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Production Strategy of Functional Oligosaccharides from Lignocellulosic Biomass Using Enzymatic Process: A Review

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Abstract Lignocellulosic biomass was previously known as agricultural waste, and its only use was in the production of compost. Over time, this waste has evolved into one of the raw materials used in the production of biofuels or bioethanol, an alternate substrate for enzyme manufacturing, and so on. Due to the abundance of lignocellulosic biomass and its potential derivate compounds, scientists have devised a process from lignocellulose fractions (cellulose and hemicellulose) to high-value cello-oligosaccharides (COS) and xylo-oligosaccharides (XOS). These oligosaccharides provide a variety of health benefits, including anti-diabetic and prebiotic characteristics. However, due to slow reactions and low yields, the manufacturing of these oligosaccharides remains an issue. As a result, in this paper, we discuss the possibilities of a multi-step process and the use of thermostable cellulases for improve COS dan XOS production from lignocellulosic biomass. We also mention from the potential pretreatment process to the "adsorption-separation" method for separating β-glucosidase from thermostable cellulases produced for the manufacture of XOS and COS from lignocellulosic biomass

Keywords: Cellulase, COS, Lignocellulose, Multi-Step, Thermostable, XOS.

Overview

Lignocellulosic biomass was formerly known as waste from agricultural products and its utilization was only up to the manufacture of compost. Along with the times, this waste has developed into one of the raw materials in the manufacture of biofuels or bioethanol by simultaneous saccharification and fermentation (Itelima et al. 2013). Some study has looked into using lignocellulose biomass (such as rice straw) as an alternative substrate for the production of thermostable enzymes (Yuansah et

al. 2019). Due to the abundance and its potential derivate compounds to explore, lignocellulosic biomass is attractive to study, especially as a substrate for the production of high-value cello-oligosaccharides and xylo-oligosaccharides.

Lignocellulose is formed by three major biopolymer components: cellulose, hemicellulose, and lignin (Pointner et al. 2014). Cellulose and hemicellulose are a carbohydrate that consists of glucose, xylose, mannose, arabinose, and galactose (Wang et al. 2016). When the homopolysaccharide, cellulose, is hydrolyzed partially, it will release cello-oligosaccharides and a little amount of glucose. While heteropolysaccharide, hemicellulose, will release some oligosaccharides (such as xylo-oligosaccharides (XOS), manno-oligosaccharides (MOS), arabino-xylo-oligosaccharides (AXOS), etc.) and monosaccharides (Saville and Saville 2018; Yuansah 2019) . The conversion process from lignocellulosic biomass to high-value oligosaccharides can be carried through various pretreatment processes and enzymatic hydrolysis. However, enzymatic conversion of lignocellulosic biomass, particularly the cellulose fraction, is considered a slow hydrolysis reaction.

The proper pretreatment techniques will optimize the oligosaccharides yield from a lignocellulose complex structure that is highly resistant to enzymatic hydrolysis (Zoghlami and Paës 2019). Before entering the enzyme hydrolysis phase, several inhibitors such as lignin and other polyphenolics must be handled in the pretreatment step (Qin et al. 2016; Zoghlami and Paës 2019). The pretreatment procedure breaks down the crystalline structure of cellulose and reduces the degree of polymerization, removing all lignin and breaking down hemicelluloses (Baruah et al. 2018). Proper pretreatment procedures are necessary to acquire the highest yield of oligosaccharides from the bioconversion of various lignocellulosic substrates.

For large-scale production of oligosaccharides from lignocellulosic biomass, enzymatic hydrolysis of lignocellulose biomass at high temperatures utilizing thermostable enzymes shows exceedingly promising. Using thermophilic or thermotolerant microbes to produce thermostable enzymes, we can overcome the limitation of lignocellulose hydrolysis. When a reaction can be carried out at a high temperature using thermostable enzymes, it is quicker, more efficient, and less prone to contamination (Giovannoni et al. 2020). As a result of the stability of thermostable enzymes, more specialized products and fewer by-products are produced, providing for a longer hydrolysis time and greater flexibility in the

69 configuration process (Giovannoni et al. 2020; Vasconcellos et al. 2015). The
70 thermostable cellulases used produced by thermostable enzymes in the process
71 should be separated from the β-glucosidase leaving only the endoglucanase and
72 exoglucanase acting on the substrate. This multi-step process improved the total
73 COS yield accumulated in each step by approximately 51.78% with the hydrolysis
74 of approximately 75.56% (Chu et al. 2014). According to the literature, adequate
75 pretreatment and a multi-step method using thermostable cellulose optimize
76 enzymatic activity and product yields of XOS and COS. As a result, this article
77 begins with a brief description of lignocellulose sources and their potential as
78 enzyme substrates for functional oligosaccharides production. With the idea that
79 suitable pretreatment and a multi-step enzymatic process enhanced product yields,
80 the possible source as enzymatic substrates seeks to make it a little easier for
81 researchers to find the optimal source for the manufacture of COS and XOS.
82
83 **Lignocellulose Source for Functional Oligosaccharides Production**
84
85 Agricultural waste is the primary source of lignocellulose, which is made up of
86 biopolymers such as cellulose, hemicellulose, and lignin. The agro-waste product
87 lignocellulose consists of cellulose (40-50%), hemicellulose (20-30%), and lignin
88 (10-25%) (Maheshwari 2018; S. Sharma et al. 2019). Because of their availability
89 and abundance of essential polysaccharides, cellulose (consisting of D-glucose) and
90 hemicellulose (consisting of glucose, mannose, xylose, arabinose, galactose, etc.),
91 agricultural waste such as rice straw (Z. Hu et al. 2021; A. L. Li et al. 2018), wheat
92 straw (Collins et al. 2014; Shrivastava et al. 2014), sugarcane bagasse (de Souza et
93 al. 2013; Guilherme et al. 2015), cacao pod (Lu et al. 2018), sago pith residue
94 (Husin et al. 2019), corncob (M. Li et al. 2014; Silva et al. 2015), apple pomace
95 (Hijosa-Valsero et al. 2017), and red algae (Rabemanolontsoa and Saka 2013) can
96 be potential candidates as an enzymatic substrate to produce xylo- and cello-
97 oligosaccharides.
98 The main component of lignocellulosic biomass is cellulose, which is the
99 most widespread and easily accessible carbohydrate polymer on the planet and a
100 primary polysaccharide element of plant cell walls (Maheshwari 2018). Cellulose
101 is a homopolysaccharide, a carbohydrate biopolymer composed of monomeric ß-
102 D-glucopyranose linked together by a 1,4 glycosidic bond. The structure of the

