or gastric juice, suggesting that these xylose oligomers are used by intestinal bacteria [50].

The concentration of xylopentaose and xylohexaose remained practically constant over 48 h of hydrolysis of CHX. When SSX was used, xylopentaose and xylohexaose concentrations increased linearly with time. The concentration of short-chain XOS peaked after 48 h of hydrolysis for both substrates. Most xylanases are sterically hindered by xylan

substituents and therefore act preferentially on unsubstituted xylan molecules, whereas some endoxylanases act preferentially on branched xylans [6].

Both arabinoxylan sources (SSX and CHX) generated linear XOS with similar DP profile after determined enzymatic hydrolysis time (Figure 3.5). The oligosaccharides may find application as functional food ingredients or soluble substrates for xylanase or enzyme mixtures. We highlight that the holocellulose extraction step was crucial to obtain high-purity arabinoxylan extracts (and, subsequently, XOS) from agroindustrial wastes.

4.4. Conclusion

The holocellulose extraction of SS and CH biomass prior to alkaline treatment with moderate conditions (Treatment 2) provided high arabinoxylan extraction yielded a high-purity product with negligible concentrations of lignin and good susceptibility to enzymes action for XOS production. In addition, the recovered solid after alkaline treatment is rich in cellulose, with possible process integration for use of this polysaccharide. The enzymes combination (G11 + GH51 + CE1) presented a synergistic effect in both arabinoxylan sources providing high XOS yields with similar XOS degree polymerization profiles and low concentration of monosaccharides after 48 h of hydrolysis, which could also implies in a possible simplification and cost reduction in the purification stage for their use in food and pharmaceutical relation applications.

Conflict of interest

The authors state that they have no conflict of interest.

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Capítulo 5

Xylooligosaccharides production by commercial enzyme mixture from agricultural wastes and their prebiotic and antioxidant potential

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Xylooligosaccharides production by commercial enzyme mixture from agricultural wastes and their prebiotic and antioxidant potential

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Highlights

Endoxylanase + α-LArabinofuranosidase + Feroloyl esterase mixture provides high XOS release.
Agricultural wastes provide XOS with high prebiotic potential.
XOS show considerable antioxidant potential.
Great potential to be use in food industry as functional ingredients.

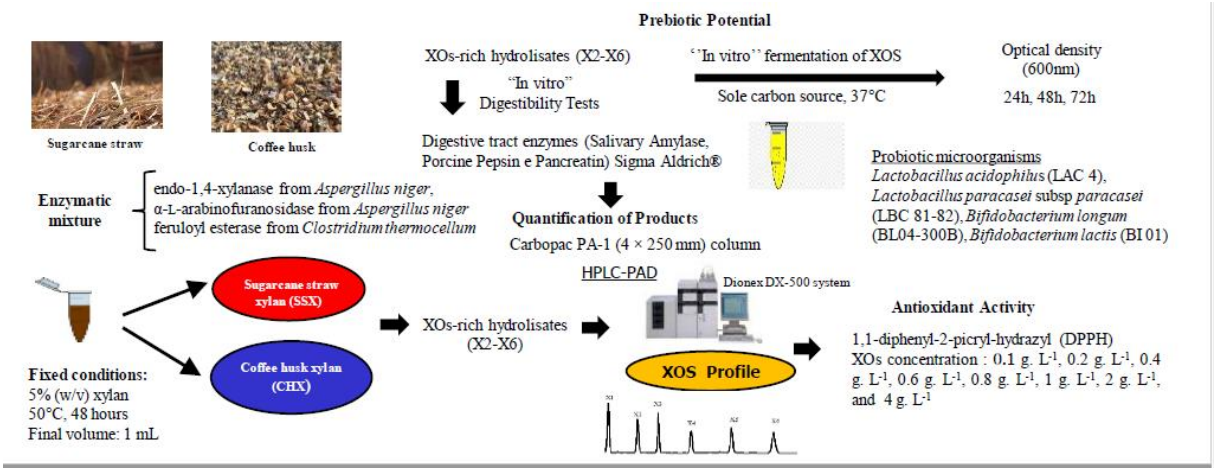
Abstract

Advancement in industrial biotechnology offers potential opportunities for economic utilization of agro industrial biomass for the production of value-added products. Xylooligosaccharides (XOS) are non-digestible food ingredients with prebiotic properties for selectively promoting the growth of probiotics providing many health benefits and several applications on food and pharmaceutical industry. The present study deal with enzymatic production of XOS from xylan extracted from different agroindustrial wastes, namely sugarcane straw (SS) and coffee husk (CH) using an optimized enzymatic mixture with endo-xylanase (GH11), α-L-arabinofuranosidase (GH51) and Feruloyl Esterase (CE1). The XOS profile concentration was quantified by HPAEC-PAD using standard (X2-X6) from Megazyme®. The efficient enzymatic mixture achieved a high total XOS concentration using SS xylan (10.23 ± 0.56 g/L) and CH xylan (8.45± 0.65 g/L). Three of four probiotic cultures of *Lactobacillus* and *Bifidobacterium* tested were able to utilize XOS produced from agricultural wastes and showed remarkable growth in the media containing XOS, consuming preferentially the X2 and X3 fractions as the sole source of carbon. The XOS produced also exhibited a considerable

resistance to hydrolysis of digestive enzymes, as well as an concentration dependent anti-oxidant activity achieving until 78 % in a XOS concentration of 2 g. L^{-1}. Thus, the results showed that XOS produced from these agricultural residues have great prebiotic potential and good antioxidant activity; therefore, it can be used in food-related applications as functional ingredients.

Key-words: xylooligosaccharides, prebiotic, coffee husk, sugarcane straw

Graphical abstract



5.1. Introduction

Active research on converting agricultural by-products into value-added products has been purposed to produce different biomolecules of great economic value; prebiotic xylooligosaccharides (XOS) is one of them (Gullon et al., 2010). The depolymerized products of xylan such as xylose and XOS have potential applications in food, pharmaceuticals, feed formulations, and agricultural purposes. XOS is a non-digestible food ingredient, made up of D-xylose units linked by β-1, 4 glycosidic bonds, has a lower degree of polymerization (DP 2–10) (Jaypal et al., 2013).

XOS production by autohydrolysis or hydrothermal processing of lignocellulosic biomass results in hydrolysates containing a large variety of undesirable components, such as xylose and lignin, demanding a complex purification step (Zhu et al., 2006). Thus enzymatic hydrolysis is generally preferred by food industries due to it does not produce undesirable by-products neither requires special equipment operating at high-temperature and high-pressure conditions, in contrast to autohydrolysis (Akpinar et al., 2009). XOS can be produced by

endoxylanases, which hydrolyze the β-1,4-glycosidic linkages in xylan. Actually knowledge about the xylanolytic activity, optimum pH and temperature, thermal stability, and kinetic parameters of a commercial xylanase preparation is pivotal to achieve relevant XOS yield as well as feasible large-scale production. Comprehensive studies of the mode of operation ratio of enzymes in cocktails can lead to positive results for rational designing of enzymatic mixtures with better efficiencies which are also cost effective promoting symergistic effect on hemicelullose conversion (Ávila et al., 2020, Goldbeck et al., 2014).Furthermore it is known that complex substrates provide from lignocellulose materials contain large amounts of arabinoxylan and lignin which are not easily degraded by endo-xylanases without prior or simultaneous treatment with arabinofuranosidases and feruloyl esterases (Ávila et al., 2020).

XOS are used in food industries and are incorporated into many food products with different applications such as sweeteners, stabilizers, emulsificants and functional ingredients (Gullon et al. 2010). One of the most important applications of XOS is as functional foods, since they are considered prebiotic compounds, due to capacity to promote beneficial health effects for their selective metabolism by bifidobacterias and lactobacillus, increasing the production of volatile fatty acids, and anti-gastric ulcer activity (Vázquez et al., 2000). These agents are considerable prebiotic when presented a resistence a digesbility enzymes, since the fiber should being able to act in the intestine as nutritional ingredients that beneficially affect the host, selectively stimulating the growth and activity of one or more beneficial intestinal bacteria, improving host health (Singh et al.,2015).Considering the tremendous health benefits that XOS offer, research on it though has relevance; it is a challenge to make it available at economic price to large number of consumers particularly in developing countries. For this reason every step in product preparation that contributes to the total price needs to be thoughtfully evaluated (Parajó et al., 2004). Furthermore “In vitro” tests are fundamental to determine the efficiency and prebiotic potential of oligosaccharides, making it easier to understand the next research steps, such as “in vivo” tests (de Figueiredo et al., 2020).

In general terms, it is believed that the regular consumption of prebiotics protects against the development of the so called western diseases, including diabetes, cardiovascular disease, colon cancer,obesity, abnormal lipid metabolism and chronic inflammatory diseases (Vázquez et al., 2000). Oxidative stress plays an important role in the worsening of these pathological conditions. For this reason, at least in theory, the capability of XOS to act as antioxidants could have beneficial effects in maintaining good health (Ashwine et al., 2019). The antioxidant activity of XOS is generally attributed to the presence of ester-linked

hydroxycinnamic acid derivatives, such as ferulic acid, coumaric caffeic, syringic acid residues and methyl glucuronic acid ramifications on the xylan chain (Vieira et al., 2020). Rao & Muralikrishna (2006) showed that the presence of sugars with uronyl or acetyl groups impact strong antioxidant activity to cereal oligosaccharides. It has been also reported that carboxyl groups increase antioxidant activity of cell wall polysaccharides (Pristov, Mitrovic, & Spasojevic, 2011). In a similar way, Malunga & Beta. (2015) reported that the antioxidant capacity of XOS obtained from arabinoxylan is correlated to their degree of ferulic acid substitution.

The purpose of this work was to produce XOS by enzymatic hydrolysis of different xylan sources (sugarcane straw and coffee husk) using commercial enzyme mixtures as well as to study its ability to stimulate the growth of some recognized probiotic microbiota (microorganisms *Lactobacillus acidophilu*s LAC 4, *Lactobacillus paracasei* LBC 81-82, *Bifidobacterium longum* BL04-300B, *Bifidobacterium lactis* B101). Additionally some digestibility tests of XOS produced were performed using digestive enzymes involved in human metabolism, as well as the antioxidant potential in order to prove its stability during human metabolism to be used in food-related applications as functional ingredients.

5. 2. Materials and Methods

5.2.1. Raw materials

Sugarcane straw was kindly supplied by the Brazilian Bioethanol Science and Technology Laboratory (CTBE/CNPEM), Campinas, SP, Brazil and the coffee husk was donated by COXUPÉ LTDA, Guaxupé, MG, Brazil. Sugarcane straw (SS) and coffee husk (*Coffea arabica*) (CH) contained 75% and 57% moisture (wet base), respectively. Both biomass were washed with water at room temperature to remove excess soil and dust (Ávila, Forte & Goldbeck, 2018). Samples were oven-dried at 80°C to at least 10% moisture (dry base), ground in a cutting mill, and sieved to retain particles in the range of 0.3-0.7 mm of diameter (Ávila et al., 2020).

5.2.2. Chemical Composition determination

Cellulose, hemicellulose and lignin content in SS and CH were determined according to Sluiter et al. (2008). Representative samples of 300 mg were hydrolyzed in two steps: 72% H_2SO_4 for 60 min at 30°C followed by dilution to 4% H_2SO_4 for an additional 60 min at 121°C. The samples were then quenched in ice and filtered. The cellulose and hemicellulose contents of the filtrates were determined by high-performance anion exchange-pulsed amper-ometric

detection chromatography (HPLC–PAD) with a Dionex DX-500 (Sunnyvale, CA, EUA) system using a CarboPacPA 1 column (4 mm × 250 mm). The monosaccharide contents found in the hydrolysates were converted to percentage of polysaccharides. The ash content was determined by burning 1 g of the sample in a tarred crucible for 6 h using a muffle furnace at 600°C until reaching constant weight, according to the NREL/TP-510-42622 procedure (Sluiter et al., 2005a). The extractives content were mensured using Soxhlet extractor with ciclohexane + ethanol solution (1:1) according to the NREL/TP-510-42619 procedure (Sluiter et al., 2005b). The composition analysis results of both substrates were obtained from the mean of three independent replicates.

5.2.3. Arabinoxylan Extraction

The materials were pre-incubated at 70°C for 16 h in 400 mL of deionized water and after reward drained. Then an additional step was performed to extract the holocellulose by treatment of the biomass with sodium chlorite under acidic conditions, according to Wise et al (1946) procedure. This step was performed in order to delignify the samples and increase the efficiency of the enzymatic hydrolysis process of the extrated xylans. After this process, 250 mL of a 24%(m/v) KOH and 1% (m/v) $NaBH_2$ solution was added for reaction with 8% (m/v) holocellulose biomass (sugarcane straw or coffee husk) during 3 h at 35°C. The liquid fraction was filtered with gauze until no solids were observed in the liquid phase. The arabinoxylan in the liquid fraction was precipitated by the mixture of 60% (v/v) ethanol, 6.7% (v/v) acetic acid and 33.3% (v/v) arabinoxylan solution. The precipitated material (arabinoxylan) was centrifuged at 4000g for 15 min. This material was washed 5 times with 1 volume of ethanol solution (50% ethanol, 50% deionized water and 0.5% EDTA): 1 volume arabinoxylan, before being dried at 60°C (Zilliox & Debeire, 1998; Akpinar et al., 2009, Figueiredo et al., 2017).

5.2.4. Commercial enzymes and determination of enzyme concentration

The enzymes used in the experimental design were endo-xylanase- *Aspergillus ninger* (GH11), α-L-arabinofuranosidase - *Aspergillus ninger* (GH51) and feruloyl esterase - *Clostridium thermocellum* (CE1), all required from Megazyme®. The enzyme concentration was determined according to the method proposed by Lorry (1951).

5.2.5. Enzyme activity measurements

The α-L-arabinofuranosidase activity was determined according to the method conducted by da Silva et al. (2010), using 100 μL of 10 mM ρ-nitrophenyl-a-l-arabinofuranoside, 200 μL of 0.5 M sodium acetate buffer pH 5.0, 650 μL of Milli-Q water,

and 50 μL of the appropriately diluted enzyme solution. After incubating the reaction mixture at 45°C for 10 min, the reaction was stopped by adding 500 μL of 1 M Na_2CO_3. The concentration of liberated ρ-nitrophenol was measured at 420 nm. One unit of α-L-arabinofuranosidase was defined as the amount of enzyme that released 1 μmol of ρ-nitrophenol at 45°C in 1 min. Endo-1,4-xylanase activity was assayed against 500 μL of 2% soluble xylan from Birchwood (Sigma–Aldrich) and 0.1 mL of appropriately diluted supernatant, which were mixed and reacted at 45°C for 30 min in 50 mM of sodium acetate buffer pH 5.0, in a total volume of 1 mL (da Silva et al., 2011) The resulting reducing sugars were analyzed using a dinitrosalicylic acid (DNS) assay (Miller, 1959). One unit of xylanase was defined as the amount of enzyme required to produce 1 μmol of reducing sugars in 1 min at 50°C. The feruloyl esterase activity was per-formed using the substrate naphthylacetate according to the method previously described by Koseki et al. (2007). One enzyme unit was defined as the amount of enzyme that released 1 μmol α- naphthol per minute under the assay conditions.

5.2.6. Enzymatic hydrolysis

The present study employed optimized enzymatic mixtures of three purified enzymes: endo-xylanase (GH11), α-L-arabinofuranosidase (GH51) and feruloyl esterase (CE1), all required from Megazyme proposed in a previous study for each substrate studied: 0.63 mg.$(g_{substrate})^{-1}$ CE1 for both extracted xylans, 5 and 6 (mg).$(g_{substrate})^{-1}$ of GH11, and 2.70 and 0.80 (mg).$(g_{substrate})^{-1}$ of GH51 for sugarcane straw xylan and coffee husk xylan substrate. Enzymatic micro-assays were carried out in 1.5 mL Eppendorf tubes using a 2–20 μL micropipette (Pipetman G P20G, Gilson). The reaction mixtures contained 50 mg of substrate, a combination of commercial enzymes as described by Goldbeck et al. (2014, 2016) and sodium phosphate buffer (0.1 M, pH 5.0) to complete the final volume of 1 mL. Samples were incubated at 50°C for 48 h under agitation (1000 rpm). After this period, the enzymes were inactivated at 99°C for 15 min and samples were centrifuged (12.000*g* for 15 min at 4°C), and the supernatant was collected for subsequent analysis.

5.2.7 *In vitro* digestibility tests

The *in vitro* resistance of XOS produced by enzymatic hydrolisis of sugarcane straw xylan (SSX) and coffee husk xylan (CHX) to the three simulated digestive fluids: simulated salivary fluid (SSF), simultaed gastric fluid (SGF) and simulated intestinal fluid (SIF). The tests were performed in an Dry Bath (Model: DBH-S, Loccus Biotecnologia, Brazil) at 500 rpm, maintained at 37 ± 1 °C, acording with the protocol of Minekus et al. (2014). Initially, the

XOS- rich hydrolysates were added to SSF of 50 : 50 (w/v), where salivary amylase was added to achieve 75 U/mL, followed by $CaCl_2$ to achieve 0.75 mM in the final mixture. 5 mL of oral fase (sample +SSF) was incubated for 2 h with 5 mL of SGF, the pH was adjusted to 3 with hydrochloric acid and monitored. SGF stock solution was prepared by the addition of 0.3 mol/L sodium chloride and pepsin 2000 U/mL in distilled water. Then 5 mL of gastric phase (sample + SGF) were mixed with 5 mL of SIF, the pH was adjusted to 7 with sodium hydroxide and monitored. The samples were incubated at 37 °C for 2 h under continuous stirring. SIF was prepared with SIF stock solution with addition of 0.3 mol/L sodium chloride, 800 U/mL pancreatin and 10 mM bile salt, suspended in distilled water. The XOS profile concentration was mensured before and after each step of digesbility.

5.2.8 *In vitro* fermentation of XOS

5.2.8.1. Microorganisms

The freeze-dryed cultures of probiotic pattern microorganisms *Lactobacillus acidophilus* (LAC 4), *Lactobacillus paracasei* subsp *paracasei* (LBC 81-82), *Bifidobacterium longum* (BL04-300B), *Bifidobacterium lactis* (BI 01) packed in envelopes sealed, were kindly provided by Danisco®, Brazil.

5.2.8.2. Growth Medium

The prebiotic activity of XOS mixture was studied by growing probiotic bacterias using the method described elsewhere with necessary adaptations in media supplemented with XOS mixture as the carbon source (Jagtap et al., 2017). The XOS fermentation experiment was carried out in three different media without any carbon source (negative control), glucose as carbon source (Positive control) and XOS mixture as carbon source (test). The growth media (basal media) was prepared by adding Peptone (10 g/L), MnSO4 (100 mg/L), MgSO4 (50 mg/L), NH4 Cl (2gm/L), KH2PO4 (2 g/L), Tween80 (1 mL/L). Bifidobacterium cultures were grown in modified medium with the addition of L-cysteine. Filter sterilized 0.35 gm% XOS and glucose were added as carbon sources for test and positive controls respectively in the basal medium. However, the control media did not contain any carbon source. The medium was prepared at pH 6.7 and autoclaved for 20 min at 121 °C.

The active inoculum of probiotic culture to be tested was inoculated each in negative control, test and positive control and incubated at 37°C for 24, 48 and 72 h. The grown of probiotic cultures in XOS was monitored by considering different parameters such as optical density at 600 nm, decline in pH and XOS profile consumption. The aliquot was centrifuged at

12,000 rpm for 5 min and the supernatant was collected to analyze the residual XOS profile. The increase in optical density and decline pH parameters were employed to demonstrate indirectly the cell growth and a suggest increase of fermentation activity of probiotic bacteria respectively. Since the pH drop is a striking characteristic behavior of the fermentation activity of probiotic bacteria due to the short chain fatty acids production (Singh et al., 2015).

5.2.9 Determinant of antioxidant potential

5.2.9.1.Total antioxidant capacity

Spectrophotometric evaluation of total antioxidant capacity (TAC) through the formation of a phosphomolybdenum complex was carried out according to Prieto et al. (1999). An aliquot of 1000 μL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was mixed with 100 μL of the xylooligosaccharide hydrolysate prepared in the cocentration gradient 0.05 g. L^{-1} to 1 g. L^{-1} . The reaction mixtures were kept in the dark for 90 min at 90 °C. The samples were cooled to room temperature and the absorbance was measured at 695 nm. A control reaction was prepared with 1000 μL of the reagent solution and 100 μL distilled water. The results were expressed as the absorbance of the samples.

5.2.9.1.DPPH radical-scavenging assay

Antioxidant activity of XOS was determined according to the procedure described by Bian et al. (2013). briefly, an aliquot of sample (1 mL), prepared in the concentration gradient of 0.1 g.mL-^{1}, 0.2 g.L^{-1}, 0.4 g.L^{-1}, 0.6 g.L^{-1}, 0.8 g.L^{-1}, 1 g.L^{-1}, 2 g.L^{-1}, and 4 g.L^{-1} thereafter was added to 10μL of a solution of freshly prepared 1,1-diphenyl-2-picryl-hydrazyl (DPPH 0.1 mM) in absolute ethanol. The mixture was incubated in dark up to 30 min at room temperature; absorbance (A_{sample}) was checked against a suitable blank at 517 nm. The control ($A_{control}$) was carried out with water instead of the sample solution (1:1 dilution of DPPH), while ethanol was used as the blank. Antioxidant activity of the sample (AO) was expressed as the percentage disappearance of DPPH, according to the following equation:

AO activity % = $(A_{control} - A_{sample})/A_{control.}$

5.2.10. Monosaccharaides and XOS quantification

Monosaccharaides (glucose and xylose) and XOS quantification was performed by high performance anion exchange (HPLC–PAD) using the supernatants that resulted from the enzymatic hydrolysis of the sugarcane mixtures. Chromatographic analysis was performed on

a Dionex DX-500 (Sunnyvale, CA, EUA) with a CarboPac PA1 column (4 mm × 250 mm), a CarboPac PA1 guard column (4 mm × 50 mm), and an electrochemical detector, adopting a linear gradient of A (NaOH 100 mM) and B(NaOH 100 mM; NaOAc 300 mM). The integrated peak areas were adjusted based on standards purchased from Megazyme® (Bray, County Wicklow, Ireland): xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

5.2.11. Statistical Analysis

Experiments were performed in triplicate, and results are presented as mean and standard deviation. Tukey's test at the 5% probability level ($p < 0.05$) was performed using Statistica® 8.0 software.

5.3. Results and Discussion

5.3.1. Arabinoxylan extraction and Chemical characterization

The method described by Zilliox & Debeire (1998) was considered reference in this work and was adapted for sugarcane straw and coffee husk for arabinoxylan extraction. Figure 5.1 shows the proximate composition of the agroindustrial wastes used in this study.

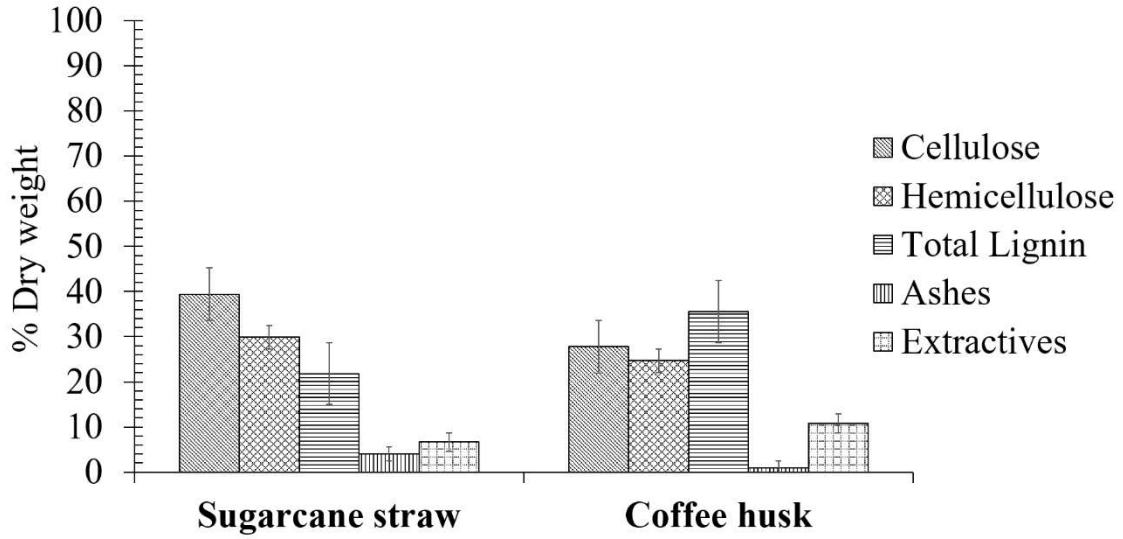


Figure 5.1. Chemical composition of agroindustrial wastes: sugarcane straw and coffee husk. Values represent mean values (from triplicate experiments); bars indicate maximum and minimum values.