103 cellulose chain consisted of 500-1400 ß-D-glucopyranose (D-glucose) molecules
104 that packed together to form microfibrils. A macrofibril (cellulose fibrils) is formed
105 up of microfibrils that have been joined together. Because of the presence of
106 cellulose fibrils, lignocellulosic biomass is particularly resistant to enzymatic
107 hydrolysis (Zoghlami and Paës 2019). Cello-oligosaccharides can be manufactured
108 by hydrolyzing cellulose using β-glucosidase deficient cellulases (Chu et al. 2014).
109 Hemicellulose is the second most common substance after cellulose (Maheshwari
110 2018). Hemicellulose is a heteropolysaccharide made up of several different
111 carbohydrate monomers, particularly pentoses sugar units such as xylose and
112 arabinose, hexoses sugar units such as mannose, glucose, and galactose, along with
113 acylated sugar which is represented in varying ratios in diverse materials (Dionisi
114 et al. 2014; Maheshwari 2018). In hardwoods, xylan is the most common
115 biopolymer, while in softwoods, glucomannan is the most abundant (Dionisi et al.
116 2014). Hemicellulose can be partially hydrolyzed and degraded into
117 xylooligosaccharides (XOS) utilizing hemicellulases (Moser et al. 2014).
118
119 **Enzyme Inhibitors in Lignocellulosic Biomass**
120
121 The presence of inhibitors is one of the challenges in using lignocellulosic
122 components as substrates in enzymatic hydrolysis. In lignocellulosic waste,
123 inhibitors are derived from lignin, phenolic groups, sugars, and their derivatives (B.
124 Hu et al. 2016; Qin et al. 2016). Inhibitors can form naturally and accumulate in
125 biomass, or form as a result of a process. The majority of phenolic compound
126 derivatives inhibit the enzyme activity of amylase and cellulase (Table 1) (Desseaux
127 et al. 2018; González-Bautista et al. 2017). However, some inhibitors can also act
128 as enzyme activators for various enzymes. *T. reesei* cellulase enzyme, for example,
129 is activated by certain concentrations of ferulic, syringic, sinapic, and vanillic acids
130 (Stamogiannou et al. 2021). Knowing the substance's potential inhibitors allows
131 you to decide what steps to take to solve the problem. Avoiding materials with few
132 inhibitors will help the enzymatic process. The lower the inhibitor content of the
133 substances, the milder the pretreatment required. Nonetheless, the use of resistant
134 materials is highly desirable to bring about a significant change in waste treatment
135 (Jönsson et al. 2013).
136

137 Table 1. Enzyme Inhibitors in Lignocellulosic Biomass

Inhibitor	Source	Inhibited Enzymes	Ref
Crude Phenolic	Sugarcane Bagasse, apple pomace, pecan	CMCase, xylanase, α-amylase, α-glucosidase	(Feng and Kong 2022; González-Bautista et al. 2017; Hijosa-Valsero et al. 2017)
p-coumaric acid	Corn stover, Cocoa pod husk	Cellulase, xylanase	(Chen et al. 2020; Lu et al. 2018)
Quercetin	Cocoa pod husk, Cocoa powder, *Mucuna pruriens* seed	Cellulase	(Lu et al. 2018; Qin et al. 2016; Rai et al. 2017; Sorrenti et al. 2020; Stamogiannou et al. 2021)
Kaempferol	Water lily	α-glucosidase, cellulase	(Mugaranja and Kulal 2020; Qin et al. 2016; Stamogiannou et al. 2021)
Tannins / Tannic Acid	Oak Bark, Acorn Caps	ß-glucosidase, cellobiohydrolase, endoglucanase	(Jönsson et al. 2013; Liu et al. 2021; Majewska et al. 2022; Mhlongo et al. 2015)

Gallic Acid	Douglas fir, Black wattle, Cocoa pod husk, *Mucuna pruriens* seed	ß-glucosidase, endo-ß-1,4-xylanase	(Jönsson et al. 2013; Lu et al. 2018; Mathibe et al. 2020)
Vanillic acid	Douglas fir, Black wattle, vanilla orchid	endo-ß-1,4-xylanase, α-amylase, α-glucosidase	(Aleixandre et al. 2022; Gallage and Møller 2015; Mathibe et al. 2020)
Vanillin	Cocoa powder, vanilla orchid	Endocellulase, exocellulase (cellobiohydrolase), ß-glucosidase. Amyloglucosidase, xylanase	(Gallage and Møller 2015; Hidayatullah et al. 2020; Sorrenti et al. 2020)
Syringic acid	Grass hay, Oak woodchip	α-amylase, α-glucosidase	(Aleixandre et al. 2022; Ziolkowska et al. 2020)
Syringaldehyde	Grass hay, Oak woodchip	Endocellulase, exocellulase (cellobiohydrolase), ß-glucosidase	(Ziolkowska et al. 2020)
Trans-cinnamic acid	*Cinnamomum cassia* bark (Chinese cinnamon)	Endocellulase, exocellulase (cellobiohydrolase), ß-glucosidase	(Qin et al. 2016; Stamogiannou et al. 2021)
Hydroxybenzoic acid	Cocoa powder	Endocellulase, exocellulase (cellobiohydrolase), ß-glucosidase	(Sorrenti et al. 2020)

Catechin	Tea, Cocoa pod husk, Cocoa powder, *Mucuna pruriens* seed	α-amylase	(Lu et al. 2018; Rai et al. 2017; Sorrenti et al. 2020; Sun et al. 2016)

138
139
140 **Pretreatment Techniques to Improve Enzymatic Hydrolysis Process**
141
142 There are several pretreatment strategies available to optimize the oligosaccharides
143 yield from lignocellulose biomass conversion. Because of its complex structure, the
144 crystallinity of cellulose, and porosity, lignocellulose is highly resistant to
145 enzymatic hydrolysis (Zoghlami and Paës 2019). Various inhibitors, such as lignin
146 and other polyphenolic compounds, must be treated in the pretreatment step before
147 entering the enzyme hydrolysis phase (Qin et al. 2016; Zoghlami and Paës 2019).
148 The objective of the pretreatment process is to break down the crystalline structure
149 of cellulose and minimize the degree of polymerization, eliminating all lignin and
150 breaking down hemicelluloses (Baruah et al. 2018). Proper pretreatment strategies
151 are required to obtain the greatest yield of oligosaccharides from the bioconversion
152 of different lignocellulosic substrates (Table 2).
153 The pretreatment process can result in the removal of lignin and the
154 destruction of the microcrystalline structure of cellulose, as well as the formation
155 of oligosaccharides and monosaccharides. For example, liquid hot water
156 pretreatment generates sugar and oligosaccharides, but the concentration of the
157 sugar is quite low (Huang et al. 2017). The hemicellulose concentration was
158 drastically reduced, although only minimal alterations in cellulose and lignin were
159 identified (Antczak et al. 2022). Alkali pretreatment can significantly reduce lignin
160 while producing fewer unwanted inhibitors. It also consumes little energy.
161 However, there remains a possibility that this pretreatment will result in
162 unrecovered salt (Jinyu Tan et al. 2021). Acid pretreatment also can disrupt the
163 lignocellulose and amorphous cellulose structures. Unfortunately, in contrast with
164 alkali pretreatment, this pretreatment consumes a large amount of energy and

165 generates a large number of byproducts (Solarte-toro et al. 2019; Jinyu Tan et al.
166 2021). Nowadays, research using greener technology using organic solvents such
167 as ionic liquid (IL) (choline, pyridine, chloride, acetate, and imidazole) and deep
168 eutectic solvent (DES) that are more favorable with enzymes and microbes.
169 However, these approaches still face challenges because solvent recovery of iLs
170 will cost more than other solvents while DESs have disadvantages such as high
171 viscosity and toxicity (Jinyu Tan et al. 2021; Zhao et al. 2022). To improve the
172 effectiveness of lignocellulosic waste pretreatment, a combination of pretreatment
173 techniques is often used.
174
175 Table 2. Pretreatment Techniques for Different Lignocellulosic Biomass

Biomaterials Substrate	Pretreatment Strategy	Efficiency / Yield	Ref
Rice Straw	*Acid*: Ammonia 20.93%; reaction time 48 h; temperature 42.74°C	± 13.91 g/L of fermentable glucose (± 87.24%)	(Kim et al. 2013)
Barley Straw	*Combination*: 2 g/100 g DM xylanase and polyethylene glycol (PEG) 4000	glucose yield 86.9%, xylose yield 70.2%, and acetone-butanol-ethanol (ABE) yield 135 g/kg pretreated straw	(M. Yang et al. 2015)
Sugarcane Bagasse	*Alkali:* NaOH (0.4M; t: 7 min)	Lignin was reduced from 31.71% to 12.07%. The difference between raw bagasse and processed bagasse is 19.64%.	(Z. Zhu et al. 2016)

	Combination: Dilute NaOH 1-3% + Commercial Enzyme Primafast 200	22.75–38.84% of saccharification / conversion	(Thite and Nerurkar 2019)
	Combination: Alkali + *B. safensis* M35	15.6% of saccharification / conversion	(Thite and Nerurkar 2019)
Sugarcane Straw	*Steam Explosion*: T: 200 °C; P: 15 bar; t: 10 min	Soluble XOS yields > 35 % (w/w) and fermentable glucose yields > ~78	(B.Brenellia et al. 2022)
Sago Pith	*Enzymatic:* 0.25 mL Cellulose Enzyme Complex (618 CMC U/g and 139 PNPG U/g); Substrate Concentration 10% (w/w); t: 24 hours	71.36% (w/w sago pith, db)	(Pinyo et al. 2016)
Sago Pith Waste (Hampas)	*Enzymatic*: 71.4 U/g Dextrozyme amylase and 20 FPU/g Acremonium cellulase; Substrate Concentration 0.09 g/mL	67.0 g/L fermentable sugar	(Husin et al. 2019)
	Combination: microwave hydrothermal hydrolysis	43.8% glucose and 40.5% ethanol yield	(Thangavelu et al. 2014)