Our results were in agreement with literature data showing that sugarcane straw (SS) is composed of 24–29% total hemicellulose, 19–25% total lignin, 38–50% cellulose, and 1–7%

ash (Ávila, Forte & Goldbeck, 2018; de Figueiredo et al. 2017; Gouveia et al. 2009). The proximate composition of coffee husk (CH) was similar to that reported by de Carvalho Oliveira et al. (2018). CH composition is greatly influenced by crop variety, cultivation conditions, and processing methods (Navya & Pushpa 2013), which explains the differences found between them and previous studies (Bekalo & Reinhardt 2010; Gouvea et al. 2009). The wide availability of SS and CH, added to their high hemicellulose content, make them interesting substrate materials for industrial-scale production of XOS (de Figueiredo et al. 2017).

Extracts were composed of heterogeneous polymers of pentose and hexose sugars (Table 5.1). Sugarcane straw xylan (SSX) had higher arabinose and xylose concentrations than coffee husk xylan (CHX). On the other hand, CHX had a greater content of glucan (4.19%) than SSX (2.14%) (Table 5.1). This result is in agreement with the high initial cellulose content found in CH biomass (Figure 5.1). Akpinar et al. (2009) found that xylose concentrations in tobacco stalk, cotton stalk, sunflower stalk, and wheat straw xylan varied from 85–95%, similar to those observed in this study for SSX and CHX. The concentration of xylans in SSX and CHX was higher than that reported in the literature for other substrates because xylans were extracted from holocellulose rather than from raw lignocellulosic biomass (de Figueiredo et al. 2017; Akpinar et al. 2009). Fractionation reduced the initial lignin content by 87.49 and 75.73% for SSX and CHX, respectively. This step was crucial for obtaining high-purity xylan extracts. Several studies have shown that delignification is necessary for an effective hemicellulose conversion after enzymatic hydrolysis (Yang & Wyman 2004; de Figueiredo et al. 2017).

Table 5.1 Chemical characterization of extracted Arabinoxylan from sugarcane straw (SSX) and coffee husk (CHX).

Xylan	% (Dry base)			
	Glucan	**Total Lignin**	**Arabinose groups**	**Xylose**
SSX	2.14 ±0.44a	0.45 ± 0.07a	3.04 ± 0.36a	90.67 ± 0.72a
CHX	4.19 ±0.78b	0.26 ± 0.05b	1.42 ± 0.20b	76.13 ± 0.56b

5.3.2 Enzymatic Hydrolysis

The conversion of xylan fraction from lignocelullosic biomass into liquid fuels and other chemicals can be achieved using multi-enzymatic systems. Rational designing of enzymatic mixtures with better efficiencies which are also cost effective promoting symergistic effect on hemicelullose conversion (Ávila et al., 2020, Goldbeck et al., 2014). Thus in this study

the enzymatic mixture endo-xylanase (GH11), α-L-arabinofuranosidase (GH51) and feruloyl esterase (CE1) optimized in the previous study to assessed the combinatorial effects of enzymes on XOS production using a central composite rotatable design experiments (data no shown) was employed in the present work to XOS production.

The enzymatic mixture was efficient providing satisfactory parameters values of XOS production for both substrates, such as the total XOS concentration around 10.23 and 8.45 g L^{-1} (Table 5.2), XOS yields of 205 and 169 mg g^{-1} substrate, and production rates of 4.27 and 3.52 mg g^{-1} h^{-1} for SSX and CHX, respectively. Compounds obtained from chemical or autohydrolysis processes generally have a high degree of polymerization, different from XOS produced by enzymatic processes. Furthermore, enzymatic hydrolysis has high specificity and minimal production of undesirable by-products (Ávila et al., 2020, Jain et al. 2014). Previous studies have successfully produced XOS by enzymatic hydrolysis of xylan extracted from lignocellulosic biomass Brienzo et al. (2010) obtained XOS yields of 2.8–6.4 mg mL^{-1} using pretreated sugarcane bagasse, and Samanta et al. (2012) produced 2.8 mg mL^{-1} XOS using *Sehima nervosum* grass. These yields are considerably lower than those achieved in the current study, suggesting that both Agroindustrial wastes (sugarcane straw and coffee husk) are potential sources of xylan for the production of xylooligosaccharides as well as the xylan extraction directly from its holocelullose source can improve XOS yield.

Table 5.2. Xylooligosaccharides profile after enzymatic hydrolysis of extracted xylan from sugarcane straw (SSX) and coffee husk (CHX).

Substrate	XOS Profile ($g.L^{-1}$)						
	X1	**X2**	**X3**	**X4**	**X5**	**X6**	**Total XOS**
SSX	0.36± 0.01	3.45 ± 0.12	2.65 ± 0.21	2.43 ± 0.04	0.97 ± 0.15	0.73 ± 0.04	10.23 ± 0.56
CHX	0.21 ± 0.03	3.04 ± 0.14	2.18 ±0.23	2.15 ± 0.06	0.57 ± 0.18	0.51 ± 0.04	8.45 ± 0.65

Where XOS corresponds to the xylooligosaccharides: xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

The data shown in Table 5.2 revealed the ability of the enzyme mixture to release short-chain XOS (X2, X3 and X4), along with smaller concentrations of X1, X5 and X6 for both substrates. A similar carbohydrate profile was observed by Manisseri & Gudipati (2010) in the hydrolysis of wheat bran by purified xylanases and by Goldbeck et al. (2014, 2016) in the hydrolysis of sugarcane bagasse by purified hemicelluloses. XOS with a low degree of polymerization (2 to 4) are preferred for food-related applications (Van Loo et al. 1999), as they

are more easily metabolized by probiotic bacteria and have increased prebiotic activity (Van Loo et al. 1999; Reddy & Krishnan 2016).

4.3.3 Evaluation of Prebiotic potential

4.3.3.1. *In vitro* Digestibity of xylooligosaccharides

The digestibility tests of oligosaccharides had the purpose of simulating the gastrointestinal tract to prove their resistance to the action of enzymes found in the digestive system (Fig.5. 2 A–B). There are few recent articles assessing the ability of prebiotics to reach the intestine intact and the minority of them evaluate the digestibility of XOS.

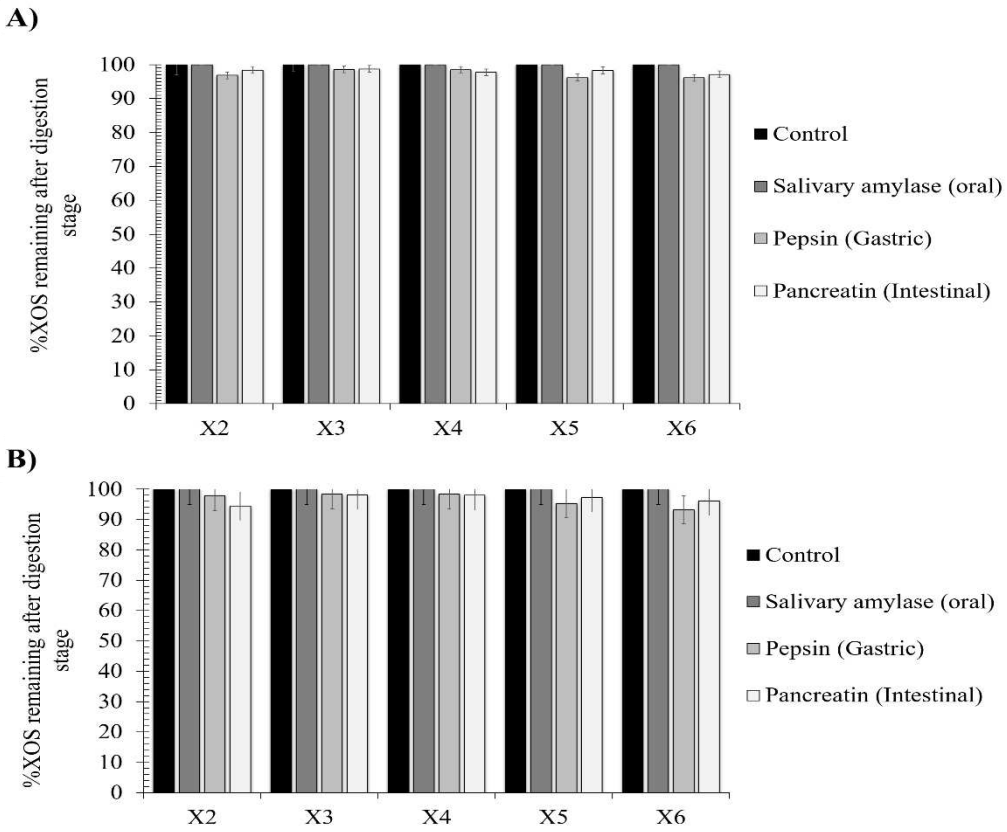


Figure 5.2. Digestibility profile of xylooligosaccharides from sugarcane straw xylan source (A), xylooligosaccharides from coffee husk source (B) by enzymes present in the gastrointestinal tract after 4 h of incubation at 37 °C. (X2-xylobiose, X3-xylotriose, X4-xylotetraose, X5-xylopentaose, X6- xylopentaose). Results are mean ± SD of three replicates.

The X2 and X3 fraction provided from SSX presented the higher resistance to digestibility after 4 h in pancreatin with a remaining percentage of 98.45 % and 98.77 % respectively (Fig 5.2A). While the remaining fractions provided from CHX was 94.44% and

98.17% respectively (Fig 5.2B). Which consequently promoted a slight higher total digestibility percentage (5.62 ± 0.60 %) than XOS provided from SSX (4.55 ± 0.6) (Table 4.3).

Table 5.3. Digestibility percentage of xylooligosaccharides from sugarcane straw xylan source $XOS_{(SSX)}$ and coffee husk xylan source $XOS_{(CHX)}$.

Substrate	% Digestibility			
	Oral	Gastric	Intestinal	Total Digestibility
$XOS_{(CHX)}$	-	3.33 ± 0.69	2.36 ± 0.72	5.62 ± 0.60
$XOS_{(SSX)}$	-	2.30 ± 0.69	2.26 ± 0.73	4.55 ± 0.67

The X4-X6 fractions also presented high resistance to digestibility for both xylan source. Then the tests proved the resistance of XOs to the action of digestive enzymes, in accordance with one of the criteria used for the classification of prebiotic ingredients (Oku, Tokunaga, & Hosoya, 1984).

A recent recent study also carried out "in vitro" digestibility tests showed similar results with resistance of xylooligosaccharides to the action of digestive enzymes. According to the study comercial XOs with DP 2-4 did not show any degradation when incubated with salivary amylase enzyme and suffered only 2% of degradation in the X2 and X3 in 4 h by pancreatin and 4% in the X4 and X3 in gastric juice (de Figueiredo et al., 2020).

The evaluation of the digestibility of oligosaccharides by enzymes is essential in order to test their ability to reach the intestine intact, where they serve as substrate for the fermentation by probiotic bacteria (de Figueiredo et al., 2020; Mussatto & Mancilha, 2007).

5.3.3.2. *In vitro* growth and fermentation of XOS by probiotics

The *in vitro* fermentation tests were carried out to demonstrate the XOS of different xylan agroindustrial waste source ability to stimulate the growth of beneficial bacteria. Many probiotic species of Bifidobacteria and Lactobacillus are capable of catabolizing a variety of monosaccharides and non-digestible oligosaccharides released by hydrolysis of plant polysaccharides (Oku, Tokunaga, & Hosoya, 1984). In this study, the ability to use XOS as prebiotics was tested against two Bifidobacterium species (*B. longun and B. lactis*) and two species of Lactobacillus (*L. acidophilus* and *L. paracasei*).

None of the probiotic bacteria grew in medium without a carbon source and all probiotic cultures showed higher growth in medium containing glucose, as expected with the negative and positive control respectively. With the exception of *B. lactis* all the probiotic

cultures was able to utilize XOS produced from agricultural wastes and showed remarkable growth in the media containing XOS as the sole source of carbon which was evident from the increase in OD600 at 72 h and the drop in the pH was also observed as a result of growth of probiotics cultures (Table 5.4).

Table 5.4. The parameters pH and density optical (OD) during the growth of probiotics on XOS prepared by bioconversion of agricultural waste xylan from sugarcane straw $XOS_{(SSX)}$ and coffee husk $XOS_{(CHX)}$ and glucose (positive control).

Probiotic		$XOS_{(SSX)}$		$XOS_{(CHX)}$		Glucose	
		pH	$OD_{(600\ nm)}$	pH	$OD_{(600\ nm)}$	pH	$OD_{(600\ nm)}$
Bifidobacterium lactis	Initial	6.70	0.03± ≥0.01a	6.70	0.02 ± ≥0.01f	6.70	0.03 ± ≥0.01a
	24h	6.70	0.03 ± ≥0.01a	6.70	0.02 ± ≥0.01f	5.10	0.71 ± 0.02l
	48h	6.70	0.03 ± ≥0.01a	6.70	0.02 ± ≥0.01f	4.80	1.69 ± 0.02m
	72h	6.70	0.03 ± ≥0.01a	6.70	0.02 ± ≥0.01f	4.50	2.00 ± 0.03n
Bifidobacterium longun	Initial	6.70	0.02 ± ≥0.01a	6.70	0.02 ± ≥0.01f	6.70	0.02 ± ≥0.01f
	24h	5.40	0.23 ± 0.04bg	6.40	0.28 ± 0.02gi	5.00	1.56 ± 0.05o
	48h	5.00	0.62 ± 0.03c	6.10	0.43 ± 0.03e	4.70	1.87 ± 0.02p
	72h	4.80	0.86 ± 0.05d	5.80	0.77 ± 0.02h	4.30	1.89± 0.03p
Lactobacillus acidophillus	Initial	6.70	0.03± ≥0.01a	6.70	0.03 ±≥0.01a	6.70	0.03 ± ≥0.01a
	24h	5.80	0.21 ± 0.04b	5.70	0.20 ± 0.03b	4.80	1.34 ± 0.03q
	48h	5.40	0.40 ± 0.06e	5.30	0.48 ± 0.04ej	4.60	1.78 ± 0.02r
	72h	5.20	0.46 ± 0.03e	5.20	0.52 ± 0.02j	4.50	1.97 ± 0.04n
Lactobacillus paracasei	Initial	6.70	0.02 ± ≥0.01f	6.70	0.02 ± ≥0.01f	6.70	0.02 ± ≥0.01f
	24h	5.80	0.25± 0.02bg	5.50	0.32 ± 0.04i	5.20	1.21 ± 0.02s
	48h	5.20	0.45± 0.05e	5.20	0.65 ± 0.02ck	5.00	1.79± 0.03t
	72h	5.00	0.59± 0.06c	4.80	0.68 ± 0.02kl	4.80	1.94± 0.02u

Means followed by the same letter in the same line and column did not differ by the Tukey test at the 5% significance level. Values represent mean values ± SE. (from duplicate experiments).

In contrast, studies showed that *B. lactis* was able to grown in commercial XOs (Longlive - China) reaching a relatively high growth rate (Crittenden et al., 2002), but this result was not observed in the present study for *B. lactis* BI-01. Regarding the growth the of *L. acidophilus* in terms of the OD600 at 72 h although it was observed a remarkable grown, it was not observed a considerable ability to grow on media containing XOS as sole source from both xylan source, since the presented absorbance values (0.461± 0.030) and (0.523 ± 0.020) was considerable lower than in medium containing glucose (positive control) (1.971 ± 0.020) (Table 5.4). According to Jagtap et al. (2017) the inability of the some cultures to grow well on media containing XOS as sole source of carbon could be correlated to lack of β-xylosidase activity. In

contrast *B. longum* and *L. paracasei* presented better and considerable capability to grow on XOS derived from sugarcane straw xylan and coffee husk xylan sources, respectively (Table 4). In the previous study Crittenden et al. (2002) studied and demonstrated the ability of *B. longum* to ferment XOs 70 (Suntory - Japan) and xylose, which is in accordance with this work.

The XOS profile from agroindustrial xylan sources obtained before and after the fermentation with the probiotic cultures that presented growth on XOS derived from sugarcane straw xylan and coffee husk xylan sources revealed that the all the cultures that presented a remarkable grow was able to consume mainly X2 and X3 fractions and a slight consumption the X4 and X5 (Figure 5.3).

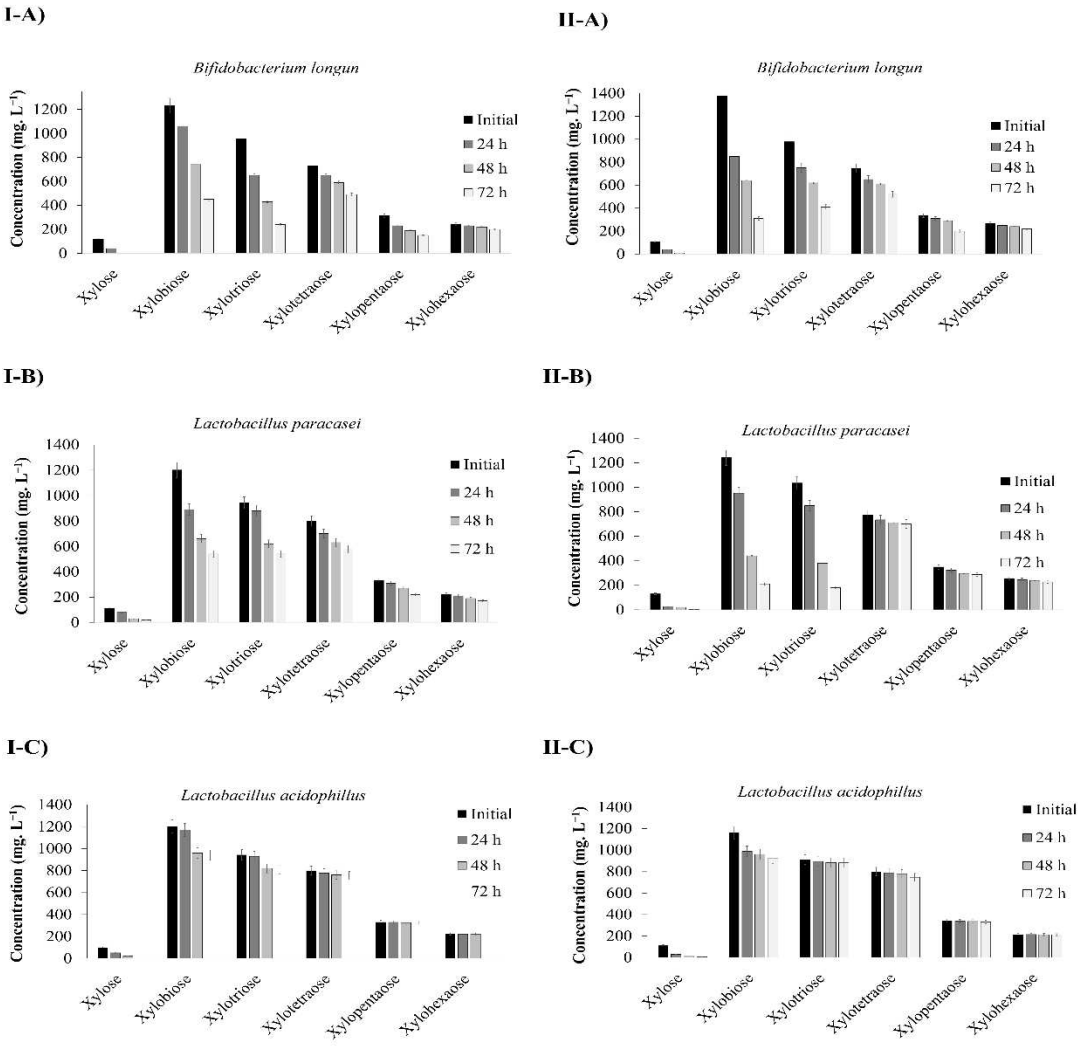


Figure 5.3. Consumption profile of xylooligosaccharides from the xylan of sugarcane straw (I) and coffee husk (II) after *in vitro* fermentation by *Bifidobacterium longum* (A), *Lactobacillus paracasei* (B) and *Lactobacillus acidophilus* (C). Values represent mean values (from duplicate experiments); bars indicate maximum and minimum values.

The experimental results have to be interpreted with the consideration that the concentrations of low and intermediate-DP oligosaccharides can be affected in two ways: consumption by bacteria and generation from XOS of higher DP. Keeping this idea in mind, the fast decrease of the concentrations of XOS with DP 2 and 3 confirms that these compounds are the preferred substrates. Compounds with DP 4 and 5 were also suitable carbon sources, but showed a slower utilization kinetics. These findings are in agreement with literature reported for the fermentability of XOS derived of agroindustrial wastes with defined DP distribution (Crittenden et al., 2002). Thus the good prebiotic activity of the derived XOS in the present study reveals the feasibility of using it as a functional food ingredient.

5.3.4 Antioxidant Potential

5.3.4.1. Total antioxidant capacity

The total antioxidant capacity (TAC) is based on the reduction of Mo^{+6} to Mo^{+5} by antioxidant compounds and the formation of green Mo^{+5} complexes with a maximum absorption at 695 nm (Brieto et al., 1999) The TAC assay is a important parameter to evalute antioxidant potential to considers the synergistic role of all antioxidants (enzymatic and non-enzymatic) rather than the simple sum of individual antioxidants since it defines the synergistic effect between the various antioxidant compounds in the sample (Serafini & Del Rio, 2004) .

The TAC of XOS mixture obtained from 48h enzymatic hydrolysis products of mixture enzymes are shown in Fig 5.4. The XOS mixture obtained from sugarcane straw xylan (SSX) showed concentration-dependent total antioxidant activity at concentrations of 0.053–0.8 $g.L^{-1}$, and the results showed that this XOS mixture exhibited significantly higher total antioxidant activity when compared to the corresponding mixture obtained from coffee husk xylan (CHX) at the same treatment concentration, and the effect increased with increasing concentration. The highest optical density of the XOS mixture obtained was 0.92 and 0.78 to XOS mixture provide from SSX and CHX respectively (Fig 5.4). The similar optical density was detected for Zhou et al. (2018) for TAC analyses of xylooligosaccharides produced from hardwood xylan using recombinant enzyme cocktail. Due to the high TAC of XOS mixtures obtained from both xylan substrate source the analyses o DPPH free-radical was perform for further detailed of its scavenging capacity (item 5.3.4.2) .