	accelerated by carbon dioxide		
Eucalyptus chip	*Combination:* instant controlled pressure drop (DIC) (controlled steam pressure (up to 7 bar) with heat (up to 170 ◦C) during a short time)	87% (g reducing sugar/100 g pretreated biomass)	(Messaoudi et al. 2015)
Aleppo pine cone	*Combination:* instant controlled pressure drop (DIC) (controlled steam pressure (> 7 bar) with heat (up to 170 ◦C) during a short time)	74% (g reducing sugar/100 g pretreated biomass)	(Messaoudi et al. 2015)
Opium poppy waste stalks	*Combination:* Combination of DIC (P: 5 bar; t: 540s) and alkaline extraction (KOH concentration 22.17%; t: 7h; V: 53.28 mL)	26.23% hemicellulose extraction yield	(Kocabas et al. 2020)
Bamboo	*Combination:* steam explosion and green-liquor (Na_2S + Na_2CO_3)	Yields: hexoses 100.0% (% cellulose), ethanol yield of 40.1% (% cellulose)	(Gao et al. 2021)
Poplar	*Combination:* hydrothermal pretreatment (HP)	6.64% of XOS, 46.8% of fermentable	(J. Zhu et al. 2022)

	and acid hydrotropic pretreatment (AHP)	sugars (44.2% g of glucose and 2.6% of xylose), and 10.35% of Lignin nanoparticles.	
Acacia Wood	*Combination:* lime (calcium hydroxide) treatment (LT) (T: 70.9°C, t: 23.5) and hydrothermal treatment (HT) (T: 200°C; t: 10 min)	Glucose yields 73.5%	(Lee and Yu 2021)
Durian Peel	*Acid:* 2.75% H_2SO_4 (T: 127.14°C; t: 74.13 min)	53 % reducing sugars	(Panakkal et al. 2021)
Corn Stover	*Combination:* ball mill-assisted alkaline peroxide pretreatment	Yields: 69.65% XOS, 20.55% xylose, 68.94% glucose, and 21.15% gluco-oligosaccharides	(Zhang et al. 2021)

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177

178 **Potential Microbial Thermostable Enzymes Source for Lignocellulose**
179 **Bioconversion**

180

181 Enzymatic conversion of lignocellulosic biomass, particularly the cellulose
182 fraction, is typically carried out at 40-50°C, which is considered a slow hydrolysis
183 reaction. The hydrolysis process has a low sugar yield, partial hydrolysis, and a high
184 risk of microbial contamination (Patel et al. 2019). These limitations could be
185 overcome by using thermophilic or thermotolerant microbes to produce
186 thermostable enzymes for lignocellulosic bioconversion (Table 3). Thermostable

187 enzymes have the advantage of being able to be stored at room temperature for
188 longer periods than standard enzymes. Furthermore, the high-temperature process
189 should avoid microbial contamination during hydrolysis and reduce the risk of
190 contamination and enzyme activity loss during processing. Thermostable enzymes
191 also enable more stable, rapid, and efficient reactions (Giovannoni et al. 2020;
192 Vasconcellos et al. 2015).

193 For instance, Endoglucanase (EG) can be produced by *Aspergillus terreus*
194 *RWY* and *Thermobifidia fusca UPMC 901,* with optimal active conditions of 50-
195 60°C and 4.0-6.0 (R. Sharma et al. 2014; Zainudin et al. 2019). In the same
196 environment, *Aspergillus terreus* RWY can produce cellobiohydrolase (CBH) and
197 xylanases (R. Sharma et al. 2014). Xylanases from *Aspergillus fumigatus* JCM
198 10253 are stable at 50°C for 144 hours. *Aspergillus fumigatus* JCM 10253 and
199 *Geobacillus* sp. HTA426 also show cellulase activity at 50-60°C (Saroj et al. 2018).
200 Endoglucanase and endoxylosidase activities are essential for cello-
201 oligosaccharides (COS) and xylooligosaccharides (XOS) production (Barbarosa et
202 al. 2020; Cano et al. 2020).
203

204 Table 3. Potential Microorganisms for the Production of Thermostable Enzymes

No	Microbes	Fermentation Condition	Enzymes	Enzyme Activity	Enzyme Optimum Condition		Ref.
					pH	Temperature	
1	*Aspergillus terreus* RWY	Solid State; 45°C for optimal production of enzyme	*Cellulases* and *Xylanases*	*Cellulases* (Filter paper cellulase (FP) (11.3±0.65 U/g-ds), endoglucanase (EG) (103±6.4	4.0-6.0	50-60°C	(R. Sharma et al. 2014)

				U/g-ds), β-glucosidase (BGL) (122.5±8.7 U/g-ds), cellobiohydrolase (CBH) (10.3±0.66 U/g-ds)), *Xylanases* (xylanase (872±22.5 U/g-ds), β-xylosidase (22.1±0.75 U/g-ds), α-L-arabino-furanosidase (126.4±8.4 U/g-ds) and xylan esterase (907±15.5 U/g-ds)			
4	*Geobacillus* sp. HTA426	Isolated from hot spring district; optimal enzyme production at	*Cellulase*	CMCase activity: 103.67 U/mL	7.0	Optimum 60°C; Stable for 5 hour	(Potprommanee et al. 2017)

		60°C for 72 h				s incubation at range 50-70°C	
4	*Aspergillus fumigatus* JCM 10253	Isolated from Warangal district, India. Solid-State Fermentation (SSF) at 50°C for 7 days	*Extracellular Lignocellulolytic enzymes isolate*	CMCase (26.2 IU/mL), FPase (18.2 IU/mL), β-glucosidase (0.87 IU/mL), and xylanase (2.6 IU/mL) (incubation time of 144 h at 50 °C)	-	60°C for crude cellulase and 50°C for FPase, β-glucosidase and xylanase	(Saroj et al. 2018)
5	*Thermobifidia fusca* UPMC 901	Isolated from composted oil palm empty fruit; fermented on	*Endoglucanase*	CMCase: 0.9 U/mL (pH 5; T: 60 °C);	5.0	Thermal stability at 70°C (t: 24 hours) and	(Zainudin et al. 2019)

modified Tryptic Soy medium at 50°C for 24 hours	50-60°C (t: 144 hours)

Multi-Step Process and Enzymatic Hydrolysis Condition to Produce Cello- and Xylo-oligosaccharides

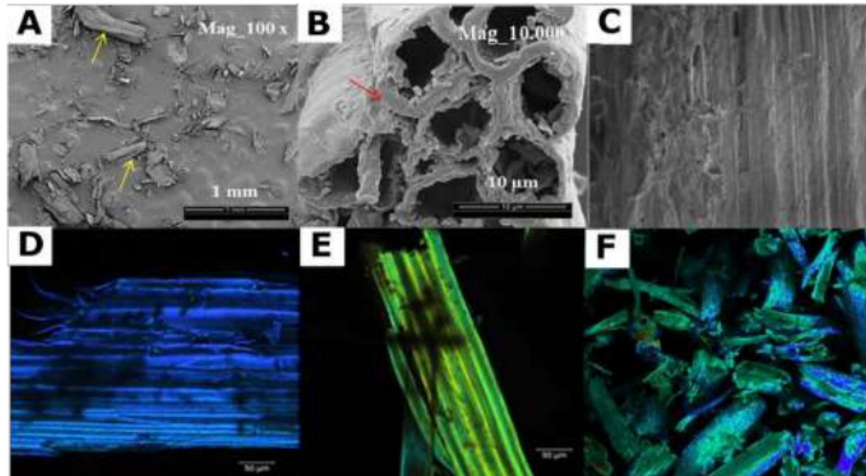
Enzymatic hydrolysis of lignocellulosic material can produce useful compounds like xylo- and cello-oligosaccharides. Both of these items have several health benefits, including prebiotic and anti-diabetic properties (Lin et al. 2016; J. Yang et al. 2015; Zhong et al. 2020). The production of cello- and xylo-oligosaccharides from lignocellulosic biomass can be carried out by multi-step enzymatic hydrolysis. Firstly, the pretreatment can be applied to the lignocellulosic biomass to destroy the lignin stealth, minimize the crystalinity of cellulose, and also break down the hemicellulose (Baruah et al. 2018).

In pretreatment I, hydrothermal pretreatment can be used to break down the crystalline structure of the lignocellulosic complex and to degrade hemicellulose. There has been no significant reduction in total lignin at this point, but there has been a change in the soluble and insoluble lignin fractions. At this point, xylo-oligosaccharides (XOS) are also being produced.

Marcondes et al. (2020) carried out hydrothermal pretreatment under optimum conditions at 182°C for 5.5 minutes in the absence of an external catalyst to produce ±43.61% xylo-oligosaccharides (XOS) and ±2.26% xylose. The XOS and xylose produce from this step accumulated in the liquid fraction while the solid fraction still consists of cellulose, xylan, and lignin.

Espirito Santo et al. (2018) observed physical changes in the lignocellulosic structure during hydrothermal pretreatment. The hydrothermal pretreatment separated the fibers on the bundle surface from the others, but there was no

231 significant difference in total lignin. Images from Field emission scanning electron
232 microscopy (FESEM) and Confocal laser scanning microscopy (CLSM) show that
233 lignin is redistributed and that no lignin accumulates on the surface. This is due to
234 variations in the soluble and insoluble lignin fractions (Figure 1).



235
236 Figure 1. SEM imaging of (a) untreated lignocellulose structure, (b) hydrothermal
237 pretreatment, fibers become more separated from the surface of the fiber bundle,
238 (c) alkaline pretreatment, lignin dissolved and pores increased; CLSM imaging of
239 (d) untreated lignocellulose structure, (e) hydrothermal pretreated, and (f) alkaline
240 pretreated lignocellulose (Ávila-lara et al. 2015; Espirito Santo et al. 2018).
241
242 After the treatment, the solid phase should enter the delignification process to
243 remove the lignin and obtain cellulosic pulp (CP) with a small amount of lignin.
244 This process will optimize the enzymatic hydrolysis because of the decreasing of
245 inhibitors (Qin et al. 2016; Zoghlami and Paës 2019). An alkaline pretreatment can
246 be chosen to remove a substantial amount of lignin while avoiding the formation of
247 undesirable inhibitor byproducts (Martín et al. 2022; Jinyu Tan et al. 2021). Using
248 a combination of alkaline sulphonation and steam pretreatment, Chu et al.
249 previously removed around 69.37% lignin. Khat et al. (2018) discovered that the
250 optimal alkaline pretreatment conditions for recovering XOS were alkaline
251 concentration, temperature, and steaming time of 12-18%, 110-120°C, and 37.5-40
252 minutes, respectively. This release greatly boosts cellulose accessibility (Ávila-lara
253 et al. 2015; Chu et al. 2018; Yuansah et al. 2019) (Figure 2).