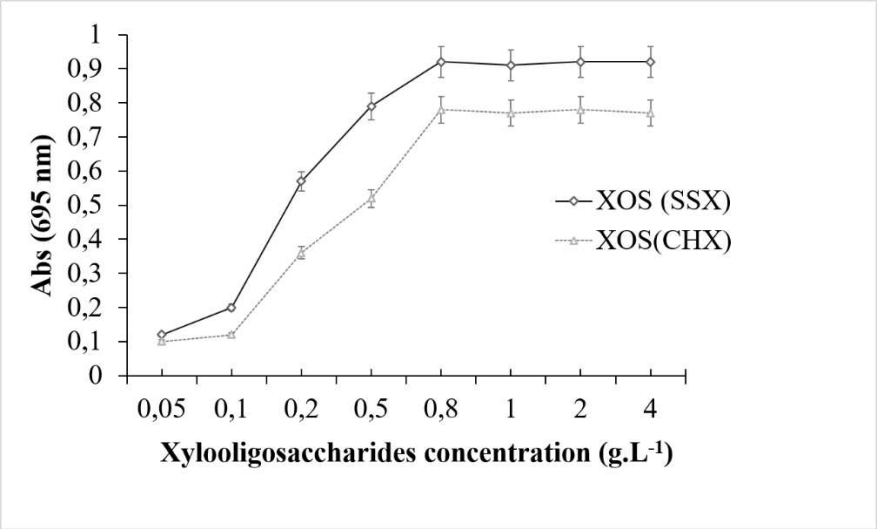


Figure 5.4. Spectrophotometric evaluation of total antioxidant capacity obtained by phosphomolybdenum complex assay of XOS obtained by enzymatic hydrolysis for 48 h from sugarcane straw xylan XOS(SSX) and coffee husk XOS(CHX).Values represent mean values (from triplicate experiments); bars indicate maximum and minimum values.

5.3.4.2. DPPH radical-scavenging activity

DPPH scavenging assay is based on the measurement of discoloration resulting from a reduction of DPPH free radical by an antioxidant. The reaction mechanism involves transfer of electron by the reducing agent to the DPPH radical. The method is a valid, easy, accurate, sensitive, and economic method to evaluate scavenging activity of antioxidants of fruits and vegetables juices or extracts, since the radical is stable and need not to be generated as in other scavenging assays (Singh & Singh, 2008). According to Vieira et al. (2020) based on previous reports the results are highly reproducible and comparable to other scavenging methods such as ABTS, for these reasons it was chosen in this present study for obtaining of more accurate analyses date.

The scavenging ability of XOS obtained from the 48 h enzymatic hydrolysis using DPPH assay is shown in Figure 5.5. The XOS from both agroindutrial wates exhibited concentration-dependent antioxidant activity at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 $g.L^{-1}$ (Figure 4.5).

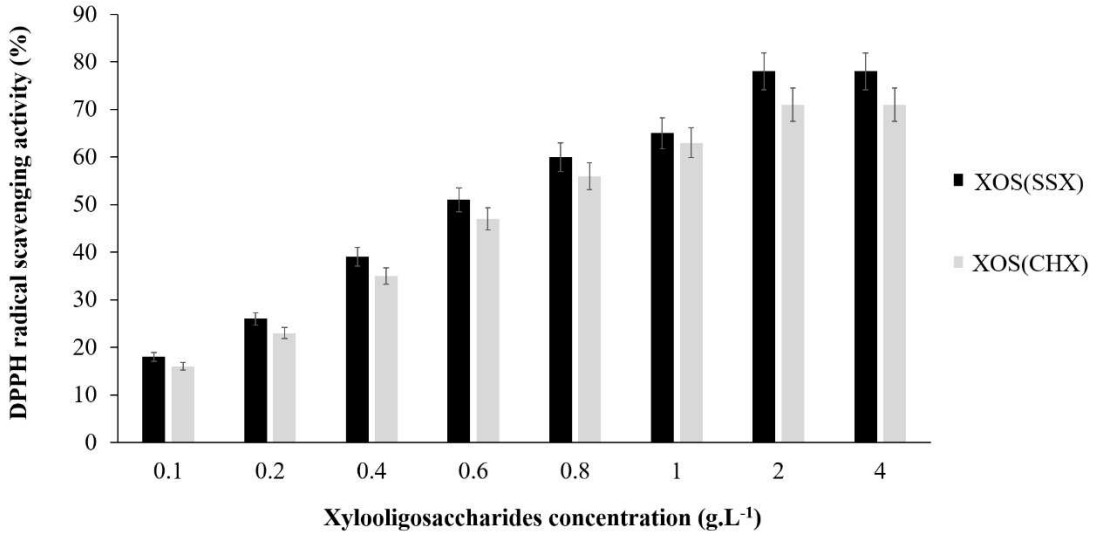


Figure 5.5. Antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl of XOS obtained by enzymatic hydrolysis for 48 h of XOS from sugarcane straw xylan XOS(SSX) and coffee husk XOS(CHX).Values represent mean values (from triplicate experiments); bars indicate maximum and minimum values.

The percent of activity gradually increased and reached 71% and 78% to XOS from sugarcane straw and coffee husk xylan, respectively at a concentration of 2 $g.L^{-1}$, after it remained constant. The reducing sugar content, the presence of phenolic groups such as ferulic acid, and the presence of uronic acid, among others, revealed to play an important role in the antioxidant properties (Eom, Senevirathne & Kim, 2012). Still further according to Vieira et al. (2020) oligosaccharides could have their antioxidant properties improved after chemical modifications, such as, carboxymethylation, acetylation, among others. The antioxidant activity exhibited by XOS in this study reached almost equal levels to that exhibited by a sugarcane bagasse XOS mixture (Bian et al., 2013) and the activity was higher than that of a maize XOS mixture as reported by Jagtap et al. (2017). The above results indicated that XOS from both source xylan can be exploited in preparing nutritional health foods.

5.4. Conclusion

Enzymatic hydrolysis of xylan from sugarcane straw and coffee husk xylan source using a enzymatic mixture resulted in high XOS production with xylobiose, xylotriose and xylooligotetraose as the major components (DP of 2, 3 and 4) identified through HPLC-PAD.

In vitro digestibility tests confirmed the resistance of XOS produced from both xylan sources to the action of enzymes found in the gastrointestinal tract and resistance to acidity of gastric juice. “In vitro” fermentation of XOS indicated the best ability of *Lactobacillus paracasei* and *Bifidobacterium longun* to grown in these oligosaccharides, consuming preferentially the X2 and X3 fractions as a source of nutrients proving their prebiotic potential. The XOS also presented a good antioxidant activity achieving until 78% in a XOS concentration of 2 $g.L^{-1}$, demonstrating thus its potential to be use in food relating applications in food industry as functional ingredients.

Acknowledgements

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Capítulo 6

Fractionating process of lignocellulosic biomass for the enzymatic production of short chain cello-oligosaccharides

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Fractionating process of lignocellulosic biomass for the enzymatic production of short chain cello-oligosaccharides

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Highlights

-Higher temperature, lesser alkali and shorter time in the efficient fractionating;

-High cellulose recovery yields from sugarcane straw and coffee husk;

-Sodium chlorite as a potential treatment for prior delignification of raw material;

-Potential effects of enzyme mixtures for short chain COS production;

Abstract

This study performed a prior delignification with sodium chlorite treatment on sugarcane straw and coffee husk waste, followed by alkaline treatment strategies; aiming at the increase cellulose recovery efficiency and the decrease of chemical consumption process. This study also explored an effective approach in the use of the cellulose recovery fraction for the enzymatic production of short chain cello-oligosaccharides, using optimal mixtures of commercial enzymes. The hydrothermal alkaline treatment using KOH concentration 4.5% w/v, displayed similar effects to treatment with moderate temperature conditions (35 °C) with high a KOH concentration (24% w/v). The cellulose extraction efficiency was significant, around 92%, and achieved delignification close to 91%. Efficient enzyme mixtures that achieved maximum cello-oligosaccharide yields of around 63.56 mg. $g_{substrate}$ and low glucose concentrations were developed. This suggests a probably cost reductions and simplification in the purification step, for future application in the different industrial fields.

Key-words*:* Lignocellulosic biomass; Cello-oligossacharides; Alkaline treatment;

6.1. Introduction

Food industries have shown a great interest in functional products (Karnaouri et al., 2019). A food product with functional properties is one that has potential health benefits for the consumer; such as increasing the absorption of a given component, promoting reducing the risk of diseases and improving their well-being (Gibson and Roberfroid, 1995). Cello-oligosaccharides (COS) are composed by D-glucose monomers with β-1,4-linkages, which, according to the degree of polymerization (DP), such as the short chain COS (DP ≤ 6) are soluble in water (Karnaouri et al., 2019). These new non-digestible short-chain oligosaccharides are considered promising due to their prebiotic properties. Thus COS have already aroused great interest from the food industry in the incorporation of these components in food formulations for the elaboration of functional foods (Otsuka et al., 2004).

In recent years, cellulose has been shown as the greatest importance biopolymer due to its wide application in different segments in the chemical industry. Furthermore cellulose is the natural substrate for cello-oligosaccharides production (Ávila et al., 2021; Tolonen et al., 2015). Therefore, from a biorefinery perspective, the agricultural lignocellulose wastes investigated in this study are interesting sources that could be a potentially cheap and abundant material to drive industrial change; in regards to fractionating into polymers for further industrial use as substrates to produce several high-value products (Khaleghipour et al., 2021; Klemm et al., 2005; Tolonen et al., 2015).

Cello-oligosaccharide production processes are classified according to the cellulose depolymerization method, which can be either chemical or enzymatic (Chu et al., 2014). Acid hydrolysis is generally not indicated due to undesirable by-products being released, such as furfural and hydroxyl methylfurfural, and high concentration of monosaccharides. Therefore, enzymatic hydrolysis is more suitable due to employ mild and controlled reaction conditions providing less by-products releasing (Carvalho et al., 2013). The cellulolytic system is constituted by complementary enzymes efficient combination employed for complete cellulose depoymerization (Lynd et al., 2020). Endoglucanases are described as enzymes of a non-processive group which cleave the cellulose generating new ends; while exoglucanases group works in the processive way, performing several hydrolysis cycles by moving on the cellulose chain, providing releasing of cellobiose (Ávila et al., 2021; Barbosa et al., 2020a).

The design of customized cellulolytic enzymatic mixtures could be a potential strategy to cello-oligosaccharides production due to provide great advantages, such as the controlled

polysaccharide cleavage breaking with less monomers produced and the ability to adapt the enzyme mixture composition to different substrates, according to their structural properties. (Karnaouri et al., 2018). Challenges arise either from the laborious techniques related to heterologous and molecular cloning production methods and protocols or the production costs of different enzymes. Otherwise, improvement the performance of the combination of commercially available enzymes for cellulose could be a promising strategy for the valorization of lignocellulosic agroindustrial wastes (Karnaouri et al., 2019; 2018).

The pretreatment processes steps are fundamental to favor the separation of main components from lignocellulosic biomass; due to the complex structural of lignocellulosic matrix. Alkaline pretreatment disruption the crystalline structure of lignocellulose biomass through the cell walls materials dissolution by dissolving hemicellulose and lignin (Khaleghipour et al., 2021). The cellulignin waste recovered after xylan alkaline solubilization treatment could be considered a potential source for COS releasing; due to its considerably cellulose content (70–80%). Therefore the utilization of these materials affords an excellent opportunity for more sustainable development process with a great value-added to agricultural sources (Chu et al., 2014; de Figueiredo et al., 2017; Karnaouri et al., 2019).

Sodium chlorite in acidic medium is a settled protocol for delignification step (Hubbell and Ragauskas, 2010). The sodium chlorite solution dissociates into highly reactive anions to break lignin into lower molecular weight compounds which are then dissolved and removed through the wash out, yielding in holocellulose material. During this reaction the cellulose recalcitrance could be partially reduced by acid hydrolysis and oxidative cleavage. The literature reports that the structure of cellulose fibers may change with different alkaline/NaClO2 treatments (e.g., time, chemical inputs) (Abdel-Halim, 2014; Rabetafika et al., 2014). Furthermore the application of alkali combined with autoclaving was also reported as an efficient strategy to provide maximum recovery of xylan from lignocellulosic materials, isolating it efficiently from the cellulose fraction (de Figueiredo et al., 2017; Khaleghipour et al., 2021). In the other hand, the cleaving of chemical linkages in lignocellulosic structure is relatively laborious and expensive, which limits fractionation of these materials into polysaccharides and their high enzymatic conversion yield. Therefore, methods that propose the reducing costs in the process for the industrial scale are important (Ávila et al., 2020a; de Figueiredo et al., 2017).

This study provided integrated fractionating process strategies using alkaline treatments of previously delignified agroindutrial materials (sugarcane straw and coffee husk)

aimed at the increase of cellulose recovery efficiency and the decrease of alkali concentration consumption process. Furthermore, this study explored an approach for short chain cello-oligosaccharides production using a mixture of commercial enzymes; demonstrating the importance of both, fractionation process and enzymes choice for further conversion of the material.

6.2. Material and Methods

6.2.1. Raw Sample

Coffee husk (*in natura*) was supplied by a local farm, Boa Esperança, MG, Brazil; and sugarcane straw (*in natura*) offered by the CNPEM, Campinas, SP, Brazil. The of excess soil and dust was removed from biomasses thorough consecutive washings with water at room temperature (Ávila et al., 2018). The samples was dried at 80 °C in a stove for 48h, then passed in a cutting mill, and sieved to obtain particles in the diameter range of 0.3-0.7 mm (Ávila et al., 2020a).

6.2.2. Cellulose extraction process

The cellulose-rich solid substrates were generated as a part of an integrated alkaline holocellulose treatment of sugarcane straw and coffee husk process, described in previous work by our research group, which had focus on the extract solubilized alkali-arabinoxylan to produce xylooligosaccharides (Ávila et al., 2020a) .

6.2.2.1. Delignification process

Lignocellulosic material was previously delignified according to Wise et al. (1946) protocol with minor modifications, using a sodium chlorite treatment to isolate the holocellulose fraction. The reaction was carried out in a 7.5% acetic acid medium containing 15 % (w/v) of solid loading biomass at 80 °C. Proximally 20 mg of sodium chlorite was weighted and inserted into the medium 4 times in 60 minutes periods. The delignified material was then filtered, washed consecutively with water and dried at 60° C in a stove for 72 h and kept at 25° C for analytical determinations (Ávila et al., 2020a).

6.2.2.2. Alkaline treatments

Alkaline treatments were applied to the holocellulose fraction in triplicate using different concentrations (4.5%, 8%, 14% and 24%) of potassium hydroxide, and under different temperatures (35 °C, 70 °C and 80 °C) for 180-360 minutes; or through steam application (121 °C) for 30 minutes. The solid loading percentage was also investigated using 8% or 15% (w/v)

(Table 6.1). The solid product was filtered, washed thoroughly with water and dried at 60 ° C in a stove for 72 h.

Table 6.1. Treatment and extraction conditions for obtaining cellulose from isolated holocellulose of sugarcane straw and coffee husk.

Treatment	Solids loading (%)	KOH (% w/v)	Temp. (°C)	Reaction time (minutes)
Treatment 1	8	24	35	180
Treatment 2	8	24	35	360
Treatment 3	8	14	70	180
Treatment 4	8	8	80	180
Treatment 5	8	4.5	121	30
Treatment 6	15	4.5	121	30

6.2.2.3. Solid recovery yield and cellulose extraction efficiency

The solid recovery yield *(Y_r)* percentage and cellulose efficiency extraction (E_c) percentage calculations were based on dry holocellulose material and cellulose content present in sugarcane straw and coffee husk samples. Hereafter, the optimal alkaline concentrations and conditions were followed to carry out the bulk cellulose extraction for subsequent cello-oligossacharides production.

The parameters were determined by the following equations:

$$Yr = \left(\frac{W_\mathrm{r}}{W_\mathrm{h}}\right) \times 100$$

where W_r is the weight of dried solid recovery material (g) and W_h is the weight of dried holocellulose material (g).

$$Ec = \left(\frac{C_\mathrm{r}}{C_\mathrm{i}}\right) \times 100$$

where C_r is the cellulose content in the recovery material (mg g^{-1}) and C_i is the initial cellulose content in the holocellulose materials (mg g^{-1}).

6.2.3. Chemical Composition determination

The chemical characterization of all samples were perform based on standard protocols of National Renewable Energy Laboratory (Sluiter et al., 2008; Sluiter et al., 2005a). The extraction contents were measured only in raw material with a ciclohexane + ethanol solution (1:1) using a Soxhlet extractor (Sluiter et al., 2005b).

6.2.4. Enzymes and determination of protein concentrate

Three enzymes were used in an experimental design template: endo-1,4-β-D-glucanase- *Aspergillus niger* (GH12), exo-1,4-β-D-glucanase *Trichoderma longibrachiatum (GH5)* and feruloyl esterase - *Clostridium thermocellum* (CE1), all acquired from Megazyme®. In addition, the cellulose enzyme cocktail Cellic® CTec2, was used to previously evaluate the enzymatic susceptibility of the cellulose provided from different treatments. The protein concentration was measured using the procedure proposed by Lorry (1951).

6.2.5. Enzyme activity measurements

Endoglucanase and exoglucanase activities were determined according to IUPAC protocols (Ghose, 1987). The resulting reducing sugars were determined using a DNS experiment method (Miller, 1959). Feruloyl esterase activity was measured in naphthyl acetate substrate, according to protocol described by Koseki et al. (2014). The amount of enzyme that released 1 μmol of α-naphthol per minute was established as the one unit of activity (Goldbeck et al., 2016).

6.2.6 Enzymatic hydrolysis and experimental design

The synergistic or antagonistic effects of enzymes on cellulose conversion to glucose and released cello-oligossaccahrides was investigated by central composite rotatable design (CCRD). The experimental design was constituted by 14 runs and 3 center points, resulting 17 experiments. Endo-1,4-β-D-glucanase, endo-1,4-β-D-glucanase and feruloyl esterase concentrations were the factors of the experimental design. The hydrolysis time and enzyme protein concentration range selection in the experiments were based on preliminary tests of kinetics parameters evaluation. Endo-1,4-β-D-glucanase concentration varied from 0.70 to 4.58 mg protein g−1 substrate. Exo-1,4-β-D-glucanase concentration varied from 0.80 to 6.68 mg protein g−1 substrate, and feruloyl esterase concentration varied from 0.14 to 3.09 mg protein g−1 substrate.

The enzymatic microassays were performed as described by Ávila et al. (2020a; 2020b) with minor modifications; the reactional médium was constituted by 0.1 M sodium phosphate buffer pH 5, including a substrate loading of 0 1% (w/v), suitable amounts of enzymes in a total volume of 1 mL. The samples were kept under agitation (1000 rpm) at 50 °C for 48 h. Then, enzymes were exposed to heat (99 °C for 15 min) for effective inactivation, centrifuged and the supernatant collected for analytical determinations.

6.2.7. Glucose and cello-oligosaccharides quantification

The glucose and cello-oligosaccharides (COS) content in the supernatant of enzymatic hydrolysis was determined by High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex DX-5000 ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA); according to the method described by Barbosa et al.(2020a;2020b). Calibration curves using commercial standards (Megazyme, Bray, Ireland) were constructed through PEAKNET software, which also provided the identification of commercial standards in samples by comparing their retention times with authentic standards.

6.2.8. Statistical Analysis

The data are presented as the mean of the triplicate analytical determinations accompanied by its respective standard deviation. Means were compared using Tukey's test ($p < 0.05$) through Statistica v. 8.0 (Statsoft, Inc., Tulsa, OK, USA). The experimental design data were analyzed with a 95% confidence level using Protimiza Experimental Design online software (http://experimental-design.protimiza.com.br/) (Rodrigues and Iemma, 2014).

6.3. Results and Discussion

6.3.1. Cellulose extraction

6.3.1.1. Chemical characterization

During the optimization process for cello-oligosaccharide production (and other by-products) using lignocellulosic waste materials, the initial raw material composition was first determined; to assess its suitability for prebiotic production. The approximate composition of the lignocellulosic materials employed in this work are presented in Table 6.2. The chemical characterization results corroborated with the literature reports, and demonstrate that the high cellulose content, makes them suitable substrate materials for COS production (Ávila et al., 2018; Carvalho Oliveira et al 2018; Bekalo and Reinhardt, 2010; Gouveia et al., 2009;Navya and Murthy Pushpa, 2013).

Table 6.2. Chemical composition (% w/w, dry basis) of untreated sugarcane straw and coffee husk biomass.

Components	Sugarcane straw	Coffee husk
*Holocellulose	70.76 ± 0.78	62.76 ± 0.67
Cellulose	39.40 ± 0.23	27.78 ± 0.78
Hemicellulose	29.86 ± 0.55	24.67 ± 0.61
Total Lignin	21.83 ± 0.91	35.58 ± 1.55
Ash	4.05 ± 0.12	0.98 ± 0.56
Extractives	6.67 ± 0.56	10.78 ± 0.78
Total	98.81 ± 2.37	99.79 ± 4.28

*Holocellulose corresponds to the estimated value of total carbohydrates (α-celulose+hemicellulose) obtained using the gravimetric method; therefore, it was not included in the total mass balance.

Alkaline treatments of lignocellulosic biomasses, such as agricultural byproducts provide their crystallinity decreasing disrupting the lignocellulose complex by dissolving hemicelluloses and lignin and by swelling cellulose (Khaleghipour et al., 2021; Klemm et al., 2005). However, it has been reported that lignin could not be completely removed through alkaline treatment (de Figueiredo et al., 2017). Therefore, to further eliminate lignin, the lignocellulosic materials were then subjected to a prior $NaClO_2$ treatment. Table 6.3 shows the chemical composition of remaining solid insoluble fractions obtained from different alkaline treatments.

Table 6.3. Chemical composition of insoluble solid recovery from sugarcane straw and coffee husk, their respective delignification percentage, and hemicellulose solubilization after alkaline treatments.

Treatments	Sugarcane straw			Hemicellulose solubilization (%)	Delignification (%)
	Cellulose (%)	Lignin (%)	Hemicellulose (%)		
Treatment 1	75. 34 ± 0.81^{a}	4.55 ± 0.27^{a}	10.53 ± 0.96^{b}	82.18 ± 0.87^{a}	91.18 ± 0.67^{a}
Treatment 2	76. 70 ± 0.81^{a}	4.55 ± 0.17^{a}	10.03 ± 0.76^{b}	82.76 ± 0.73^{a}	91.76 ± 0.57^{a}
Treatment 3	67. 31± 0.94^{c}	4.56 ± 0.23^{a}	13.40 ± 1.27^{a}	76.78 ± 0.81^{b}	91.12 ± 0.61^{a}
Treatment 4	70.94 ± 0.77^{b}	4.60 ± 0.12^{a}	13.20 ± 1.27^{a}	77.87 ± 0.84^{b}	91.65 ± 0.77^{a}
Treatment 5	75.54 ± 0.94^{a}	4.70 ± 0.12^{a}	11.17 ± 1.35^{b}	82.48 ± 0.83^{a}	91.13 ± 0.64^{a}
Treatment 6	61.10 ± 0.72^{d}	4.64 ± 0.78^{a}	14.70 ± 1.33^{a}	74.76 ± 0.83^{c}	91.20 ± 0.57^{a}

Treatments	Coffee husk			Hemicellulose solubilization (%)	Delignification (%)
	Cellulose (%)	Lignin (%)	Hemicellulose (%)		
Treatment 1	76.12 ± 0.87[a]	10.32 ± 0.15[a]	11.78 ± 0.94[b]	75.78 ± 0.81[a]	80.18 ± 0.57[a]
Treatment 2	75.56 ± 0.87[a]	10.26 ± 0.05[a]	11.55 ± 1.20[b]	76.05 ± 0.77[a]	80.98 ± 0.53[a]
Treatment 3	70.52 ± 0.87[b]	10.42 ± 0.14[a]	13.31 ± 1.03[a]	71.78 ± 0.63[cd]	80.43 ± 0.67[a]
Treatment 4	69.94 ± 0.87[b]	10.56 ± 0.13[a]	12.62 ± 1.13[ab]	72.67 ± 0.87[c]	80.21 ± 0.54[a]
Treatment 5	75.54 ± 0.87[a]	10.53 ± 0.12[a]	11.65 ± 0.89[b]	74.78 ± 0.83[b]	80.46 ± 0.83[a]
Treatment 6	63.12 ± 0.87[c]	10.61 ± 0.22[a]	12.17 ± 1.15[ab]	70.67 ± 0.81[d]	79.34 ± 0.83[a]

Means followed by the same letter in a column for the same material do not differ at $p < 0.05$ by Tukey's test.