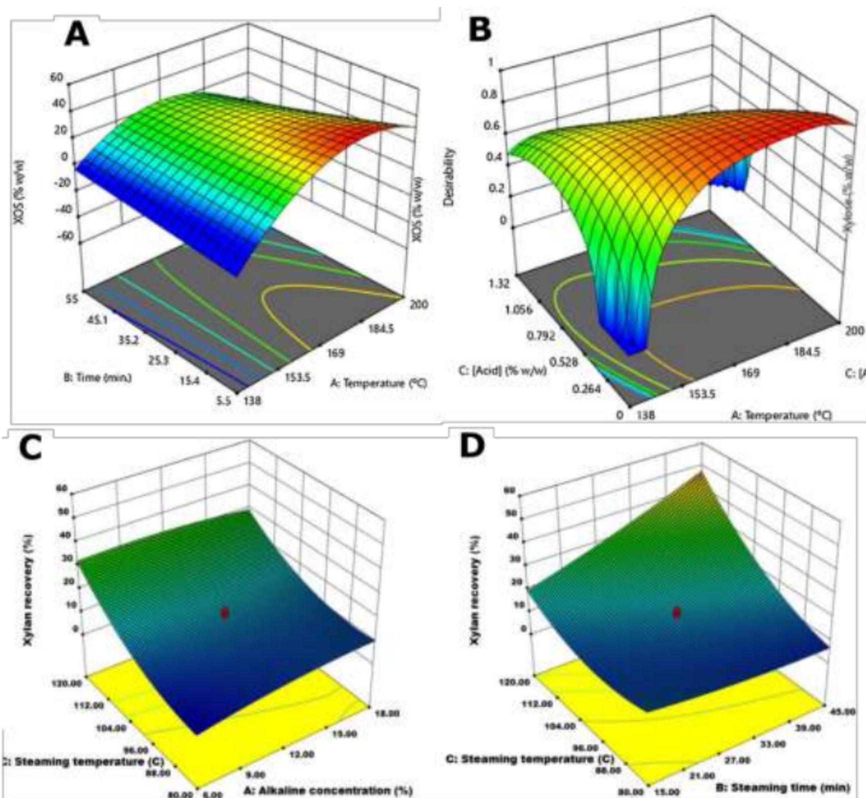


Figure 2. Hydrothermal pretreatment response surface for XOS yields (a) in the absence of acid, (b) for XOS maximum and xylose minimum yields; The alkaline pretreatment response surface (c) the effect of steam temperature and NaOH concentration, (d) steaming temperature and time (Khat et al. 2018; Marcondes et al. 2020).

Delignified cellulosic pulp can be hydrolyzed optimally by thermostable cellulases. Using thermostable cellulase enzymes to improve the process carried out under higher hydrolysis temperature might result in enhanced performance, such as lower enzyme concentration and shorter hydrolysis time. A higher temperature process is potentially lower hydrolysis charges and also more stable for longer hydrolysis time. Endoglucanase isolated from *Thermobifidia fusca* UPMC901 has been reported can maintain its activity without loss at temperature 50-60°C for 144 hours (Zainudin et al. 2019).

The thermostable cellulases used in the process should be separated from the β-glucosidases (BG) leaving only the endoglucanases (EG) and cellobiohydrolases

(CBH) acting on the substrate. β-glucosidase is effectively separated from other cellulases by carrying out the "adsorption-separation" method. In this method, the process was carried out at temperature 10°C and pH 7.0 to improve the retention of CMCase and decrease the retention of β-glucosidase. The decrease in the β-glucosidase retention rate is due to a change in pH and charge. EGs and CBHs have different isoelectric points (pI), while BGs have a pI of 8.7. When the pH is adjusted close to the pI of the BGs, the retention rate decreases. In addition, the BGs separate and accumulate in the supernatant during centrifugation. When β-glucosidase is already separated from cellulases, the process can be performed with elevated temperatures up to 50-60°C to optimize the endoglucanase activity. The hydrolysis performs with three-stage, improving the total COS yield accumulated in each step by approximately 51.78% with hydrolysis approximately 75.56% (Chu et al. 2014) (Figure 3).

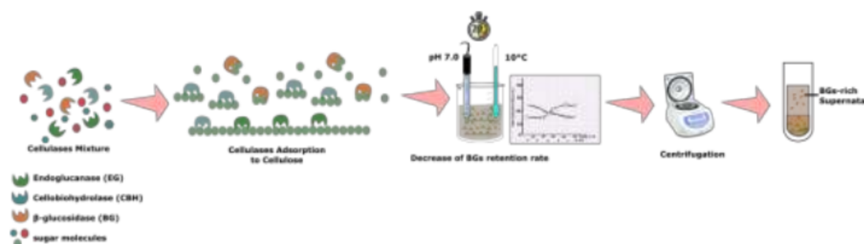


Figure 3. Schematic diagram of adsorption-separation method developed by (Chu et al. 2014).