The prior sodium chlorite pretreatment provided a significant delignification percentage, close to 91% and 80% for sugarcane straw (SS) and coffee husk (CH) biomass respectively (Table 6.3). In the acidic medium, aqueous sodium chlorite solutions dissociate into highly reactive anions. These convert lignin into small compounds, which are then dissolved and washed out, resulting in the solid permanent whiteness (Abdel-Halim, 2014; Rabetafika et al., 2014). Moreover, studies have shown that delignification is necessary for effective cellulose conversion after enzymatic hydrolysis (Goldbeck et al., 2016). The process also provided high hemicellulose solubilization (61.10-76.70%), which would not only contribute to the insoluble purity of the insoluble cellulose material, but to also perform an efficient xylan precipitation step in the soluble liquid alkaline fraction. Consequently, the insoluble materials presented high cellulose concentration (purity) value ranges (61.10–81.34%), with a relatively low residual hemicellulose range (10.53-14.70%) (Table 6.3). The residual lignin content was similar for all the applied treatments in this study; however, there were some significant differences regarding the cellulose and residual hemicellulose content for each insoluble material source. For both sources, the insoluble materials provided from treatments 3, 4 and 6 presented significant difference of treatments 1, 2 and 5, while these three were similar. (Table 6.3).

6.3.1.2. Solid recovery and cellulose extraction efficiency

The insoluble solid recovery yield (Yr), after the alkaline solubilization process from holocellulose isolated materials, was from 62 to 72%. The cellulose extraction (Ec) efficiency of treatments 1 and 2 from sugarcane straw (SS) were statistically similar; while for the materials from coffee husk (CH), the application of steam, lower reagents and solid loading concentration (Treatment 5) also provided similar efficiency (Table 6.4). However, increased holocellulose solid loading concentration to 15% in treatment 6 did not favor the recovery yield or efficiency data for any source. Extracts from treatment 6 presented a considerably viscosity

(data not shown), which may impede the KOH catalytic activity (Ávila et al., 2020a; de Figueiredo et al., 2017).Treatments 3, 4 and 5 presented lower solid recovery yield (Yr) values, despite having higher cellulose efficiency extraction (Ec) values than treatments 1, 2 and 5 for both SS and CH. The lower Yr values after treatment can be justified by the lower residual hemicellulose content; since there was also significant hemicellulose solubilization in the alkaline solution (Table 6.3).

Table 6.4. Insoluble solid recovery yield (Y_r) and Cellulose extraction efficiency (E_c) of sugarcane straw and coffee husk after alkaline treatments.

Treatments	Sugarcane straw		Coffee husk	
	Y_r (%)	E_c (%)	Y_r (%)	E_c (%)
Treatment 1	63.78 ± 1.56^a	92.80 ± 1.65^a	64.43 ± 1.23^a	86.83 ± 0.82^a
Treatment 2	62.45 ± 0.85^a	92.50 ± 0.76^a	64.07 ± 1.02^a	85.71 ± 0.68^b
Treatment 3	67.31 ± 0.96^b	84.35 ± 0.75^b	67.07 ± 0.87^b	83.74 ± 0.62^c
Treatment 4	70.94 ± 1.10^c	89.81 ± 0.62^c	66.56 ± 0.89^b	82.42 ± 0.46^d
Treatment 5	61.36 ± 0.87^d	91.43 ± 0.62^d	64.75 ± 0.78^a	86.60 ± 0.57^a
Treatment 6	70.45 ± 0.76^c	82.94 ± 0.71^e	72.14 ± 0.82^d	80.81± 0.67^e

Means followed by the same letter in a column do not differ at $p < 0.05$ by Tukey's test.

The evaluated treatments showed a significant reduction in the alkali reagent and processing time. The alkaline hydrothermal treatments (Treatments 5 and 6) showed reductions of higher than 78% in KOH concentration per gram of extracted cellulose, compared to treatments 1 and 2. Furthermore, processing times were reduced by up to 12x using hydrothermal conditions (121°C), compared to the treatments using mild temperature conditions. However, an increase from 35 °C (Treatments 1 and 2) to 70 °C, 80°C (Treatments 3 and 4) and 121 °C (Treatments 5 and 6) resulted in an increase of used energy. Treatments 3, 4, 5 and 6 had reduced chemical inputs in relation to treatments with mild conditions (Treatments 1 and 2).

Similar KOH treatments in sugarcane bagasse were presented by de Figueiredo et al. (2017), although aiming the arabinoxylan exytraction. The authors also reported a considerable reduction of 75% in all chemicals used, compared with a reference method using KOH in a high concentration (24% w/v). The literature also reports the successful use of KOH, compared to NaOH, for other biomasses such as rice straw and corn pretreatment under enzymatic hydrolysis of the cellulose (Kaar and Holtzapple, 2000; Ong et al., 2010). Sharma et al. (2013) evaluated the KOH concentration of 0.5% at 21 °C and 12 h and verified that it was sufficient for good digestibility of the cellulose. Although this processing time was longer than the employed in this present work, the alkaline concentration was lower.

Therefore, treatments 1, 2 and 5 achieved better cellulose extraction results, based on higher cellulose percentage and similar residual hemicellulose and lignin content. However, the application of hydrothermal alkaline conditions under lower solid loading concentrations (Treatment 5) appears more suitable. This is due to a considerable time reduction from 6h (Treatment 2) to 0.5 h; and reduced alkali reagent consumption, from 24% (Treatments 1 and 2) to 4.5%. Despite this treatment has displaying potential for a cost reduction strategy, a proper economic evaluation is still necessary to verify the economic viability for industrial scale application.

6.3.1.3. Extracted cellulose as a substrate for cellulase enzymatic activity

The enzymatic extract Cellic®-CeteC2 (Noyozymes) was used to evaluate the enzymatic susceptibility of different substrates from sugarcane straw (SS) and coffee husk (CH) (Table 6.5).

The substrates evaluated were the insoluble solid fraction obtained after the sodium chlorite and KOH treatments performed in this study. All substrates obtained had considerably lower enzymatic activity values than amorphous commercial cellulose substrate such as caboxymethyl cellulose (CMC) (603.57 ± 10.21 UI mL^{-1}). However, they did present higher values than Avicel microcrystalline cellulose (10.65 ± 0.56 UI mL^{-1}), which suggests considerable crystallinity of the cellulose recovery material. The cellulose chain could also probably be protected, in some regions, by residual hemicellulose and presence of lignin. Unproductive adsorption could occur because of the presence of lignin, resulting in enzyme unavailability for the required reaction (Ko et al., 2015). The enzymatic activity is determined with excess of substrate and, in this case, lignin was probably not an interfering factor; since total content was minimal and similar between the substrates. However, the residual hemicellulose content might have affected the results as treatment 6, which provided substrates (SS6 and CH6) had the highest residual hemicellulose content for both sources (Table 6.3), also displayed lower enzymatic susceptibility. In comparison, substrates used for treatments 1, 2 and 5 for both sources (SS1, CH1, SS2, CH2, SS5 and CH5) had lower residual hemicellulose content and displayed higher enzymatic activity values.

Table 6.5. Enzymatic activity determination for commercial cellulolytic cocktail Cellic®-CeteC2 (Novozyme); using the insoluble cellulose recovered thorough alkaline and chlorite treatments (1, 2, 3, 4, 5 and 6) of sugarcane straw (SS) and coffee husk (CH), carboxymethil-cellulose (CMC) and Avicel microcrystalline cellulose.

Substrate	**Activity** (UI/mL)
SS1	25.56 ± 1.22^{a}
SS2	25.45 ± 1.32^{a}
SS3	23.45 ± 1.12^{b}
SS4	21.32 ± 1.15^{b}
SS5	25.67 ± 1.10^{a}
SS6	20.56 ± 1.12^{b}
CH1	20.56 ± 1.16^{b}
CH2	20.23 ± 1.28^{b}
CH3	20.45 ± 1.17^{b}
CH4	19.05 ± 1.21^{c}
CH5	20.54 ± 1.11^{b}
CH6	18.65 ± 1.02^{d}
Avicel	10.65 ± 0.56^{e}
CMC	603.54 ± 10.21^{f}

Means followed by the same letter in a column for the same material do not differ at $p < 0.05$ by Tukey's test.

Therefore, based on not only enzyme susceptibility but also on chemical input and reaction time, the substrates obtained using hydrothermal alkaline conditions (SS5 and CH5) were selected to carry out bulk cellulose extraction for subsequent cello-oligosaccharides production.

6.3.2. Cello-oligosaccharides production

Cello-oligossacharides (COS) are a recent investigated group of oligosaccharides of great importance for industrial sector, which have been shown applications in different fields of the chemical and feed industries; currently they have been studied as potential prebiotic compounds (Ávila et al., 2021; Karnaouri et al., 2019). However, the COS production is still less studied than glucose releasing process, due to be a fundamental carbon source for bioprocesses and important derived of platform chemical within the biorefinery (Cano et al., 2020). For this reason, most of the commercial cellulolytic cocktails have been developed with high β-glucosidase activity and low endoglucanse and exoglucanase activities. It is important to be taken that β-glucosidase activity hampers the COS releasing, since this kind of enzyme hydrolyses glycosidic bonds within cellobiose, yielding high amounts of glucose (Chu et al.,

2014). Thus, purified commercial enzymes were employed in this study as a strategy for optimizing COS production; and to modulate the activity for each specific enzyme to evaluate synergistic actions between them. Although there are no reports of synergistic interaction between cellulases associated with esterases in the production of COS, Goldbeck et al. (2016) was successful in studying the application of Feruloyl esterase-*Clostridium thermocellulun* with hemicellulases. Therefore, it was included as a variable in this study, since residual lignin and hemicellulose was found in the obtained substrates.

The combined effects were evaluated using an experimental design strategy (Central Composite Rotational Design, CCRD) in order to obtain the ideal combination and protein concentration in the mixture. The planning included three independent variables: endoglucanase (GH12), exoglucanase (GH5) and Feruloyl Esterase (CE1) (Megazyme®). The CCRD matrix (Table 6.6) corresponds to the experimental designs with 3 central points and 6 axial points, totaling 17 experiments. The response variables were the total COS concentrations released after 48 h with DP from 2 to 6. It was noted that the selected cellulose recovered substrate from sugarcane straw (SS5) was the most favorable for COS production, achieving considerably higher yields than those obtained from coffee husk (CH5) (Table 6.6).

Table 6.6. Central composite rotatable design matrix and experimental results of cello-oligosaccharides (COS) production from the selected recovery cellulose substrate from sugarcane straw (SS5) and coffee husk (CH5) using enzyme mixtures.

Enzymes (mg protein).($g_{substrate}$)$^{-1}$			**SS5**	**CH5**
GH12	**GH5**	**CE1**	**COS (mg).($g_{substrate}$)$^{-1}$**	**COS (mg. $g_{substrate}$)$^{-1}$**
1.0 (-1)	0.4 (-1)	0.68 (-1)	35.60	29.66
2.5 (+1)	0.4 (-1)	0.68 (-1)	53.00	37.63
1.0 (-1)	1.6 (+1)	0.68 (-1)	37.80	34.15
2.5 (+1)	1.6 (+1)	0.68 (-1)	68.70	42.98
1.0 (-1)	0.4 (-1)	2.0 (+1)	48.00	30.20
2.5 (+1)	0.4 (-1)	2.0 (+1)	47.40	40.73
1.0 (-1)	1.6 (+1)	2.0 (+1)	53.40	33.60
2.5 (+1)	1.6 (+1)	2.0 (+1)	68.80	45.63
0.5 (-1.68)	1.0 (0)	1.34 (0)	33.03	26.40
3.0 (+1.68)	10.0 (0)	1.34 (0)	69.60	45.70
1.75 (0)	0.08 (-1,68)	1.34 (0)	43.00	35.66
1.75 (0)	2.0 (1.68)	1.34 (0)	60.00	42.18
1.75 (0)	1.0 (0)	0.23 (-1,68)	58.70	37.73
1.75 (0)	1.0 (0)	2.43 (+1,68)	58.20	41.98
1.75(0)	1.0 (0)	1.34 (0)	67.60	42.13
1.75 (0)	1.0 (0)	1.34 (0)	67.40	42.28
1.75 (0)	1.0 (0)	1.34 (0)	67.10	42.03

Where GH12, GH5 and CE1 **(mg protein/$g_{substrate}$)** corresponds to endoglucanase, exoglucanase and Feruloyl Esterase, respectivelly. COS corresponds to the sum of total cello-oligossaccharides concentrations: cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentose (C5) and cellohexaose (C6).

This may be justified by the higher proportion of available cellulose in the reaction medium, and the lower percentage of lignin in the solid fraction from SS treatment (Table 6.3). Although the lignin content is low, it can still adsorb enzymes during the process, significantly interfering with enzymatic hydrolysis processes (Goldbeck et al., 2016). Several strategies have been studied in order to increase enzymatic hydrolysis efficient by impeding exposure of the lignin surface due to its great capacity to adsorb cellulases (Ko et al., 2015).

Good reproducibility was observed for both experiments, as the tests numbered 15, 16 and 17 (central points) presented highly similar results (Table 6.6). Models that reflect COS release were generated based on the CCRD results, and the coefficients were evaluated at $p < 0.05$. The second-order coded models for COS production by enzymatic hydrolysis of selected sugarcane straw and coffee husk cellulose recovery substrates (SS5 and CH5), respectively are represented by the fallowing equations:

$$COS_{SS5}\ (mg).(g_{substrate})^{-1} = 67{,}45 + 9{,}12\ x_1 - 5{,}97\ x_1^2 + 5{,}37\ x_2 - 5{,}91\ x_2^2 - 3{,}45\ x_3^2 + 3{,}69\ x_1\ x_2 - 4{,}19\ x_1\ x_3$$

$$COS_{CH5}\ (mg).(g_{substrate})^{-1} = 42{,}23 + 5{,}26\ x_1 - 2{,}43\ x_1^2 + 2{,}13\ x_2 - 1{,}41\ x_2^2 + 0{,}94\ x_3 - 1{,}08\ x_3^2$$

where x1, x2 and x3 correspond to endoglucanase (GH12), exoglucanase (GH5) and Feruloyl Esterase (CE1) (mg protein/$g_{substrate}$), respectively.

In order to verify the validity of the obtained mathematical models, an analysis of variance (ANOVA) was performed (Syplementary material, Tables S6.1 and S6.2). The F values were calculated for the regression, considering all studied enzymes, were much higher than the F critical values for both experiments (approximately 8x for COS production through SS5 cellulose and 20x for CH5). In addition, the percentage of variance (R^2) explained by the models was greater than 95%.

Figure 6.1 represents the CCRD-generated contour curves for the optimal range of COS production enzyme concentrations (DP 2-6) after 48 h. As shown in the contour curves, we observe a synergistic action between CE1 with the other evaluated enzymes. In this case, significant interactions between GH5 and GH12, as well as between GH5 and CE1 were

observed, to produce COS using the selected cellulose recovery substrates from the two biomasses (SS5 and CH5); achieving maximized yields using low protein enzyme concentrations.

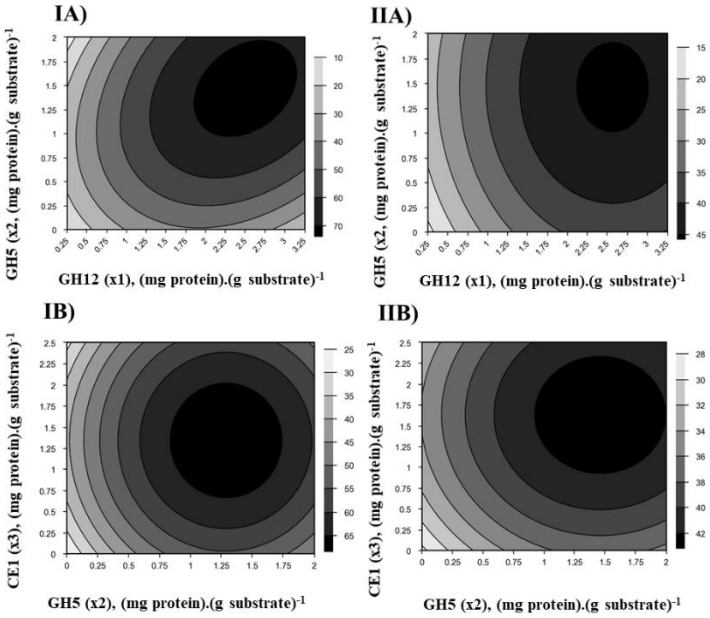

Figure 6.1. Contour curve plots of CCRD results, illustrating optimum concentrations for endoglucanse (GH12) and exoglucanase (GH5) (A), exoglucanase and feruloyl esterase (B); and for the release of COS from the selected cellulose recovery substrate from sugarcane straw (SS5) (I) and coffee husk (CH5) (II).

The model validations were performed using the enzymatic combinations (GH12 + GH5 + CE1) under optimized conditions through experimental tests in triplicate, which resulted in yields of 63.56 ± 0.56 mg. gsubstrate^{-1} and 41.78 mg. gsubstrate^{-1}, using the selected cellulose recovery substrates SS5 and CH5, respectively. The results predicted by the obtained models were close to the experimental results, demonstrating that our strategy to develop efficient enzymatic mixtures for COS production was optimal. From the optimized enzyme concentration, the COS profile obtained along with the hydrolysis times of 12, 24 to 48 h were investigated (Figure 6.2). The results demonstrated a considerable ability of the enzymatic mixtures to release short-chain COS (C2-C6), mainly cellobiose (C2) with very low glucose (C1) concentrations. Regarding hydrolysis times, although a higher production yield was observed in 48 h, the higher production rate was observed after 12 h (Figure 6.3). This could be justified by the high C2 production, which might have inhibited exoglucanase and endoglucanase enzymes during longer hydrolysis times. It has also been reported that cellobiose

could inhibit the accesses of cellulases, by forming an enzyme–saccharides complex and have a potential impact on enzymatic hydrolysis yields (Chu et al., 2014).

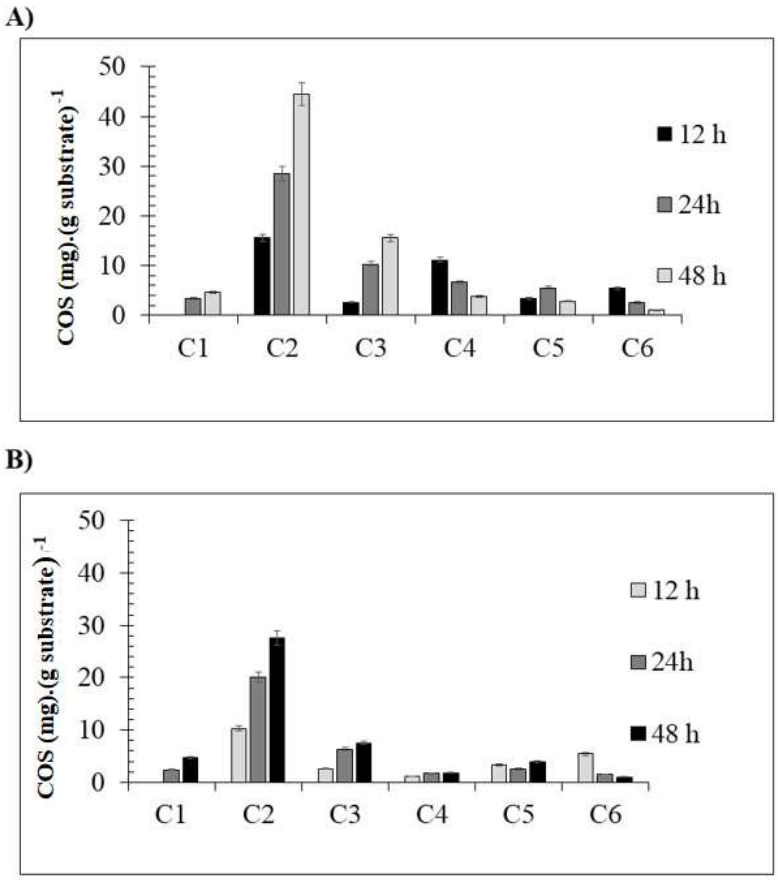

Figure 6.2. Cello-oligosaccharide profiles obtained from the selected cellulose recovery substrate from sugarcane straw (SS5) (A) and coffee husk (CH5) (B) after 12, 24, and 48 h of enzymatic hydrolysis. Concentrations were calculated using a standard curve for glucose (C1), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5) and cellololohexaose (C6).

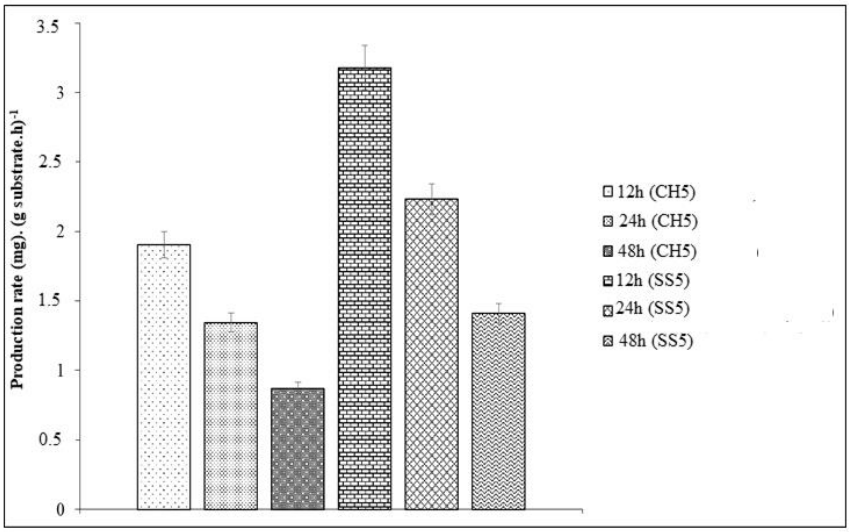

Figure 6.3. Production rates of cello-oligosaccharides through enzymatic hydrolysis of the selected cellulose recovery substrate from sugarcane straw (SS5) and coffee husk (CH5) after 12h, 24h and 48h.

Although the optimized range, in relation to enzyme concentrations was determined, the concentrations of short-chain COS were considerably lower in relation to other reports of oligosaccharides provided from the other lignocellulose polysaccharide sources, such xylo-oligosaccharides(Ávila et al., 2020a; Zhou et al., 2019). However, the values obtained are significant when compared with the few reports in the literature about COS production from lignocellulose materials. Amongst the few reports, Barbosa et al. (2020b, 2020c) was highlighted; using a similar strategy to optimize COS production, they achieved approximately 60 mg/g of hydrothermally pretreated SS using a combination of heterologous expressed cellulases. These results were very similar to those obtained in this study. Therefore, COS production from lignocellulose materials is promising and not yet fully explored, as different enzyme mixtures can still be evaluated for increased production and possible synergistic effects; with the aim of developing efficient and low cost enzymatic cocktails to produce these functional compounds.

6.4. Conclusion

The hydrothermal alkaline treatment (Treatment 5) showed a similar effect to treatment with moderate conditions using high KOH concentration (Treatments 1 and 2) with great cellulose content and considerable hemicellulose solubilization in the alkaline medium; along with possible process integration for obtaining both polysaccharides (cellulose and

hemicellulose) with a considerably reduced of alkali reagent input. The enzyme combination (GH5 + GH12 + CE1) displayed potential effects in both cellulose sources, achieving considerable COS yields and low glucose concentrations. This could also result in a probably simplification and consequent cost reduction at the purification step for future food and chemical industry applications.

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Capítulo 7

Cello-oligosaccharides production from multi-stage enzymatic hydrolysis by rich cellulose agroindustrial wastes and their prebiotic activity

In submission to Carbohydrate Polymers

Cello-oligosaccharides production from multi-stage enzymatic hydrolysis by rich cellulose agroindustrial wastes and their prebiotic activity

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Abstract

Cello-oligosaccharides (COS) are very recent promising water-soluble dietary fibers. An efficient approach to their synthesis is throught multi-stage enzymatic hydrolysis of cellulose obtained from previous delignified lignocellulosic biomass through "adsorption" of endoglucanese enzyme in the substrate aiming to obtain a high yield of COS production. A three-stage enzymatic hydrolysis of cellulose extracted from agroindustrial wastes (sugarcanae straw and coffee husk) with the 1,4-β-endoglucanase cellulase (GH12) was proposed totalizing 48 hours of process. Analysis indicated that the removal of hydrolysis products (glucose and cello-oligosaccharides) at each stage improved cello-oligosaccharides enzymatic hydrolysis production. The COS concentration released in-stage enzymatic hydrolysis were significantly improved, which were approximally 65% higher than single-stage hydrolysis. The COS were examined for growth promotion of probiotic strains and they showed up stimulation of cell density for *Lactobacillus acidophilus* and *Lactobacillus brevis,* but were less efficient with *Bifidobacterium sp*. This study shows the COS as selectively functional carbohydrates with prebiotic potential and demonstrates their efficient enzymatic production using multi-stage strategy.

Key-words: Cello-oligosaccharides, Lignocellulosic biomass, Enzymatic hydrolysis, Prebiotics

7.1. Introduction

Non-digestible oligosaccharides (NDOs) are oligosaccharides with a degree of polymerization (DP) of 2–9 that are resistant to digestion by human gastric and pancreatic enzymes. Cello-oligosaccharides (COS) are NDOs defined as saccharides consisting of glucose by β-1,4-linkages and important functional oligosacchaides in the feed and food field mainly due to the prebiotic potential application. Therefore these molecules could be present as substrates for probiotic microorganisms that produce important metabolites, recently called postbiotics, which are associated with most health-promoting effects. However, there is still limited information available for their large-scale production (KARNAOURI et al., 2019).

Cellobiose and other cello-oligosaccharides (COS) can be obtained by acid and enzymatic hydrolysis of the insoluble cellulose. Enzymatic hydrolysis was described for many authours as more attractive due to the relatively mild reaction condition and the easier control of the polysaccharide cleavage breaking (BARBOSA et al., 2020). During their production process the oligosaccharides and monomers are formed which may cause an inhibition of the enzymes involved (CHU et al., 2014). It has been reported that cellobiose may inhibit the binding of cellulases enzyme–saccharides complex (YU et al., 2012), and further have an impact on hydrolysis efficiency. Aiming to alleviate the product inhibitions on enzymes, multi-stage enzymatic hydrolysis strategies have been perfomed, in which enzymatic hydrolysis was separated into some stages, and the product formed was removed after each stage (CHU et al., 2014). According to some authors the multi-stage enzymatic hydrolysis could effectively remove product inhibition and significantly enhance hydrolysis efficiency (CHU et al., 2014 and YU et al., 2012).

Endoglucanases (EG) are key enzymes for the production of COS in many ways. Firstly, a class of them, namely processive, that have higher preference toward glycosidic bonds near the end of cellulose chains, are able to release soluble COS (mainly C2 and C4) before detaching from the substrate (WILSON AND KOSTYLEV, 2012). In addition, individual cellulolytic activity of EG has been related to rapid and efficient liquefaction of cellulose-rich lignocellulosic materials, under high dry matter loadings, which is of out most importance in order to achieve high COS yields (KARNAOURI et al., 2019; KARNAOURI et al., 2018).

This study explore an effective approach for cello-oligosaccharides production by multi-stage enzymatic hydrolysis of cellulose obtained from alkali- recovery celulose from agorindustrial wastes (sugarcane straw and coffee husk) using an endoglucanase (G12) enzyme. Firstly, the, multi-stage enzymatic hydrolysis by endoglucanase was assessed, aiming to reduce

the product inhibition at each stage to improve the COS yields. Secondly the COS production were assessed for growth promotion of a representative selection of probiotic *Lactobacillus* and *Bifidobacterium* strains. In addition digestibility tests of COS produced also were performed using digestive enzymes involved in human metabolism, in order to prove its stability during human metabolism in order to evidence their emerging prebiotic potential.

7.2 Material and Methods

7.2.1. Solid cellulose rich substrates

The solid rich cellulose substrates previously characterized were provided from a integrate process of alkali treatment of holocellulose from sugarcane straw and coffee husk described by Ávila, Martins and Goldbeck (2021). The raw materials were previously delignified according to Wise et al. (1946) protocol, using a sodium chlorite treatment to isolate the holocellulose fraction. Then the alkaline treatment was applied to the holocellulose fraction in triplicate using a 4.5% of potassium hydroxide, and under 121°C throught steam application for 30 minutes. The solid product was filtered, washed thoroughly with water and dried at 60 ° C in a stove for 72 h. The main composition of the result cellulose substrates as follows (dry weight basis): cellulose (75.21, 73.54%); hemicellulose (11.17, 11.65%); lignin (4.70, 10.61%), for substrates from sugarcane straw and coffee husk, respectively.

7.2.3. Enzymes and their activity measurements

Endo-1,4-β-D-glucanase- *Aspergillus ninger* (GH12), required from Megazyme® was used in the enzymatic hydrolysis experiments. The filter paper activity and CMCase activities were determined according to IUPAC recommendations (GHOSE et. al. 1987). The resulting reducing sugars were analyzed using a DNS assay (MILLER, 1959).

7.2.5. Multi-stage hydrolysis of cellulose rich substrates for cello-oligosaccharides production

Multi-stage hydrolysis experiments with prior CMCase enzymes activity adsorbed in the substrate were carried out to alleviate the product inhibition to cello-oligosaccharide production as described by Chu et al. (2014) with some modifications. The cellulases with dosage of 15 FPU was prior adsorbed in the substrates using cellulose consistency of 5% (2.5 g cellulose per 50 ml reaction mixture) in Na_2HPO_4–citric acid buffer pH 7, shaking at 150 rpm at 10°C for 30 minutes. After adsorption, the supernatant was collected at 3000 rpm, for 10 min for determination of CMCase activity. Then the multi-stage hydrolysis was conducted in 250

ml Erlenmeyer flasks at 50°C using 50 mM citrate buffer (pH 4.8) with shaking at 150 rpm. The enzymatic hydrolysis included experiments with three-stages including 6 + 18 + 24 hours, totalizing 48 hours. At the end of each stage, the solid substrate was separated from the liquid phase by centrifugation. The fresh buffer (pH 4.8) with the same volume of the removed supernatant was added to the solid residue to carry out the next stage hydrolysis. The supernatants of hydrolysis experiments were analyzed for soluble sugars production.

7.2.6. *In vitro* digestion tests

The *in vitro* resistance of COS produced by enzymatic hydrolysis of sugarcane straw cellulose and coffee husk cellulose to the three simulated digestive fluids: simulated salivary fluid (SSF), simulataed gastric fluid (SGF) and simulated intestinal fluid (SIF). The tests were performed in an Dry Bath (Model: DBH-S, Loccus Biotecnologia, Brazil) at 500 rpm, maintained at 37 ± 1 °C for 4h and then immersed in a boiling water bath for 10 min to deactivate the enzyme, acording with the protocol of Minekus et al. (2014). As a control, COS and were incubated under the conditions described in the following subsections (7.2.7.1 to 7.2.7.3), but without the addition of digestive enzymes. Remaining oligosaccharides were evaluated by Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) as described in the 7.2.6 section.

7.2.6.1. Digestion by amylase present in human saliva

The COS-rich hydrolysates were added to SSF electrolyte stock solution of 50:50 (w/v), where α-amylase (Sigma Aldrich) was added to achieve 75 U/mL, followed by $CaCl_2$ to achieve 0.75 mM in the final mixture.

7.2.6.2 Digestion by artificial gastric juice

The oral fase (sample + SSF electrolyte stock solution) was added to SGF electrolyte stock solution of 50:50, the pH was adjusted to 3 with hydrochloric acid and monitored. SGF electrolyte stock solution was prepared by the addition of 0.3 mol/L sodium chloride and porcine pepsin (Sigma Aldrich) 2000 U/mL in distilled water.

7.2.6.3. Digestion by porcine pancreatic enzymes

The gastric phase (sample + SGF electrolyte stock solution) was added to SIF electrolyte stock solution of 50:50, the pH was adjusted to 7 with sodium hydroxide and monitored. SIF stock solution with addition of 0.3 mol/L sodium chloride, 800 U/mL pancreatin and 10 mM bile salt (Sigma Aldrich), suspended in distilled water.

7.2.7. “In vitro” fermentation of COS

7.2.7.1. Microorganisms

The freeze-dryed cultures of probiotic pattern microorganisms *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Bifidobacterium longum*, *Bifidobacterium bifidum, Bifidobacterium animalis* subsp. *lactis* were obtained from Tropical Culture Collection (CCT) by André Tosello Foundation, Campinas, SP, Brazil.

7.2.7.2. Growth medium

The COS fermentation experiment by growing probiotic bacterias was carried out in three different medium: without any carbon source (negative control), only glucose as a carbon source (Positive control) and COS as an increment in a glucose carbon source (test). The growth medium to *Lactobacillus* strains was the proposed by de Man, Rogosa and Sharpe (1961) (MRS). *Bifidobacterium* cultures were grown in Reinforced Clostridial medium (RCM) prepared by adding Sodium acetate (3g/L), Agar (0.5 g/L), Starch soluble (1 g/L), L-cysteine (0.5 g/L) and Peptone (10 g/L) at 37°C. Filter sterilized 0.10 gm% of COS and 2 g/L of glucose were added as carbon sources in the basal medium. However, the control media did not contain any carbon source. The medium were prepared at pH 6.7 and autoclaved for 15 min at 121 °C. The active inoculum of probiotic culture to be tested was inoculated each in negative control, test and positive control and incubated at 30°C and 37 °C for *Lactobacillus* and *Bifidobacterium* strains, respectivelly for 12, 24 and 48 h for in anaerobioc conditions using anaerobic jar. The grown of probiotic cultures in COS was monitored by optical density at 600nm, COS profile consumption, pH and organic acids production. The aliquot was centrifuged at 12,000 rpm for 5 min and the supernatant was collected to analyze the residual COS profile. The increase in optical density were employed to demonstrate indirectly the cell growth and a suggest increase of fermentation activity of probiotic bacteria respectively (ÁVILA et al., 2020; SINGH et al., 2015).

7.3. Analytical quantification products

The content of glucose and COS in the supernatants of enzymatic hydrolysis was determined by High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex DX-5000 ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) according to the method described by Barbosa et al. (2020), with some modifications. The samples were diluted in ultrapure water, filtered through a 0.22 μm filter and injected into the column using an auto-sampler. The flow rate was

1 mL/min, and the injection volume of the samples was 100 µL. Data were acquired and processed using PEAKNET software. The glucose and COS (Cellobiose, Cellotriose, Cellotetraose and Cellohexaose) were identified in samples by comparing the retention times with authentic standards. Calibration curves were constructed using commercial standards (Megazyme, Bray, Ireland) to quantify the glucose and COS in the samples.

Supernatants from anaerobic fermentation were filtered hrough a 0.22 µm and analysed for their content in acetic, lactic, formic, propionic, and butyric acids using an HPLC (Thermo Fischer Scientific, USA) system equipped with an infrared detector (Thermo Fischer Scientific, USA) at 50°C and a Aminex HPX-87H (BioRAd California, USA) column at 50°C, using 5 mM H_2SO_4 as mobile phase at 0.6 mL/min. Total short chain fat acids (SCFA) were calculated as the sum of acetic, propionic, formic, and butyric acid concentrations. Calibration curves were constructed using analytical standard solutions (Sigma–Aldrich, USA).

7.3. Results and Discussion

7.3.1 Enzymatic hydrolysis

Prior multi-stages enzymatic hydrolysis experiments it was performed the adsorption of CMCase activty in the rich cellulose substrates from sugarcane straw and coffee husk at cellulose consistency of 5%, pH 7.0 at 10°C for 30 min. The adsorption of CMCase activity in the substrates were 92.78 ± 0.56% and 89.78 ± 0.98% for substrates provided from sugarcane straw and coffee husk, respectively. By conducting multi-stage enzymatic hydrolysis, a significant increment in the cello-oligosaccharides yield around 65% was obtained within the same reaction time (48 h), compared to the single-stage enzymatic hydrolysis experiment (Table 7.1).

Table 7.1 Cello-oligosaccharides production by enzymatic hydrolysis multi-stages.

Enzymatic hydrolysis strategies/substrates	Cello-oligosaccharides (mg.) ($g_{celulose}$)			
Sugarcane straw	**The first stage**	**The second stage**	**The third stage**	**COS (DP 2-6)**
6+ 18 +24h	28.87 ± 0.45	48.78 ± 0.32	7.78 ± 0.23	85.43 ± 1.00
48h	51.78 ± 0.87	-	-	51.78 ± 0.87
Coffee husk				
6+ 18 +24h	21.58 ± 0.57	32.89 ± 0.63	5.89 ± 0.34	60.36 ± 1.54
48h	47.89 ± 0.67	-	-	47.89 ± 0.67

The elimination of sugars in the hydrolysis system, the product inhibition caused by sugars accumulation was greatly reduced, which resulted in a much higher cello-oligosaccharides

production for both substrates. Chu et al. (2014) enhanced an increment of 51.78% to 75.56% in the COS production yields for single to three-stages of enzymatic hydrolysis, respectively using a cellulase cocktail in a previous work. The increment obtained in this work was significant higher, it could be explained by the use of a purified endoglucanse enzyme activity and totally absence of β-glucosidade activty. The oligosaccharides and monomers that are formed certainly caused an inhibition of the enzyme involved. Especially, the produced of cellobiose that have inhibition on exoglucanases and endoglucanases. It has been reported that cellobiose may inhibit the binding of cellulases by formingan enzyme–saccharides complex and its removing between stages in the enzymatic hydrolysis experiments has an great impact on hydrolysis efficiency in the COS production (CHU et al. 2014; YANG et al., 2010).

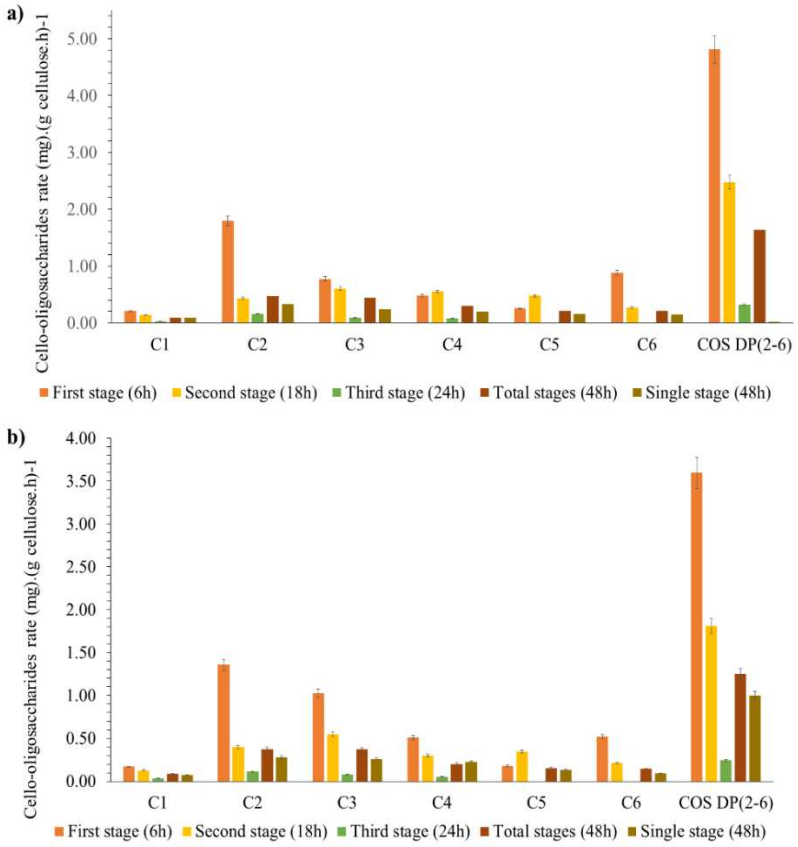


Figure 7.1. Cello-oligosaccharides profile rate in single and multi-stage enzymatic hydrolysis from cellulose rich substrate from sugarcane straw (a) and coffee husk (b).

Figure 7.1 shows that both enzymatic hydrolysis strategies (multi or single stage) released mainly celobiose (C2) and smaller concentrations of glucose (C1), from both substrates. The kinetics COS profile from multi-stage enzymatic hydrolysis experiment presented a higher hydrolysis rate in the first 6 h, mainly the celobiose fraction. However in the last 24 hours of the process a very small increment of cello-oligosaccharides production was

obtained. It was also possible to observe a considerable increment in all the fractions (C2, C3, C4, C5 and C6) on the total multi-stage process in relation the single stage enzymatic hydrolysis process in the use of both substrates (Figure 7.1). The reasons for the slower and limited conversion of one-stage enzy-matic hydrolysis can be originated from various aspects, and product inhibition was reported as a significant factor, which affected the rate-reducing kinetics of enzymatic hydrolysis (YU et al., 2012; CHU et al., 2014).

Thus according to the results of three-stage hydrolysis listed in Table 7.1 a concept of three-stage hydrolysis to alleviate the product inhibition at each stage, contributed to improving the cello-oligosaccharides rate production in the result from both residual cellulose delignified wastes, which also showed a great potential in terms of supply the production of this prebiotics in the local area and offer a new application for these industrial wastes.

7.3.2. Prebiotic potential

7.3.2.1. *In vitro* digestibility of cello-oligosaccharides

The digestibility tests of cello-oligosaccharides had the purpose of simulating the gastrointestinal tract to prove their resistance to the action of enzymes found in the digestive system. There is not anyone recent articles assessing the ability of COS to reach the intestine intact and the majority of them evaluate the digestibility of FOS or XOS. The celo-oligosaccharides produced from both sources did not show any degradation when incubated with salivary amylase enzyme and suffered the greatest degradion values by pancreatin around 9% in the C2 and C3 from sugarcane straw cellulose (Figure 7.2a) and 8% in the C4 and C3 from coffee husk cellulose in gatric juice (Figure 7.2b). The results were very similar to obtained by de Figueiredo et al. (2020) for commercial FOs DP 2−3 kestose (GF2) and nystose (GF3) which showed resistance to the amylase enzyme and degradation of 10% in GF2 in 4 h by porcine pancreatin and 11% in GF3 on gastric juice. Therefore, the results of this present work were positive for COS classification as prebiotic ingredients and proved their resistence to the action of digestive enzymes, in accordance with one of the criterias used for the classification of prebiotic ingredients (OKU, TOKUNAGA and HOSOYA, 1984). The evaluation of the digestibility of oligosaccharides by enzymes is essential in order to test their ability to reach the intestine intact, where they serve as substrate for the fermentation by probiotic bacteria. In this sense, the prebiotics inhibits the proliferation of pathogenic bacteria in the intestinal colon (de FIGUEIREDO et al., 2020).

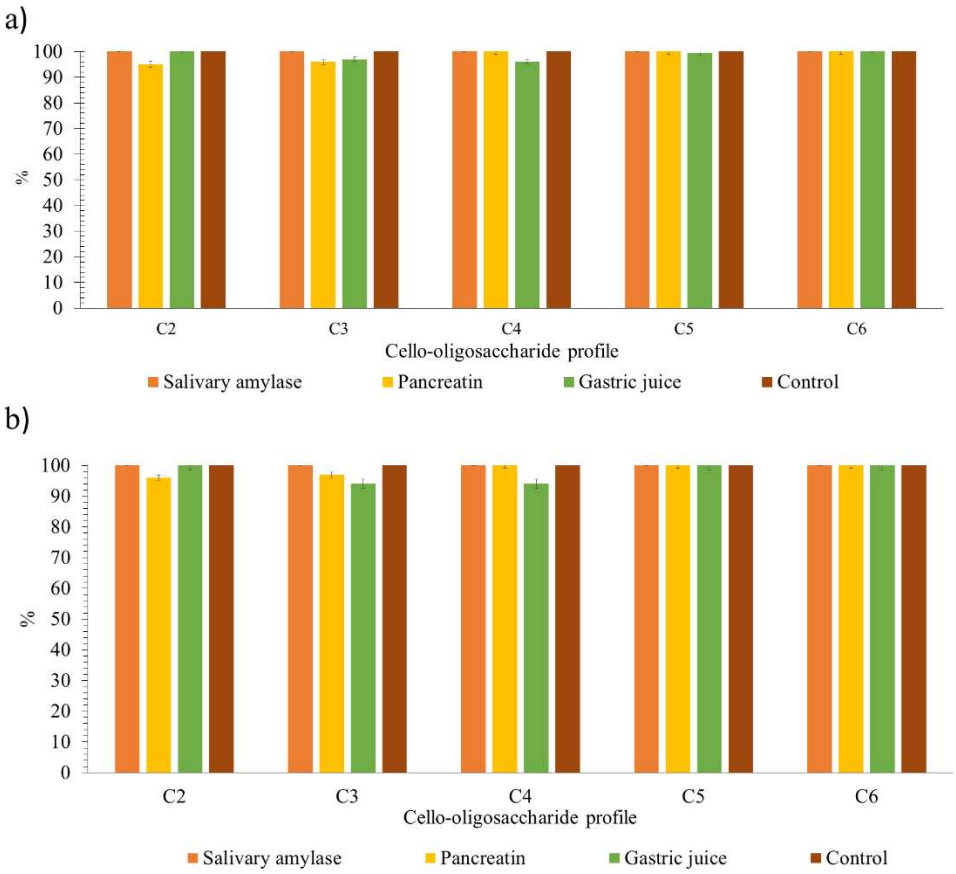


Figure. 7.2. Digestibility profile of cello-oligosaccharides from cellulose substrate from sugarcane straw (a) and coffee husk (b) by enzymes present in the gastrointestinal tract after 4 h of incubation at 37 °C. C2 cellobiose, C3-cellolotriose, C4-cellotetraose, C5- cellopentaose, C6-cellohexaose. Results are mean ± SD of three replicates.

7.3.2.2. *In vitro* fermentation of cello-oligosaccharides

The *in vitro* fermentation tests were carried out to demonstrate the COS ability to stimulate the growth of beneficial bacteria. The strains used were (*Bifidobacterium spp. and Lactobacillus spp.*) frequentlly considered in the studies that evaluate compound prebiotic potential. We chose *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Bifidobacterium longum*, *Bifidobacterium bifidum, Bifidobacterium animalis* subsp. *lactis* strains, which were recommended as a probiotic claimed for the maintenance of intestinal microflora. Unlike previous studies that used the COS in individual DP or COS from enzymatic hydrolysate containing a large amount of cellobiose, the current study focused on the potential prebiotic effect of COS mixtures with DP centered at 2−6.

Results of the growth analysis by optical density (OD600) are summarized in Figure 7.3.