This multi-step process using thermostable enzymes has the potential to improve the yield of COS and XOS production, decrease the use of enzyme concentration, quick hydrolysis time, and eliminate the risk of contamination. Furthermore, the gradual separation of products in the system in a multi-step process minimizes the inhibition of enzyme action due to product accumulation, allowing for a higher total yield of the desired product (Chu et al. 2014; Patel et al. 2019). Chu et al. (2014) developed a strategy for COS production using a cellulase enzyme deficient BG by performing the "adsorption-separation" method and multi-step enzymatic hydrolysis repeated three times. The product from each stage is separated from the process to avoid enzyme inhibition. The hydrolysis scheme gradually hydrolyzed the substrate over 6 hours, 6 hours, and 12 hours, yielding COS of 20.40%, 15.53%, and 15.85%, respectively, for a total yield of 51.78%.

Because a large portion of COS will be produced through enzymatic processes, cellulase inhibitors such as xylan and its derivatives must be separated at each stage to achieve optimal yields. This product can be made using a multi-step enzymatic hydrolysis process. Unlike COS, XOS will accumulate during pretreatment stages I, II, and the first stage of enzymatic hydrolysis (Figure 4). Product separation at each stage of multi-step enzymatic hydrolysis keeps XOS from being hydrolyzed further to the monosaccharide xylose. Several studies from Hao et al. (2022) and Zhang et al. (2021) used xylanase-cellulase enzymes in multi-step enzymatic hydrolysis. According to the findings, XOS only detected in the first step of enzymatic hydrolysis. Further hydrolysis revealed only monosaccharide accumulation (Table 4).

For the separation process, all of the soluble oligosaccharide-rich products from the multiple stages were collected and thin layer chromatography (TLC) can be used to separate COS, COS, sugars (disaccharides and monosaccharides), and other compounds (phenolic compounds and lignin derivatives) (Figure 4).

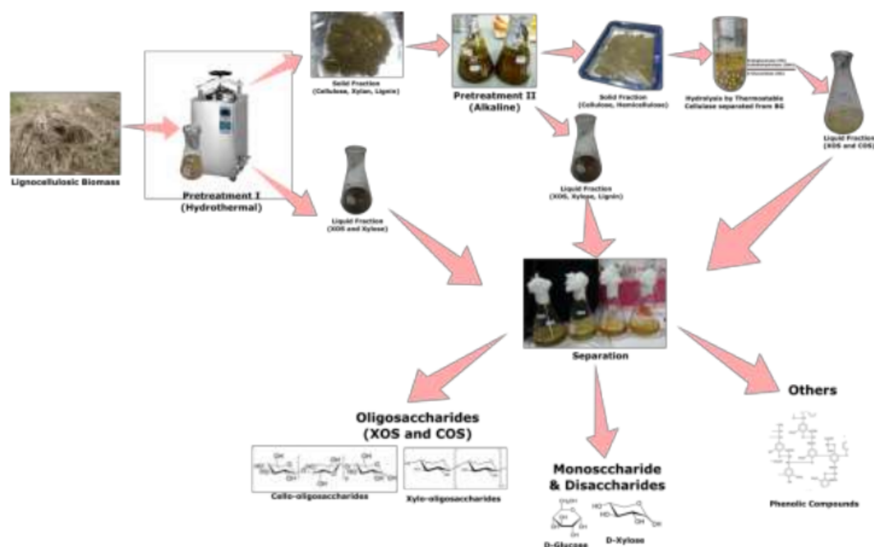


Figure 4. Schematic diagram of multi-step enzymatic hydrolysis strategy for COS and XOS production

323 Table 4. Comparison of XOS and COS production yields from multi-step
324 enzymatic hydrolysis strategies

Source	Enzymes	Strategy	Product	Hydrolysis Yields (%)			Total Yields (%)	Ref.
				1st stage	2nd stage	3rd stage		
Corncob	Cellulase deficient BGs	6+6+12 h	COS	20.40	15.53	15,85	51.78	(Chu et al. 2014)
	Xylanase - Cellulase	24+72 h	XOS	27.8	nd	n/a	27.8	(Hao et al. 2022)
Corn stover	Xylanase - Cellulase	8 +72 h	XOS	69.65	nd	n/a	69.56	(Zhang et al. 2021)

325 n/a: not applicable / not identified / not observed
326 nd: not detected

327 **Bioactivities of Cello- and Xylo-Oligosaccharides**

328

329 Cello-oligosaccharides (COS) can act as prebiotics by promoting the growth of
330 good microbiota in the gut, such as *Clostridium butyricum*, *Lactococcus lactis*,
331 *Lactobacillus paracasei*, and *Lactobacillus rhamnosus*, whereas xylo-
332 oligosaccharides (XOS) stimulate the bacterial groups Bifidobacilli and
333 Lactobacilli (Khat-udomkiri et al. 2020; Zhong et al. 2020). COS and XOS
334 fermentation by this group of beneficial bacteria can increase the production of
335 short-chain fatty acids (SCFA), which play an important anti-inflammatory role by
336 increasing immune cell chemotaxis and acting as an antimicrobial by disrupting
337 osmotic and pH balance (Jian Tan et al. 2014). XOS also has anti-diabetic activity
338 in type 2 diabetic rats by regulating blood glucose levels and increasing glucose
339 tolerance (Khat-udomkiri et al. 2020) (Figure 5).