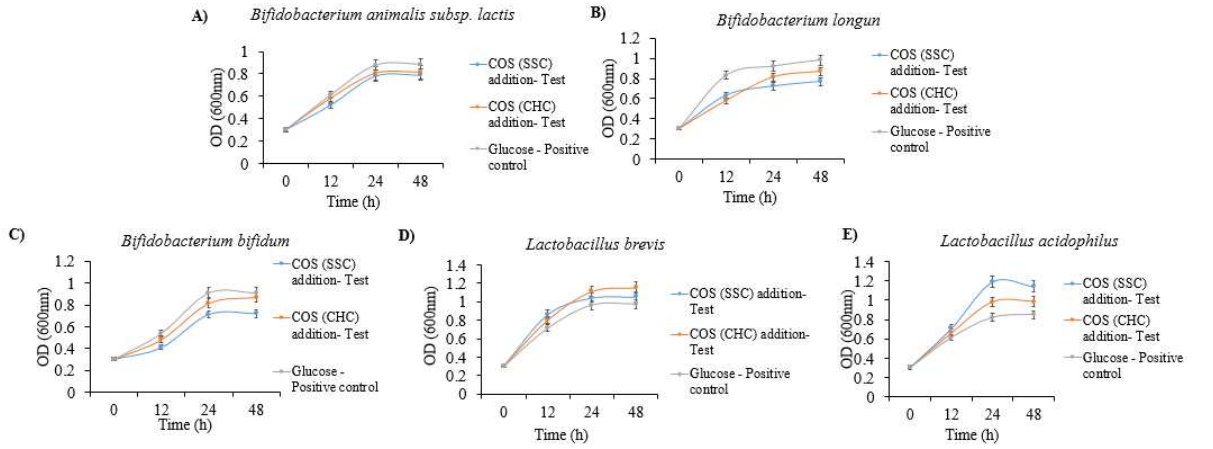


Figure 7.3. The density optical (OD) parameter during the growth of probiotics *Bifidocaterium subsp. lactis* (A), *Bifidobacterium logum* (B), *Bifidobacterium bifidum* (C), *Lactobacillus brevis* (D), and *Lactobacillus acidophilus* (E) on glucose with COS addition prepared by bioconversion of agricultural waste cellulose from sugarcane straw COS(SSC) and coffee husk COS(CHC) and in glucose source as a positive control.

Lactobacillus strains showed preference for COS increment in glucose medium, being able to ferment with better efficiency than in glucose medium as a sole carbon source and consumpted preferentially the C2 and C3 fractions (Figure 7.4). On the other hand, the *Bifidobacterium* strains did not present significative increment in the growth in the medium with COS increment, and presented only glucose consumption (Figure 7.4). Similar trends was found by Zhong et al. (2020), acoording to the authors the corresponding (β-1,4-specific) transporters were rarely found in the *Bifidobacterium* strains. The observed growth of these strains on COS might be explained by the non specific uptake mediated by unrelated transporters with the promiscuous specificities. Collectively, the presence of a system comprised of suitable transporter and/or catabolic enzymes may be required for bacteria to efficiently grow on the COS.

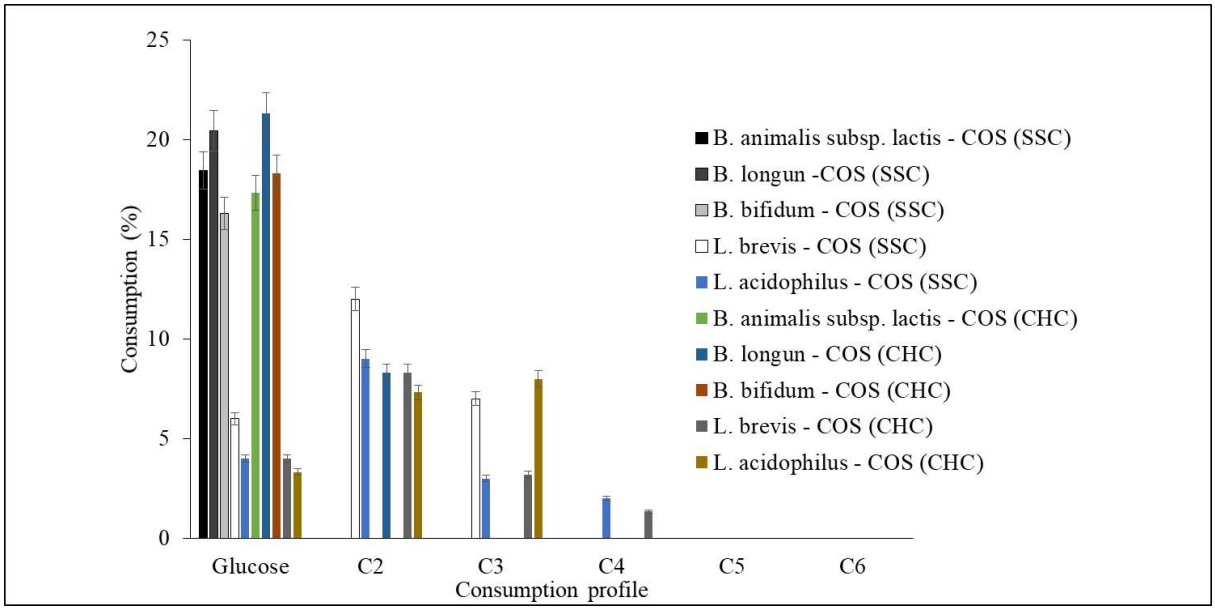


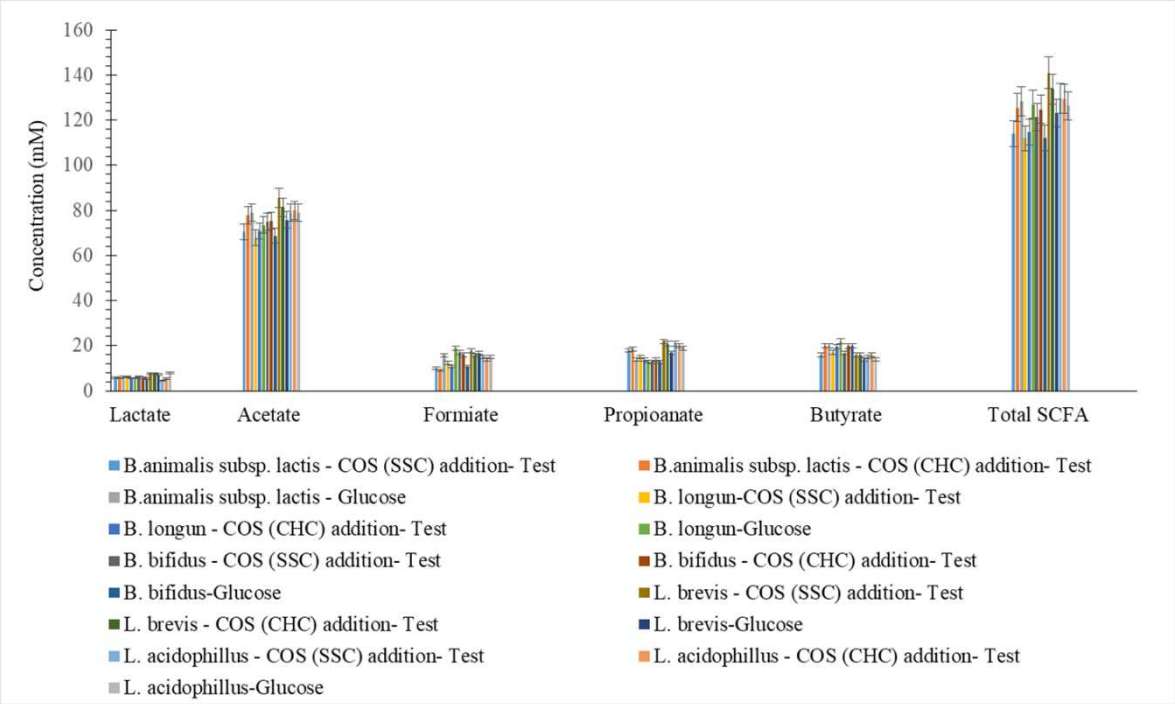
Figure 7.4. Consumption profile of glucose and cello-oligosaccharides, cellobiose- C2, cellotriose- C3, celotetraose- C4, cellopentaose- C5 and cellohexaose- C6 from the cellulose of sugarcane straw (SSC) and coffee husk (CHC) after *in vitro* fermentation tests with probiotic strains.

7.3.2.3. Organic and short chain fat acids production

The concentrations of short-chain fatty acids in samples were analysed at the final fermentation assays (48h). It must be taken into consideration that the structure of oligosaccharides (degree of polymerization, purity, presence of substituents, and/or type of linkages) can affect to fermentation pattern, including the product profile (RINNINELLA et al., 2019; ZENG et al., 2014). Figure 7.5 shows the concentration of lactate, fat short chain acids (SCFA) and the pH evolution of the media during the fermentation assays. The evolution in pH values was similar in experiments carried out with COS increment from both sources sugarcane straw (SSC) and coffee husk (CHC), decreasing from 6.70 to 4.51 and 4.52, respectively (Figure 7.5 II). Increased acidity during fermentation is an indicator of substrate utilization and organic acid production. The significant drops in pH with COS increment (pH drops were in the ranged from 2.03 to 2.19) demonstrated that the COS increment substrates tested were readily by probiotic bacterias. Similar decreases in pH (decreases in pH 2.0 units) were obtained by Álvarez et al. (2020) when purified xylooligosaccharides were used *in vitro* experiments inoculated with human fecal microbiota. This acidification is a positive result as it hinders the proliferation of some pathogenic microorganisms, preventing the accumulation of toxic compounds such as ammonia or biogenic amines (YOO et al., 2012). In the evolution of pH through fermentation, two stages are distinguished at the first stage, where this decrease is

significant (early 12 h) due to the growth and/metabolic activity of probiotic bacteria, and then remains relatively constant (early 24h).

I)



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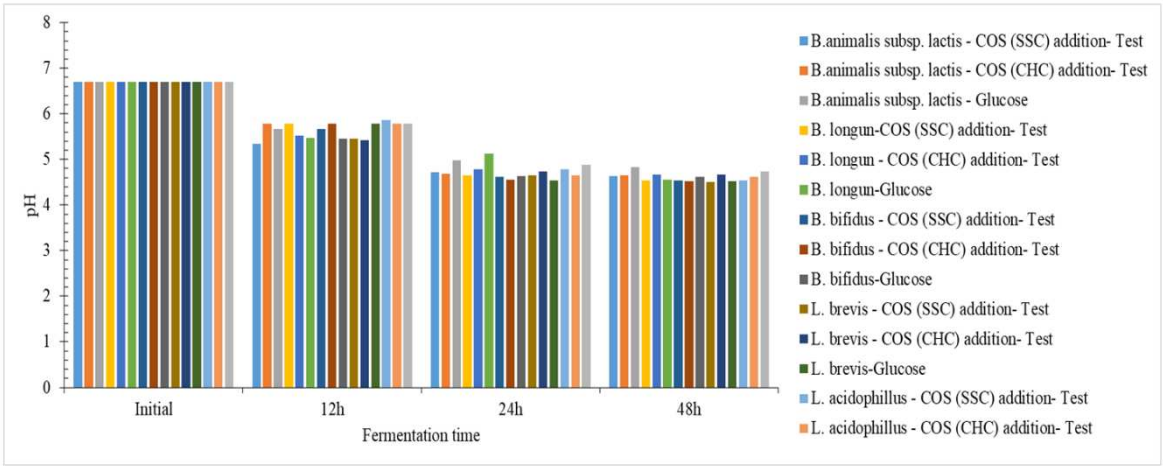

Figure 7.5. Concentration (mM) of (SCFA) short chain fat acids (acetate, formiate, propionate and butyrate) and lactate after 48h of fermentation (I), pH values during the fermentation assays (II) using only glucose (positive control), COS (SSC) and COS (CHC) addition as a carbon source.

Similar results were reported by Gullón et al. (2014), where the decrease of pH was observed mainly during the first 12h of fermentation. As expected, a mixture of organic acids mainly made up of acetic, butyric, and propionic acids were generated in the media due to the

fermentation of substrates. These results are in agreement with the data reported by Álvarez et al. (2020). After 48h of incubation, the maximun total concentrations of SCFA in the media were 141.02 mM and 133.79 mM, for COS increment from SSC and CHC, respectively. All the carbon sources evaluated in this work induced lactate production in the range of 4.43 to 7.88 mM. The reason for the production is that some bacteria can consume lactate generating other metabolites such as acetate, butyrate, and propionate. The most lactate concentration was detected in glucose from *Lactobacillus acidophillus* assay (7.88 mM), agree to the decrease in pH around 1.96 units. Acetate was the most abundant organic acid produced during the fermentation, regardless of the carbon source used, accounting until 62.38%, of total SCFA. The highest concentrations of this acid were achieved at 48 h (85.49 mM and 81.36 mM from COS increment from CHC and SSC, respectively). The predominant formation of acetic acid was similar to that reported by several authors when used other oligosaccharides derived from lignocellulosic biomass (ÁLVAREZ et al., 2020, RUIZ et al., 2017; DÁVILA et al., 2019). Butyrate was the second compound relevant metabolic product found in the fermentation medium for *Bifidobacterium* strains. However for the *Lactobacillus* strains the proprionate was the predominant metabolic product found after 48 h of incubation. The values observed for butyrate and propionate in this study were higher than those obtained with other substrates such as oligosaccharides from vine shoot reported previously (ÁLVAREZ et al., 2020; DÁVILA et al., 2019). Butyrate is considered one of the most essential colon metabolites as it has multiple positive functions. For instance, it was reported to induce the differentiation and apoptosis inhost cells, it is used as an energy source for colonocytes, and italso has anti-inflammatory properties (FERNÁNDEZ et al., 2016).It was demonstrated that rectally administered can relieve the symptoms of inflammatory bowel disease (ROBERFROID et al.,2010). Propionic acid is another compound with positive health effects. The liver used it, and there is a close relation between propionic acid and the inhibition of lipid andcholesterol synthesis (VAN DEN ABBEELE et al., 2018). Moreover, butyrate and propionate were reported to stimulate mucins production, which are glycoproteins required for themaintenance of the mucous layer that protects the intestinalepithelial tissue (ROBERFROID et al., 2010). Finally, the highest amount of formic acid produced was using only glucose as a carbon source (18.8 mM) in *Bifidobacterium longum* assay. The production of formic acid is related to the formation of extra ATP, necessary for *Bifidobacterium longum* growth on sugars metabolized slowly (VANDERMEULEN et al., 2004).

7.4. Conclusion

The multi-stage enzymatic production was a efficient strategy to alleviate the product inhibition at each stage and to contribute to improving the cello-oligosaccharides production in use the both rich cellulose substrates (sugarcane straw and coffee husk), which presented a great potential in terms of supply the production of this emerging prebiotic oligosaccharide. The prebiotic potential of short chain soluble COS with DP 2−6 was confirmed by the resistance of COS to the action of enzymes found in the gastrointestinal tract and resistance to acidity of gastric juice. As well as by the ability of Lactobacillus strains to ferment preferencially these oligosaccharides as source of nutrients for growth.

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Capítulo 8

Fractionating of functional oligosaccharides using nanofiltration serie membranes and their influence on the prebiotic activity

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Fractionating process of functional oligosaccharides using serial nanofiltration membranes and their influence on the prebiotic activity

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Abstract

Functional oligosaccharides are non-digestible by human gut enzymes and providing health benefits as fibers and prebiotics. The cello-oligosaccharides (COS) and xylo-oligosacharides (XOS) are functional oligosaccharides provided from xylan and cellulose, respectivelly present in the lignocellulosic material. In this study, we proposed to apply the nanofiltration (NF) membranes technology configured as a serial process for oligosaccharides enzymatic hydrolysate fractionation to isolate XOS or COS fractions with short degree of polymerization (DP 2-3) aiming to investigate the impact of the fractionating process in their prebiotic activity. The NF polymeric membranes NP030 (weight cut-off of 500-600 Da) and DK (weight cut-off of 150-300 Da) were employed using fixed defined operational conditions. The operation of diafiltration (DF) was also investigated and it was found that only 1-time DF in NP030 was more suitable strategy and improved significativelly the performance indices for XOS and COS with DP(2-3) in the serial membrane-based process. The short DP fractions obtained favored the cell density for probiotic strains, which presented an increasing in the optical density up to 20% and 25%, respectively, regarding the presented by their growth in the mixture of XOS or COS with DP (2-6) in the original hydrolysates from sugarcane straw. This study brought great contribution to oligosacharides fractionation using NF membranes technology, mainly due to the protocol of investigating DF influence in each oligosacharide and to also demontrate the considerably intensification of the prebiotic property of each fraction for future related application in the food and pharmaceutical industry.

Key-words: Nanofiltration, Prebiotics, Cello-oligosacharides, Xylooligosacharides

8.1 Introduction

Oligosaccharides (OS) are considered functional fibers or prebiotics containing mainly dietary carbohydrates in their composition, that provided by remnants of edible plants, polysaccharides, lignin, and associated substances resistant to digestion by endogen enzymes of humans. Carbohydrate fibers include cellulose, hemicelluloses, lignin, gums, mucilage, pectin, and other associated minor substances present in the lignocellulosic source (BHATIA et al., 2019). More than that, several insoluble and non-fermentable fibers can by hydrolyzed by enzymatic or physical-chemical process to become soluble and fermentable and present functional properties such xylo-oligosaccharides (XOS) from xylan and cello-oligosaccharides (COS) from cellulose (CANO et al., 2020). However the lignocellulose hydrolysate composition depends on the type of raw material, pretreatment, and hydrolysis conditions.These non-distible carbohydrates stimulate the growth of beneficial bacteria in the gut microbiota showing a beneficial effect on body weight control, improving blood glucose and lipid levels (WANG et al., 2011), and reducing the symptoms of colon cancer (AACHARY et al., 2015) In fact, prebiotics are substrates for probiotic microorganisms that produce important metabolites, recently called postbiotics, which are associated with most health-promoting effects (ŻÓŁKIEWICZ et al., 2020). Prebiotics are also cited among the "Top Ten Functional Food Trends", and foods containing these compounds will continue to find a welcome market (SLOAN, 2020).

In general oligosaccbarides with low degree of polymerization (DP), such as (DP 2) and (DP 3), have been presented as potential prebiotic compounds due to their faster consuption by probiotic bacterias grown and therefore are preferred for food-related applications (VAN LOO et al., 1999). In vitro assays proved that *Bifidobacterium* spp are able to utilize both X2 and X3 (SUWA et al., 1999). Therefore, the challenge of fractionation and purification of the hydrolysate is evident. XOS and COS coming from industrial wastes as a bottleneck to overcome. For this purpose, nanofiltration (NF) membranes stand out as a technological solution since that they make up generally compact, energy-efficient systems,easily operable and environmentally sustainable (DE OLIVEIRA, FRANCO and FORTE, 2021)

Specifically for XOS and COS, studies for nanofiltration (NF) membrane-based applications are limited in the literature, maninly for COS, that there are not any study. However some authors already explored the serial membrane-based fractionation of XOS from different sources such as sugarcane straw, cotton stalks and almond shells (DE OLIVEIRA, FORTE and FRANCO, 2021; AKPINAR et al., 2007; SINGH et al., 2019). Also, it is possible to find some

interesting publications that describe the processing of liquors resulting from hydrolytic treatment followed by enzymatic reaction. For example, membranes were applied to XOS obtained from enzymatic hydrolysis or autohydrolysis of xylan-containing materials (VEGAS et al., 2006). The diafiltration (DF), which is the addition of a solvent (usually water) to the feed tank for concentration of a product in the retentate side or recovery in the permeate side have been used a strategy to improve the performance of concentration in the studies for membrane-based applications. De Oliveira, Forte and Franco (2021) demonstrated that 1-time DF operation for each nanofiltration (NF) membrane for XOS hydrolysate improve the performance indices for long XOS degree polymeriazation (DP), achieving final values of 3.7 g/L of concentration, 20.9% purity, and 46.0% yield from sugarcanes straw. Other recent study have also already evaluated the benefits of DF operation, for example, in the extraction of dextran (DÍAZ-MONTES et al., 2020).

This study proposes to evaluate a serial nanofiltration (NF) membrane-based process in the fractionation of enzymatic hydrolysates from xylan and cellulose from sugarcane straw rich in xylo-oligosaccharides (XOS) and cello-oligosaccharides (COS), respectivelly using a set of series NF membranes. The main objective of the process was to obtain fractions with a lower degree of polymerization (DP) and investigate the influence of the process on the prebiotic potential of the short XOS or COS DP fractions. It is important to highlight that although in this study it was applied fixed defined reported operational conditions such as temperature and pressure in the membranes, the diafiltration (DF) operation was investigated in the process as a strategy to improve indices for XOS or COS with short DP (2-3) fractions (concentration, purity and yield).

8.2. Material and Methods

8.2.1. Sugarcane straw hydrolysate liquor

Delignified sugarcane straw hydrolisate rich in XOS or COS was prepared using optimal mixture enzymes concentrations defined in the previous work by Ávila, Martins and Goldbeck (2021) (Chapter 4 and Chapter 6 of this thesis, respectively). The both hydrolysates were reproducted in a higher scale using a 250 mL enrlemeyer flasks at 150 rpm, at 50 °C, pH 5, for 48 h. Then, enzymes were exposed to heat (99 °C for 15 min) for effective inactivation, centrifuged and the supernatant collected for analytical determinations. Due to the considered low concentration of COS obtained in the previous work (Chapter 6 and 7), the COS rich

hydrolysate was previously concentrated in 3 times using retroevaporation equipament. The main composition of the both hydrolysates liquors are presented in the Table 8.1.

Table 8.1. Carbohydrate composition of hydrolysates rich in XOS and COS

Compounds	g/L	
	XOS rich hydrolysate	**COS rich hydrolysate**
Xylose	0.32 ± 0.52	-
Xylobiose	3.78 ± 0.87	-
Xylotriose	1.98 ± 0.56	-
Xylotetraose	1.56 ± 0.87	-
Xylopentaose	0.72 ± 0.34	-
Xylohexaose	0.54 ± 0.32	-
Glucose	-	0.22 ± 0.12
Cellobiose	-	0.74 ± 0.21
Cellotriose	-	0.34 ± 0.32
Cellotetraose	-	0.23 ± 0.12
Cellopentaose	-	0.46 ± 0.32
Cellohexaose	-	0.25 ± 0.12

8.2.2. Oligosaccharides fractionating process

The XOS and COS fractionation strategy was perfomed using the same procedures and optimal operational conditions defined by de Oliveira, Forte and Franco (2021) in serial nanofiltration (NF) membranes (Table 8.2).

Table 8.2. Operational conditions in the nanofiltration (NF) membranes.

NF Membrane	MWCO (Da)	pH	Temperature (C°)	Pressure (bar)
1st NP030	500-600	4.6	20	40
2nd DK	150-300	4.6	50	20

MWCO, molecular weight cut-off

The hydrolysates provided with XOS or COS was applied as feed solution to a 1st NF membrane NP030 (MWCO of 500-600 Da) retaining XOS or COS with higher degree of polymerization (DP) between 4-6, while permeating XOS or COS with lower DP between (2-3) and monosaccharides, xylose or glucose. Then, the permeate from 1st NF membrane was applied as feed solution to a 2nd NF membrane DK (MWCO of 150-300 Da), retaining XOS or COS with DP between 2-3 while permeating xylose or glucose. For the experiments, NF membranes were submitted in an experimental device according to Figure 8.1.

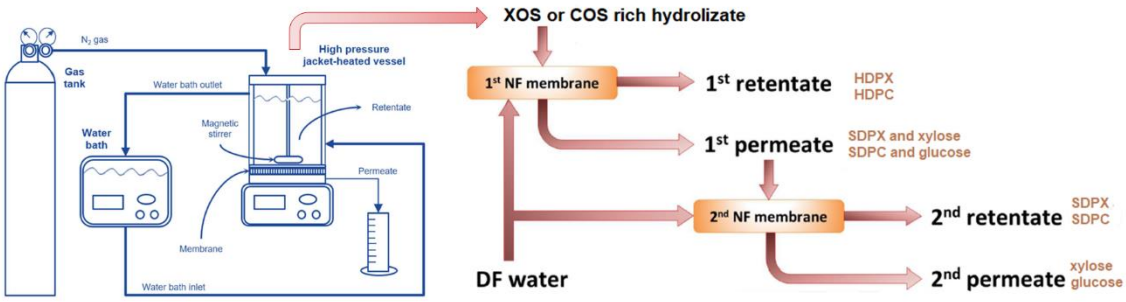

Figure 8.1. Schematic fractionating experiments for oligosaccharides using serial nafiltraiom membranes. Where SSHX- **s**ugarcane straw xylooligosacchahrides hydrolysate, SSHC- sugarcane straw cello-oligosaccharides hydrolysate, SDPX- short degree of poymerization xylooligosaccharides, SDPC- short degree of polymerization cello-oligosaccharides, HDPX- high degree of polymerization xylooligosaccharides, HDPC**-** high degree of polymerization cello-oligosaccharides (Adapted from DE OLIVEIRA, FORTE and FRANCO, 2021).

This device was composed by a high-pressure jacket-heated vessel under agitation of 400 rpm with effective membrane area of 15.2 cm². Initial volume of feed solution was 90 mL with volume reduction ratio (VRR) of 4 times (final collected volumes: 67.5 mL of permeate and 22.5 mL of retentate). In order to maximize XOS or COS fractionation under the NF membranes, the diafiltration (DF) operation was investigated in the experiments, where one DF assay was perfomed in both membranes and only in NP030 membrane. Experiments without DF also were performed as a control experiment. Composition of fractions were determined for feed solution and permeate. As evaluation criteria of DF experiments, it was considered the performance indices such as short degree of polymerization XOS (SDPX) and short degree of polymerization COS (SDPC) concentration (C), SDPX and SDPC purity (P), SDPX and SDPC purification factor (PF), and SDPX and SDPC yield (Y). To evaluate each membrane performace it was evaluated rejection coefficients responses of high XOS or COS depolmeryzation degree HDPX or HDPC (R_H), SDPX or SDPC (R_S), and monosaccharides glucose or xylose (Rm). Additionally, the permeate flux (J_P) was verified in each NF membrane. These parameters were calculated according to the fallowing equations described by de Oliveira, Forte and Franco (2021):

Rejection coefficient was calculated by:

$$Ri = 1 - \left(\frac{C_{\mathrm{i,p}}}{C_{\mathrm{i,f,b}}}\right) \times 100$$

Where, R_{i} is the rejection coefficient of i (%);

$C_{\mathrm{i,p}}$ is the concentration of i in permeate (g.L^{-1});
$C\mathrm{i}_{\mathrm{,f,b}}$ is the concentration of i in bulk of feed side (g.L^{-1}).

Yield was calculated by:

$$Y = \left(\frac{m_{\mathrm{SDP}}}{m_{\mathrm{T}}}\right) \times 100$$

Where, Y is the yield for SDPX or SDPC (%);

$\mathrm{M_{SDP}}$ is the total weight of SDPX or SDPC in sample (g);

$\mathrm{M_T}$ is the total weight of XOS or COS in the hydrolysate (g);

Permeate flux was calculated by:

$$JP = \left(\frac{\Delta \mathrm{V}_P}{A_{\mathrm{m}\Delta \mathrm{t}}}\right) \times 100$$

Where, J_{P} is the permeate flux (L.m-^{2}.h^{-1});

A_{m} is the effective membrane area (m^2);

ΔV_{p} is the volume permeated (L);

Δt is the interval of time (h);

Purity was calculated by:

$$P = \left(\frac{m_{\mathrm{SDP}}}{m_{\mathrm{solids}}}\right) \times 100$$

Where, P is the purity for SDPX or SDPC (%);

m_{SDP} is the total weight of SDPX or SDPC (g);

m_{solids} is the total weight of solids (g).

Purification factor was calculated by:

$$PF = \left(\frac{\mathrm{P}}{P_{Hydrolisate}}\right) \times 100$$

Where, *P* is the purity for SDPX or SDPC (%);

$P_{Hydrolysate}$ is the purity of SDPX or SDPC in the hydrolysate (g).

8.2.3. Prebiotic potential

8.2.3.1 Microorganisms

The freeze-dryed cultures of probiotic pattern microorganisms *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Bifidobacterium longum*, *Bifidobacterium bifidum, Bifidobacterium animalis* subsp. *lactis* were obtained from Tropical Culture Collection (CCT) by André Tosello Foundation, Campinas, SP, Brazil.

2.8.2. Growth medium

The COS fermentation experiment by growing probiotic bacterias was carried out in different medium: with XOS or COS original hydrolysate, with XOS or COS short degree polymerization (2-3) SDPX or SDPC and only in glucose as a carbon source. The growth media (basal media) to *Lactobacillus* strains was the proposed by de Man, Rogosa and Sharpe (1961) (MRS). *Bifidobacterium* cultures were grown in Reinforced Clostridial medium (RCM) prepared by adding Sodium acetate (3g/L), Agar (0.5 g/L), Starch soluble (1 g/L), L-cysteine (0.5 g/L) and Peptone (10 g/L). Filter sterilized 0.35 gm% of COS or XOS added as carbon sources in the basal medium. The medium was prepared at pH 6.7 and autoclaved for 20 min at 121 °C. The active inoculum of probiotic culture to be tested was inoculated and incubated at 30°C and 37 °C for *Lactobacillus* and *Bfidobacterium* strains, respectivelly for 12, 24 and 48 h in anaerobioc conditions through the use of anaerobic jar.The grown of probiotic cultures in COS was monitored by considering different parameters such as optical density at 600 nm and COS profile consumption. The aliquot was centrifuged at 12,000 rpm for 5 min and the supernatant was collected to analyze the residual COS profile. The increase in optical density were employed to demonstrate indirectly the cell growth of probiotic bacteria (ÁVILA et al., 2020).

8.2.4. Analytical quantification products

8.2.4.1. Monosaccharides and oligosaccharides

The content of glucose, xylose, COS and XOS in all the samples was determined by High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric

Detection (HPAEC-PAD) using a Dionex DX-5000 ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) . The samples were diluted in ultrapure water, filtered through a 0.22 μm filter and injected into the column using an auto-sampler. with a CarboPac PA1 column (4 mm × 250 mm and an electrochemical detector adopting a linear gradient of A (NaOH 100 mM) and B(NaOH 100 mM; NaOAc 300 mM) (ÁVILA, MARTINS and GOLDBECK, 2021, ÁVILA et al., 2020). The flow rate was 1 mL/min, and the injection volume of the samples was 100 μL. Data were acquired and processed using PEAKNET software. The xylose, glucose, COS and XOS were identified in samples by comparing the retention times with authentic standards. Calibration curves were constructed using commercial standards (Megazyme, Bray, Ireland) to quantify the xylose, glucose, COS and XOS in the samples.

8.2.4.2. Organic and short chain fat acids

Supernatants from anaerobic fermentation were filtered hrough a 0.22 μm and analysed for their content in acetic, lactic, formic, propionic, and butyric acids using an HPLC (Thermo Fischer Scientific, USA) system equipped with an infrared detector (Thermo Fischer Scientific, USA) at 50°C and a Aminex HPX-87H (BioRAd California, USA) column at 50°C, using 5 mM H_2SO_4 as mobile phase at 0.6 mL/min. Total short chain fat acids (SCFA) were calculated as the sum of acetic, propionic, formic, and butyric acid concentrations. Calibration curves were constructed using analytical standard solutions (Sigma–Aldrich, USA).

8.3. Results and Discussion

8.3.1. Oligosaccharides fractionating

In order to obtaining a short degree of polymerization oligosaccharides a fractionating process using serial nanofiltration was performed using defined operational conditions purposed in a previous work by de Oliveira, Forte and Franco (2021). Accoding to the obtained rejection coefficient data presented in Table 8.3, the membranes employed in this work attended the targets previously purposed. Since in both hydrolysates NP030 membrane presented higher R_H with lower R_S and R_m values, while in DK membrane were higher R_s and R_H with lower R_m values. In addition permeate flux results showed a proportional trend with pore size of each NF membrane type (Table 8.2). However in the general way the permeate flux obtained for both membranes were considering higher for COS hydrolysate than XOS hydrolysate experiments, which can be explained by the higher XOS concentration in the hydrolysate composition presented (Table 8.1).

Table 8.3. Flux permeate (Jp) and Rejection coefficients (Rs, R_H and R_m) for serial nofiltration membranes.

NF membrane	SSHX			
	J_P ($L.h^{-1}.m^{-2}$)	R_S (%)	R_H (%)	R_m (%)
NP030 (1st membrane)	9.48 ± 1.32	24.46 ± 3.07	85.67 ± 4.21	3.33 ± 1.84
DK (2nd membrane)	7.78 ± 1.32	90.67 ±2.78	95.32 ±5.45	20.45 ± 3.21
	SSHC			
NP030 (1st membrane)	37.38 ± 3.21	34.43 ± 2.34	76.78 ± 4.32	5.43 ± 1.35
DK (2nd membrane)	18.37 ± 2.09	98.78 ± 3.43	96.56 ± 3.43	21.45 ± 2.32

Where SSHX- sugarcane straw xylooligosacchahrides hydrolysate, SSHC- sugarcane straw cello-oligosacchahrides hydrolysate, R_S- rejection coeficient of short degeree of polymerization oligosaccharides, R_H- rejection coeficient of high degeree of polymerization oligosaccharides, R_m - rejection coeficient of monosaccharides

Improvement of XOS and COS fractionation system, under DF operation, was estimated by the SDPX and SDPC performance indices, shown in Table 8.4.

Table 8.4. Perfomece indices of short degree of polymerization XOS (SDPX) and short degree of polymerization COS (SDPC) under diafiltration assay

Parameters	Without DF operation (Control)			DF operation in NP030			DF operation in NP030 and DK		
	Initial feed	NP030 permeate	DK retentate	Initial feed	NP030 permeate	DK retentate	Initial feed	NP030 permeate	DK retentate
Short degree of polymerization XOS (SDPX)									
C (g/L)	5.76	5.58	14.54	5.76	5.39	16.44	5.76	5.26	17.12
P (%)	64.71	77.78	83.42	67.71	88.78	92.43	64.71	84.76	95.76
PF (%)	1.00	1.2	1.29	1	1.37	1.42	1	1.3	1.48
Y (%)	100	61.87	46.32	100	89.76	44.43	100	86.43	37.32
Short degree of polymerization COS (SDPC)									
C (g/L)	1.08	0.94	2.34	1.08	0.87	2.57	1.08	0.82	2.87
P (%)	48.21	64.42	75.78	48.21	75.56	81.34	48.21	78.43	87.53
PF (%)	1.00	1.33	1.57	1.00	1.56	1.69	1	1.62	1.81
Y (%)	100	65.45	42.35	100	83.78	45.87	100	88.78	39.78

The P, and PF were increased in final DK retentate with the application of diafitration (DF) assay, demonstrating that DF operation recovered the SDPX and SDPC in NP030 permeate and concentrated them in DK retentate. However, the Y was decreased with the application of DF in DK membrane, demonstrating that DF operation caused some loss by SDPX and SDPC leaking to the DK permeate. From this scenario, the DF operation only in the NP030 membrane was chosen as the best condition for the fractionation system, since it improved the P and PF,while it did not decrease Y too much.

8.3.2. Prebiotic potential

. The time course of optical density was determined to indirectly demonstrate the cell growth of probiotic bacteria. It was possible to observe a considered increment in the final OD values considering the growth of *Bifidobaterium* spp in a mixture xylo-oligosaccharides before the fractionating process. In highlight *Bifidobaterium animalis* that presented the lowest grown in medium with initial XOS mixture, presenting a final OD value around 20% higher with the SDPX obtained by the fractionating process (Figure 8.2).

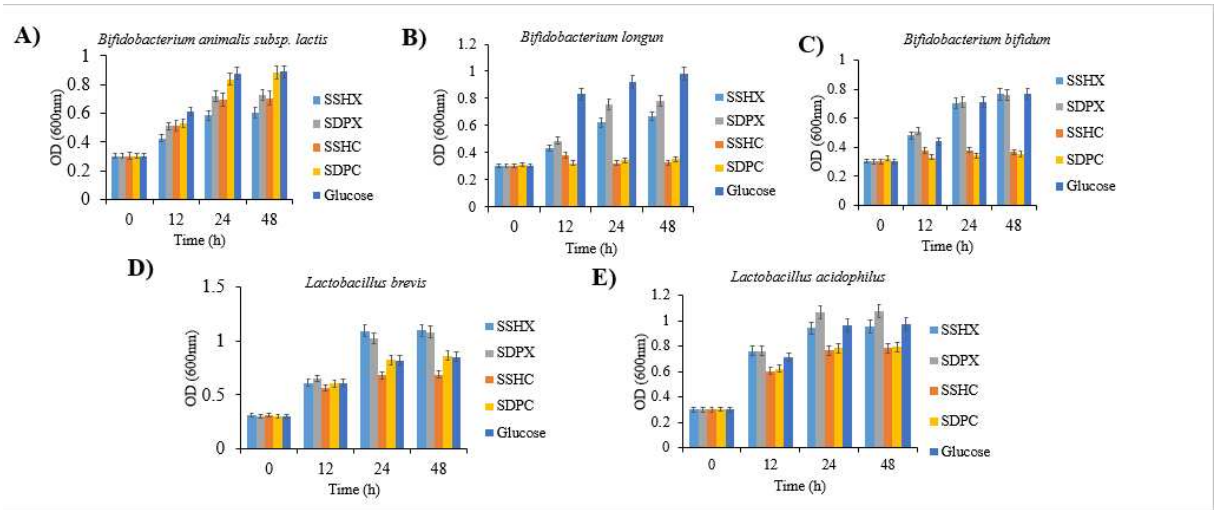

Figure 8.2. The density optical (OD) parameter during the growth of probiotics *Bfidocaterium subsp. Lactis* (A), *Bfidobacterium logum* (B), *Bifidobacterium bifidum* (C), *Lactobacillus brevis* (D), and *Lactobacillus acidophilus* (E) on XOS hydrolysate from sugaracane straw (SSHX), COS hydrolysate from sugarcane straw (SSHC), fractioneted XOS with short degree of polymerization (SDPX), fractionated COS with short degree of polymerization (SDPC) and on glucose as sole carbon source.

This result is in accordance with what was obtained by Ávila et al. (2020) and de Figueiredo et al. (2020) that evidenced a preferential consumption of the X2 and X3 in a mixture of xylo-

oligosacharides. Regarding to COS experiments, it was possible to observe a significant increment in the final OD values up to 25% in the SDPC in *Lactobacillus brevis* and *Bifidobacterium animalis*, however the other strains studied did not present notable growth in COS, only in XOS and glucose medium as a sole carbon source (Figure 8.3). It was partially justified by the possible effects of highly polymerized oligosaccharides that could be dependent on the presence and ability of bacteria that is more able to initiate degradation, followed by the possible subsequent stimulation growth (BIEDRZYCKA, BIELECKA, 2004). The results evidenced that the serial nanofiltration (NF) membranes process to obtain purified short polymerization degree oligosaccharides from xylan and cellulose (SDPX and SDPC) was an efficient strategy to intensification the prebiotic potential of both oligosaccharides, which would have been presented potential benefits to modulate the gut microbiota community stimulating particularly some probiotic bacteria strains, however, more studies with other probiotic strain and more accurate studies are needed to prove this hypothesis.

8.3.2.1. Impact of the fractionated oligosaccharides on the lactate and SCFA production after fermentation assays

The *in vitro* fermentation tests with probiotic bacterias using the oligosaccharides (XOS and COS) and their respective short degree polymerization fractions obtained after refinating process employing in serie nanofiltration membranes to evaluate the fractionating effects on their prebiotic potential. The data concerning to the lactate and short chain fat acids (SCFA) production during the *in vitro* fermentations, displayed in the Figure 8.3. Both sugarcane straw refinated and non refinated hydrolysates of XOS and COS (SPDX, SDPC, SSHX and SSHC) were metabolized by the probiotic bacterias leading to production of SCFA produced by the 48 h fermentation. For all the sources, the acetate was the main metabolic product, which accounted for about 75% of the total SCFAs, followed by propionate, butyrate, formiate and lactate. A lower lactate production was observed at assay using short degree polymerization COS (SDPC) with *Lactobacillus brevis* strain, being more abundant in the experiments containing non refinating COS hydrolysate (SSHC) medium with a *Bfidobacatererium animalis subesp. lactis* fermentation assays (1.08 versus 7.12 mM). Lactate is a transient metabolite produced by bifidobacteria and lactic bacteria which could be converted into other organic acids bydifferent bacterial species present in the gut. This explains the lower concentration found of this compound at the end of fermentation (DÁVILA et al.,

2019).

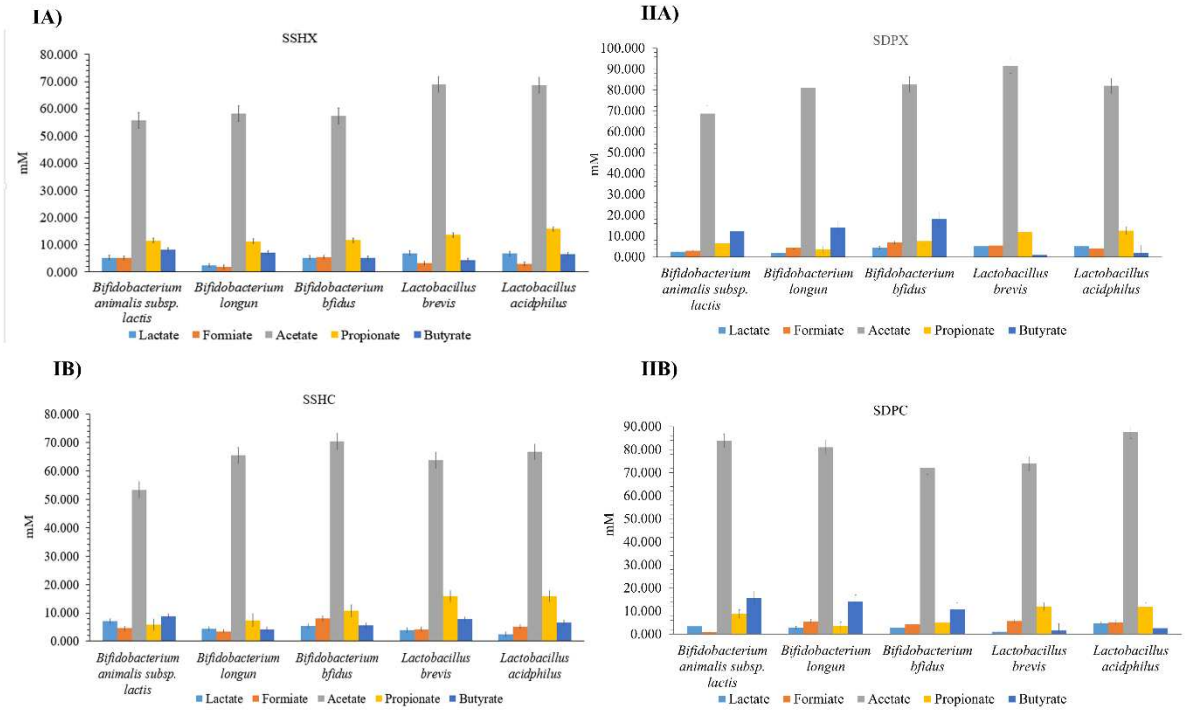

Figure 8.3. Concentration (mM) of SCFA and lactate after 48h of fermentation by probiotic bacterias using sugarcane straw xylooligosaccharides hydrolysate (SSHX) (IA), short degree of polymerization xylooligosaccharides (SDPX) (IIA), sugarcane straw cello-oligosaccharides hydrolysate (SSHC) (IB) and short degree of polymerization cello-oligosacharides (SDPC) (IIB) as sole carbon source.

High concentrations of acetate were detected in all the assays, being the levels significantly higher for the medium containing the refined oligosaccharides. After 48 h of incubation, the concentration of the produced acetate in this medium in the non fractionating hydrolysates reached a value up to 70.41 mM in SSHC, and an increase up to 30% and 24% were verified with respect oligossacharides when SDPC or SDPX were used (91.5 mM and 87.71 mM), respectivelly. The amount of acetate obtained in this work is higher than the reported in the fermentation of xylooligosaccharides from corn stover and cello-oligosaccaharides (ÁLVAREZ et al., 2020; ZHONG et al., 2020). Some studies have linked the production of acetate to different intestinal functions, including an increase of the colonic blood flow, an enhancement of the ideal motility, the regulation of the normal epithelial cell division, and it also plays an important role in the protection against genotoxic agents (SHARMA and SHUKLA, 2016; ZENG et al., 2014).

Taking into account the propionate and butyrate production, coletivelly the experiments with the refined oligosaccharides medium content fermented by *Lactobacillus* strains gave rise to a significantly higher concentration up to 15.9 mM of propionate while the medium fermented by *Bfidobacterium* strains presented significantly higher concentrations of butyrate up to 15.76 mM. The concentrations of propionate and butyrate obtained in the present investigation are similar to the observed by Gullón et al. (2014) when they evaluated the prebiotic effect of refined arabinoxylooligosaccharides from wheat bran. Multiple beneficial effects on the human health are associated with the production of propionate and butyrate from prebiotic carbohydrates. Propionate plays an important role in the prevention of cardiovascular diseases since it reduces lipogenesis and serum cholesterol level (DÁVILA et al., 2019; ÁLVAREZ et al., 2020). Butyrate acts as an anti-inflammatory agent and it is considered the preferred carbon source for the colonocytes (DÁVILA et al., 2019; ZENG et al., 2014). Furthermore, numerous studies highlight the role of both acids in the prevention and in the inhibition of colorectal cancer (CANANI et al., 2011; JAN et al., 2002; SHARMA and SHUKLA, 2016). Overall, the profile of SCFAs obtained in this work was similar to what it has been reported in previous studies in which oligosaccharides with different structural characteristics were used (ÁLVAREZ et al., 2020; DÁVILA et al., 2019).

8.4. Conclusion

Over the course of this work, we can confirm that the membrane-based process was a suitable strategy for XOS and COS fractionation, mainly to recover and concentrate oligosaccharides of short degree polymerization from hydrolyzates of sugarcane straw. In addition, the proposed methodology of serial nanofiltration membranes under one diafiltration (DF) operation was an effective system, showing that DF only in the 1st membrane (NP030) was efficacy on the performance process for these hydrolysates (concentration, purity, and yield) and presented a big potential to be considered as one of the downstream strategies for XOS and COS fractionating. The short polymerization degree fractions obtained demonstrated potential effects in the intensification of prebiotic potential of oligosaccharides and evidenced a possible modulating capacity of the microbial community due to favor specific probiotic bacterias in short degree polymerization fractions obtained.

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Capítulo 9

Discussão e Conclusão Geral

9.1. DISCUSSÃO GERAL

9.1.2. Tratamentos alcalinos para extração da arabinoxila e recuperação da Celulose

O considerável teor de hemicelulose e celulose, principalmente no que se diz respeito a fração xilana das fontes estudadas (palha da cana-de-açúcar e casca de café) impulsionou o estudo de diferentes condições de processo para solubilização/extração da hemicelulose especificamente da fração arabinoxilana com posterior recuperação da celulose. Embora exista vários relatos na literatura em relação a solubilização da hemicelulose, as condições de processo adequadas para sua devida aplicação industrial ainda não foram devidamente determinadas. As quais incluem principalmente a redução do consumo de reagentes e do tempo de processo.

Dessa forma no **Capítulo 4** aprimoramos o método descrito por Zilliox e Debeire (1998) (Tratameto 1) o qual foi considerado como referência neste trabalho e adaptado para palha de cana-de-açúcar e casca de café para extração da arabinoxilano, de forma a agregar no processo uma prévia etapa de desliginificação, de modo a extrair a holocelulose, a partir da qual foram empregados os tratamentos alcalinos. Essa etapa foi realizada com o objetivo de deslignificar as amostras e aumentar a eficiência do processo de extração e da hidrólise enzimática, promovendo uma deslignificação expressiva na ordem de 90%. Neste sentido foram realizados diversos tratamentos alcalinos, variando temperatura, carga de sólidos, concentração de hidróxido de potássio, proporção de etanol e ácido acético e tempo de extração. Com base na composição do arabinoxilano apresentado extraído da holocelulose, foi realizado a viabilidade dos tratamentos propostos para extrair a xilana recuperada da holocelulose de cada biomassa, dessa forma a eficiência da xilana extraída para cada processo foram obtidas.

A eficiência na extração da xilana (E) obtida no Tratamento 2 apresentou valores significativamente superiores em relação a todos os tratamentos realizados a partir da holocelulose extraída. Assim, o aumento do tempo de extração alcalina em relação ao procedimento tradicional com etapa adicional de deslignificação (Tratamento 1) de 3 para 6h, bem como a ligeira alteração na proporção de etanol e ácido acético usado no Tratamento 2 foi desejável para melhorar o processo de extração proporcionando um aumento significativo na eficiência de aproximadamente 4%, em comparação com o método adaptado acrescido de deslignificação (Tratamento 1). Além disso, o Tratamento 2 apresentou uma diminuição do tempo da etapa de incubação de 16 para 10h, o que representou uma diminuição de 3h no tempo total do processo, que poderia ser interessante industrialmente.

Conforme o desejável industrialmente, a carga de sólidos foi aumentada para 15% no tratamento 6, mas isso não resultou em melhor rendimento ou eficiência de extração. Os

extratos obtidos com o tratamento 6 apresentaram alta viscosidade (dados não mostrados), o que provavelmente dificultou a atividade catalítica do KOH (De Figueiredo et al., 2013). A ausência da etapa de pré-incubação também pode ter contribuído para esse resultado, embora em trabalhos anteriores, De Figueiredo et al. (2013) relataram valores de rendimento satisfatórios usando uma carga de sólidos inferior (8%) sem uma etapa de incubação anterior. Como resultado, o tratamento 6 proporcionou os menores rendimentos de extração e eficiências de extração de arabinoxilano. Altas quantidades de resíduos de sal foram obtidas após precipitação no tratamento 6, consequentemente, grandes quantidades de etanol foram usadas durante a etapa de lavagem. De Figueiredo et al. (2013) experimentaram dificuldades semelhantes na hidrólise do bagaço da cana-de-açúcar.

Nos tratamentos 5 e 6, a quantidade de KOH utilizada por grama de xilana extraída foi 80% menor que a utilizada no Método ZD. De Figueiredo et al. (2013) reduziram todos os produtos químicos usados para a hidrólise enzimática do bagaço da cana em 75% em comparação com o método de Zilliox e Debeire (1998) sem afetar os resultados. No entanto, os autores não realizaram extração de holocelulose antes da hidrólise. De Figueiredo et al. (2013) foram capazes de reduzir o tempo de reação em 27 vezes, excluindo a etapa de pré-incubação e lavando os produtos apenas 4 vezes. O tempo de reação dos tratamentos 2 e 3 foi 15 min menor que o do método de referência, pois foram realizadas 4 lavagens em vez de 5, mas as temperaturas foram maiores (70 e 80 ° C, respectivamente), aumentando a quantidade de energia elétrica utilizada no processo. Assim os resultados demonstraram que tratamentos em condições moderadas (Tratamento 2) proporcionou valores mais satisfatórios de rendimento e eficiência da extração de arabinoxilano, podendo assim proporcionar uma melhor susceptibilidade à ação enzimática para a produção de XOS.

Em realção a fração celulose no **Capítulo 6** é ressatado a sua recuperação nas frações sólidas obtidas após os tratamentos alcalinos apresentados no **Capítulo 4** mostrando dessa forma a integração do processo de fracionamento das biomassas. Os tratamentos 1, 2 e 5 obtiveram os melhores resultados de recuperação da celulose, com base na maior porcentagem de celulose e teores residuais de hemicelulose e lignina apresentados. Para recuperação da celulose a aplicação combinada de condições hidrotérmicas e alcalinas sob concentrações de uma baixa carga sólidos (Tratamento 5) foi a estratégia mais eficiente. Isso se deve a uma considerável redução do tempo de 6h (Tratamento 2) para 0,5h; e redução do consumo de reagentes alcalinos, de 24% (Tratamentos 1 e 2) para 4,5%. Embora o Tratamento 5 não tenha sido o tratamento que também tenha proporcionado o maior rendimento para extração da

arabinoxilana, o mesmo se mostrou o mais propício tanto pela redução de tempo como econômia de reagente para se obter as duas frações (arabinoxilana e celulose) de forma integrada, uma vez que os resultados obtidos para solubilização da arabinoxilana foram próximos aos obtidos no Tratamento 2, ainda que não similares estisticamente. Apesar deste tratamento se apresentar como uma potencial estratégia para redução de custos, ainda é necessário uma avaliação econômica adequada para verificar a viabilidade para aplicação em escala industrial.

9.1.2. Produção enzimática de XOS e COS

O fracionamento das biomassas proporcinou que as frações aribinoxilana e celulose obtidas das biomassas estudadas fossem aplicadas para a produção de oligossacarídeos, no caso xilo-oligosscarídeos e celo-oligosscarídeos, respectivamente. Em relação a produção de XOS observamos que quando a combinação (GH51+GH11+CE1) foi empregada foi observado um aumento considerável na produção total de XOS atingindo uma produção por volta de 10 g/L. Como também foi demostrado por análises estatísticas (analise de efeitos por Pareto) que as três enzimas apresentaram efeito significativo na liberação de X2 e X3 ($p < 0,1$), que foram os principais produtos da hidrólise, demonstrando assim um potencial efeito sinergístico para produzir X2 e X3. Os quais são os de maior interesse para a indústria alimentícia e farmacêutica devido aos relatos de seu maior potencial prebiótico. Embora GH51 e CE1 tenham apresentado efeito significativo na formulação da mistura enzimática, quando empregados de forma isolada não apresentaram capacidade de liberação de XOS. Em relação ao GH11, como esperado foi o que apresentou o maior efeito na liberação de XOS . Uma vez que também apresentou considerável capacidade de produzir uma alta concentração de XOS de forma isolada ou em associação com as demais enzimas, liberarando principalmente XOS de cadeia curta (xilobiose, xilotriose e xilotetraose) e menores concentrações de xilose, xilopentose e xilohexaose para ambos os substratos. O que é esperado por uma endo-xilanase da família GH11, a qual é capaz de clivar em regiões mais distantes das cadeias laterais, produzindo XOS de graus de polimerização diversificados (DE FREITAS, CARMONA, BRIENZO, 2019).

Em relação a produção de COS também foi possível observar uma ação sinergística entre CE1 com as demais enzimas avaliadas, no caso interações significativas entre GH5 e GH12, como também entre GH5 e CE1 foram observadas para produzir COS usando o sólido proveniente das duas biomassas (palha de cana-de-açúcar e casca de café), no entanto atingindo rendimentos bastante inferiores aos encontrados para XOS. O que hipotetizamos ser uma

possível inibição pelo principal produto liberado (celobiose), o qual pode vir a interferir nas atividades das enzimas exoglucanase e endoglucanase durante um longo tempo de hidrólise (48 horas). Dessa forma é importante enfatizar que embora a maior produção tenha sido apresentada em 48 horas, em 12 horas foi alcançada a maior taxa de produção obtida. Evidenciando desta forma que de fato a celobiose poderia inibir o acesso das celulases empregadas, formando um complexo enzima-sacarídeo gerando um potencial impacto nos rendimentos de hidrólise enzimática (CHU et al., 2014).

Dessa forma com intuito de investigar os possíveis processos de inibição pela celobiose e tentar aumentar a produção enzimática de COS, no **Capítulo 7** é mostrado uma segunda a estratégia de produção de COS por meio de um processo em multiplos estágios usando a mesma endoglucanase (GH12) empregada no **Capítulo 6**, no entanto previamente adsorvida aos substratos. O processo de adsorção das enzimas se mostrou um processo potencial para redução de custos do processos, uma vez que as enzimas mantiveram de forma eficiente a sua atividade no substrato. A etapa a hidrólise enzimática foi separada em três estágios, onde a cada estágio o produto formado era removido. De acordo com alguns autores, a hidrólise enzimática em vários estágios pode efetivamente remover a inibição do produto e aumentar significativamente a eficiência da hidrólise (CHU et al., 2014 e YU et al., 2012).

Os resultados obtidos mostraram um incremento considerável em torno de 65% do total produzido no processo com três estágios em relação ao processo de hidrólise enzimática com um único estágio. O processo apresentou maior taxa de hidrólise nas primeiras 6 h, principalmente em relação a fração celobiose. Porém nas últimas 24 horas não houve um incremento considerável na produção de COS. No entanto os resultados alcançados neste estudo (85,43 ± 1,00 mg. gsubstrato^{-1}) superou a concentração atingida no **Capítulo 6** (63,56 ± 0,56 mg. gsubstrato^{-1}), utilizando a estrtégia de misturas enzimáticas, e dessa foma se mostrou como a melhor estratégia para a produção de COS. Embora os resultados alcançados sejam bastante inferiores em realçao a quantidade obtida de XOS, estes são consideráveis quando comparados com os poucos relatos da literatura em relação a produção de COS a partir de materiais lignocelulósicos. Barbosa et al. (2020) usando estratégia semelhante otimizou a produção de COS atingindo aproximadamente 60 mg /g de palha de cana-de-açúcar pré-tratada hidrotermicaente usando combinação de endoglucanases processivas expressas heterólogas, o que foi bastante próximo dos resultados obtidos neste estudo. Uma das hipóteses confirmadas seria a inibição pelo produto (celobiose), e de certo modo a considerável recalcitrancia da celulose comparada a hemicelulose que é mais amorfa e mais facilmente hidrolisada (CANO et

al., 2020). Dessa forma a produção de COS a partir de materiais lignocelúlósicos ainda é um campo a ser explorado, uma vez que diferentes misturas de enzimas e estratégias ainda podem ser avaliadas para incremento na sua produção.

Portanto neste presente trabalho de tese a estratégia de sinergismo entre as enzimas foi uma estratégia potencial para produzir XOS. Já em relação aos COS a estrégia utilizando uma única enzima (endoglucanase - GH12) previamente adsorvida ao substrato através do processo em três estágios apresentou maior potencial para produção de COS, por reduzir o processo de inibição pela liberação de celobiose. Embora as duas biomassas (palha da cana-de-açúcar e casca de café) tenham apresentado tendências bastante similares em relação aos dados obtidos ao longo deste estudo, a palha da cana-de-açúcar se mostrou ser um substrato de maior potencial para produção de ambos os oligosscarídeos estudados, o que pode ser explicado pela mesma ser uma fonte mais abundante de ambas as fraçoes celulose e hemicelulose, além de apresentar um considerável menor teor de lignina.

9.1.3. Potencial prebiótico

Com intuito de comprovar o potencial prebiótico dos oligosscarídeos produzidos de ambas as fontes, testes de digestão e fermentação in vitro foram realizados. O primeiro estudo apresentado no **Capítulo 5** evidencia o potencial prebiótico e anti-oxidante de xilo-oligosscarídeos produzidos utilizando misturas enzimáticas otimizadas no **Capítulo 4.** De modo a comprovar sua resistência à ação de enzimas encontradas no sistema digestivo. Os XOS com grau de polimerização de 2-6 (xilobiose, xilotriose e xilotetraose, xilopentose e xilohexose) presentes no hidrolisado produzido pelo arabinoxilano em estudo não foram degradados quando incubados com a enzima amilase salivar. Porém, em relação à digestão gástrica, as frações hidrolisadas (X2, X5 e X6) apresentaram menor resistência do que outras frações (X3 e X4). Em contraste, os testes que simularam a digestão intestinal mostraram que todas as frações estudadas (X2-X6) apresentaram resistência semelhante à digestão. O que consequentemente proporcionou maior percentual de digestibilidade na fase gástrica do que na fase.

Muitas espécies probióticas de *Bifidobacterias* e *Lactobacillus* são capazes de catabolizar uma variedade de monossacarídeos e oligossacarídeos não digeríveis liberados pela hidrólise de polissacarídeos vegetais (OKU, TOKUNAGA, HOSOYA, 1984). Dessa forma utilizou-se cepas probióticas comerciais *Lactobacillus acidophilus* (LAC 4), *Lactobacillus paracasei subsp paracasei* (LBC 81-82), *Bifidobacterium longum* (BL04-300B), *Bifidobacterium lactis* (BI 01), as quais foram gentilmente fornecidas pela Danisco®, Brasil. Através deste estudo observou-se que nenhuma das bactérias probióticas cresceram em meio

sem fonte de carbono e todas as culturas probióticas apresentaram maior crescimento em meio contendo glicose, como controle negativo e positivo respectivamente. Com exceção de *B. lactis*, todas as culturas probióticas foram capazes de utilizar os XOS produzidos a partir de ambas as biomassas lignocelulósicas e apresentaram notável crescimento nos meios contendo XOS como única fonte de carbono. O que ficou evidente a partir do aumento da densidade óptica DO a 600 nm após 72 horas e a queda acentuada do pH observada como resultado do crescimento das culturas de probióticas. No entanto neste presente estudo também foi observado um expressivo crescimento em glicose, o que pode ser explicado por um processo anaeróbio ineficiente, que possivelmente deslocou o metabolismo para o consumo de glicose.

O perfil de XOS antes e após a fermentação com as culturas probióticas que apresentaram crescimento em XOS derivados da xilana tanto da palha da cana-de-açúcar como da casca de café revelaram que todas as culturas que apresentaram crescimento notável foram capazes de consumir principalmente as frações X2 e X3 e um leve consumo das frações X4 e X5. Além de atender os requisitos para confirmação do potencial prebiótico. Ainda no **Capítulo 5** também foi evidenciado o seu potencial anti-oxidante o qual foi dependente da sua concentração no meio atingindo até 78% de atividade em relação ao radical DDPH em uma concentração de até 2 g. L^{-1} de XOS, bem como ao radical molibdênio em concentraçoes na faixa de 0,053–0,8 g.L^{-1}. Esta presente atividade antioxidante pode ser atribuída à presença de hidroxicinâmicos ligados a ésteres derivados de ácidos, como ácido ferúlico, cumárico cafeico, resíduos de ácido siringico e ramificações de ácido metil glucurônico, presentes na cadeia de xilana (VIEIRA et al., 2020).

Em relação ao potencial prebiótico dos celo-oligoscarídeos, o mesmo foi investigado no **Capítulo 7** como parte do estudo partindo de uma segunda estratégia de produção enzimática de COS através de hidrolise por múltiplos estágios conforme discutido anteriormente no tópico 9.1.2. Da mesma forma que para os XOS produzidos neste estudo também foi avaliado a resistência dos COS às enzimas digestivas. Os celo-oligossacarídeos produzidos de ambas as fontes também não apresentaram degradação quando incubados com a enzima amilase salivar e sofreram os maiores valores de degradação pela pancreatina em torno de 9% nas fraçoes C2 e C3 e 8% nas fraçoes C4 e C3 na fase gástrica. No entanto neste estudo não foi possível avaliar o crescimento das cepas probióticas em meio de cultura com COS como única fonte de carbono devido a baixa concentração produzida. Dessa forma optamos por avaliar a sua atividade prebiótica como um incremento a um meio contendo glicose, com intuito de avaliar a capacidade de estimular o crescimento de cepas probióticas que em sua grande parte não

foram culturas comerciais como as estudadas anteriormente no **Capítulo 5**. As cepas estudadas (*Bifidobacterium* spp e *Lactobacillus spp*.) foram adquiridas da coleção de culturas tropicais da Fundação André Tozello. Os resultados evidenciaram uma possível preferência das cepas de *Lactobacillus* pelo incremento de COS em meio glicêmico, sendo capazes de fermentar com maior eficiência do que em meio glicêmico como única fonte de carbono, consumindo preferencialmente as frações C2 e C3. Por outro lado, as cepas de *Bifidobacterium* não apresentaram incremento significativo no crescimento em meio com incremento de COS, consumindo preferencialmente a glicose. Há poucas evidências do cresimento de COS em cepas probióticas devido ao número bastante reduzido de estudos. No entanto foi possível verificar que tendências semelhantes foram encontradas no estudo Zhong et al. (2020), de acordo com os autores os transportadores correspondentes (β-1,4-específicos) raramente são encontrados nas cepas de *Bifidobacterium*. Assim o crescimento dessas cepas em COS pode ser explicado pela captação não específica mediada por transportadores não relacionados com as especificidades promíscuas.

Conforme averiguado nos estudos descritos nos **Capítulos 5 e 7**, as cepas probióticas frequentemente encontadas na microbiota intestinal demosntraram preferencias por frações tanto de XOS e COS com grau de polimerização (GP) entre 2 e 3. Dessa forma no **Capítulo 8** foi proposto um processo de fracionamento de XOS e COS partir da celulose da palha da cana-de-açúcar a qual se mostou mais propicia a produção de ambos os oligosscarídeos. Neste estudo foi empregado membranas de nanofiltração em série de modo a isolar as frações com GP entre 2 e 3 e investigar o impacto do processo de fracionamento no seu potencial prebiótico. Dessa forma após o fracionamento dos hidrolisados, foi possível observar um considerável incremento nos valores finais de DO considerando o crescimento de *Bifidobaterium spp* na mistura de xilo-oligossacarídeos. Em destaque a cepa a *Bifidobaterium animalis subsesp*. *lactis* que apresentou o menor crescimento em meio com mistura inicial de XOS tanto nos ensaios reaalizados no **Capítulo 5**, quanto no **Capítulo 8**, apresentando um valor de DO final em torno de 20% maior quando utilizou-se a fração rica em X2 e X3 (SDPX) obtido pelo processo de fracionamento.

Em relação aos experimentos de COS o qual devido a baixa concentração produzida passou previamente por um processo de concentração por retroevaporação. E foi possível observar um incremento significativo nos valores finais de DO de até 25% quando utilizou-se a fração rica nas fraçoes C2 e C3 (SDPC) em *Lactobacillus brevis e Bifidobacterium animalis subesp lactis*, porém as demais cepas estudadas não apresentaram crescimento notável em COS, apenas em XOS e glicose como única fonte de carbono. Isso pode ser parcialmente justificado

pelos possíveis efeitos de oligossacarídeos altamente polimerizados que poderiam ser dependentes da presença e capacidade de bactérias mais capazes de iniciar a degradação (BIEDRZYCKA, BIELECKA, 2004). Portanto os resultados evidenciaram que o processo com membranas de nanofiltração em serie para fracionamento dos oligossacarídeos de curto grau de polimerização a partir de xilana e celulose (SDPX e SDPC) favoreceu o potencial prebiótico de ambos. No entanto estudos com outras cepas probióticas envolvendo análises mais acuradas são necessários para comprovação dessa hipótese. Embora o processo de fracionamento com as membranas tenha apresentado resultados positivos no potencial prebiótico de ambos os oligosscarídeos, este processo é dispedioso e dessa forma o incremento da DO obtido neste processo não justificaria o custo benefício para o processo industrial. Assim diante dos resulatdos obtidos com os hidrolisados sem as etapas de fracionamento (SSHX e SSHC) podemos apontar que os mesmos já são propícios para estimular o crescimento das cepas probióticas estudadas, não havendo portanto a necessidade do emprego dessas etapas no seu processo de obtenção visando um composto prebiótico.

9.2. CONCLUSÕES GERAIS

Diante dos resultados alcançados ao longo dos Capítulos de 4 a 8 podemos podemos concluir que:

i) A extração da holocelulose com clorito de sódio antes do tratamento alcalino em condições moderadas de temperatura (Tratamento 2) favoreceu a extração de arabinoxilano com pureza considerável e proporcinou deslignificação expressiva. Além disso os sólidos recuperados após os tratamentos alcalinos foram ricos em celulose, com possível integração de processos para aproveitamento destes polissacarídeos. No entanto para se recuperar a celulose a partir deste mesmo processo a estratégia combinada de tratamento alcalino em baixas concentrações (4.5% m/v) com processo hidrotérmico (Tratamento 5) se mostrou mais eficiente, e foi o mais propício para obtenção das frações arabinoxilana e celulose em um processo integrado.

ii) As combinações enzimáticas otimizadas apresentaram efeito sinergístico na produção de ambos oligossacarídeos. Onde foi possível alcançar altos rendimentos de XOS e baixas concentração de monossacarídeos após 48 h de hidrólise enzimática. A associação de Feroloyl esterase com hemicelulases e celulases se mostrou favorável na produção de ambos os oligossacarídeos.

iii) Os experimentos utilizando hidrólise enzimática em múltiplos estágios foi uma estratégia fovorável para reduzir o processo de inibição pela celobiose, e dessa forma se mostrou como melhor estratégia para se produzir COS.

iv) Os testes *in vitro* confirmaram o potencial prebiótico dos XOS e COS produzidos, uma vez que cepas probióticas apresentaram preferência pelo meio incrementado com COS e obtiveram um notável crescimento em meios contendo XOS como única fonte de carbono, consumindo preferencialmente as frações com grau de polimerização de 2 a 3. Bactérias probióticas do gênero *Bifidobacterium* apresentaram maior resistência ao cresimento no meio contendo os oligosscarídeos produzidos. Os oligosscarídeos também apresentaram alta resistência á ação de enzimas encontradas no trato gastrointestinal. Dessa forma atenderam os requisitos para serem classificados como oligossacarídeos prebióticos.

v) O processo de fracionamento com as duas membranas de nanolfitração em serie com operação de diafiltração apenas na primeira membrana (NP030) favoreceu o proceso de obtenção das frações tanto de XOS como de COS de menor grau de polimerização. O uso das frações retidas na segunda membrana em ensaios de fermentação com cepas probióticas demonstrou efeito na intensificação da atividade prebiótica de ambos os oligossacarídeos. No entanto ambos os hidrolisados produzidos (XOS e COS) sem as etapas de fracionamento apresentaram considerável teor de pureza e também propriedades prebióticas significativas. Dessa forma poderiam ser utilizados com intuito de proporcionar efeito prebiótico sem as devidas etapas de purificação e fracionamento, o que é uma potencial estratégia para redução de custo e viabilidade do processo para obtenção em escala industrial .

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APPENDIX I

Supplementary Material

CHAPTER 4

Table S4.1. Analysis of variance ($p < 0.1$) for xylooligosaccharides production by enzymatic hydrolysis of sugarcane straw arabinoxylan.

Source of variation	Sum of squares	Degrees of freedom	Mean square	$F_{value}/F_{critical}$	R^2
Regression	90.4	7	12.9	38.96	98.7
Residual	1.2	9	0.1		
Total	91.6	16			

Table S4.2. Analysis of variance ($p < 0.1$) for xylooligosaccharides production by enzymatic hydrolysis of coffee husk arabinoxylan.

Source of variation	Sum of squares	Degrees of freedom	Mean square	$F_{value}/F_{critical}$	R^2
Regression	87.3	4	21.8	44.51	97.35
Residual	2.4	12	0.2		
Total	89.7	16			

CHAPTER 6

Table S6.1. Analysis of variance ($p < 0.05$) for cello-oligosaccharides production through enzymatic hydrolysis of the selected cellulose recovery substrate from sugarcane straw (SS5).

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_{value}/F_{critic}	R^2
Regression	524.7	6	87.5	20.21	97.49
Residual	13.5	10	1.4		
Total	538.2	16			

Table S6.2. Analysis of variance ($p < 0.05$) for cello-oligosaccharides production through enzymatic hydrolysis of the selected cellulose recovery substrate from coffee husk (CH5).

Source of variation	**Sum of squares**	**Degrees of freedom**	**Mean square**	F_{value}/F_{critic}	R^2
Regression	2400.6	7	342.9	8.5	95.60
Residual	110.4	9	12.3		
Total	2511.0	16			

ANEXOS

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ANEXO III. Permissão para uso do artigo correspondente ao Capítulo 5

Xylooligosaccharides production by commercial enzyme mixture from agricultural wastes and their prebiotic and antioxidant potential

Author: Patrícia F. Ávila,Manoela Martins,Fátima A. de Almeida Costa,Rosana Goldbeck

Publication: Bioactive Carbohydrates and Dietary Fibre

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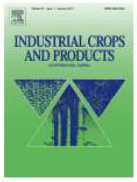

Fractionating process of lignocellulosic biomass for the enzymatic production of short chain cello-oligosaccharides

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