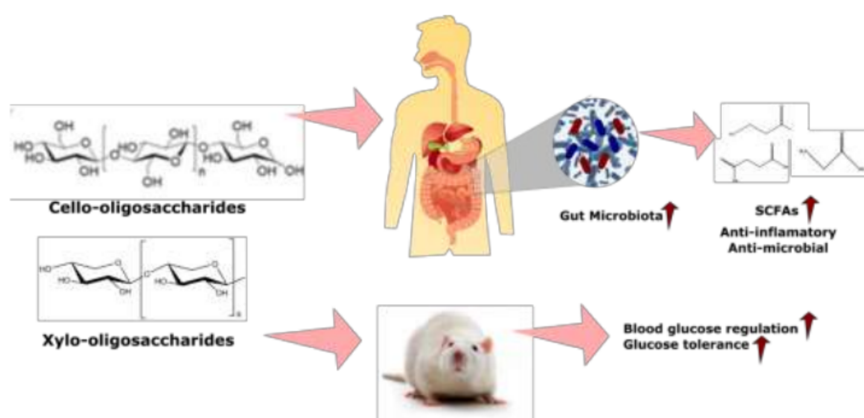


Figure 5. Bioactivities potential of COS and XOS

Challenges and Future Work

Pretreatment by-product Recovery During Production of XOS and COS

In the production process of XOS and COS, lignocellulose releases several phenolic compounds. Phenolic compounds released during the pretreatment process can be lignin derivatives or plant secondary metabolites. Vanillin is one of the valuable phenolics formed from lignin compounds that are discarded during the COS and XOS manufacturing processes. To generate the vanillin from the lignin, the liquid fraction from the pretreatment II process (Figure 4) was collected and fermented in an orbital shaker using submerged fermentation (SmF). The vanillin compound was extracted in ethyl acetate for 160 minutes from the fermented products. The vanillin was separated from other phenolic compounds, then centrifuged and filtered. The presence of vanillin in the filtrate and residue can be confirmed and quantified using LC-ESI-MS/MS. Thin layer chromatography (TLC) was used for further purification (Harshvardhan et al. 2017; Nurika et al. 2020) (Figure 6).

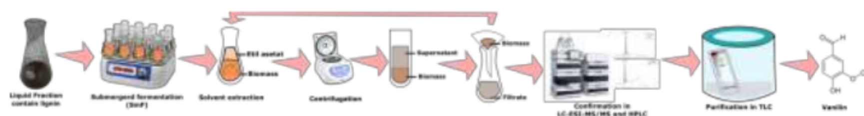


Figure 6. Shematic diagram of vanillin recovery from post-pretreatment liquid fraction in XOS and COS production

Separation of β-glucosidase Enzymes Using ‘Separation-Adsorption’ Method in High Temperature and Continuous System for COS Production

To increase the amount of COS produced, the BG enzyme must be separated from the other cellulase enzymes (endoglucanase (EG) and cellobiohydrolase (CBH)). Separating enzymes generally has a high production cost, so the adsorption-separation method developed by (Chu et al. 2014) is an alternative to separating BG from EG and CBH. The BG enzyme is separated by utilizing its isoelectric point, and decreasing the temperature results in a decrease in the retention rate of BG. When centrifuged, BG will leave the solid-liquid system and accumulate in the supernatant, while the solid residue consists of EG, CBH, and a trace of BG. Each time the product is separated from the substrate, the BG remains are gradually released in the multi-step enzymatic hydrolysis system.

To increase production capacity on a larger scale, thermostable cellulases can be induced from thermophilic microbes and used in the process (Yuansah et al. 2019). An "adsorption-separation" method can be used to separate thermostable cellulase enzymes from BGs. The thermostable cellulase-deficient BGs can be immobilized in a matrix for continuous system bioreactors (Chu et al. 2014; Pino et al. 2018) (Figure 7). The stability of immobilized thermostable enzymes will be an advantage in large-scale production, particularly in avoiding contamination and optimizing production yields. Furthermore, the enzymes used in the process can be reused, and the enzymes do not mix with the product or require further separation.

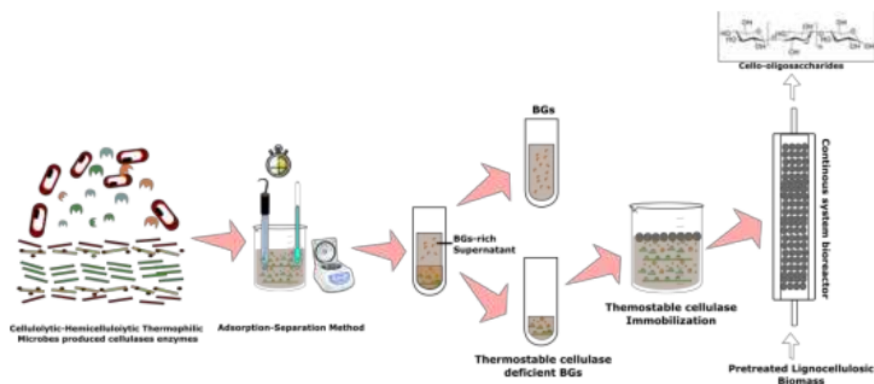


Figure 7. Schematic diagram of adsorption-separation methods to produce thermostable cellulase deficient BGs and its application in continuous system bioreactor

Conclusions

Based on the discussion thus far, it is possible to conclude that lignocellulosic biomass is one of the natural sources that can be converted into a variety of functional compounds such as XOS and COS. As previously stated, because the complex structure of lignocellulose makes it difficult to convert enzymatically and results in a slow reaction and low yield, it is critical to understand the inhibitors on the lignocellulosic source to determine strategies for carrying out enzymatic hydrolysis for COS and XOS production. Thus, it is envisaged that additional studies will be conducted to establish a more efficient technique for the production of valuable products from lignocellulose.

Declaration

Ethics approval and consent to participate not applicable

Consent for publication not applicable

Availability of data and materials not applicable

Competing Interest The authors declare that they have no competing interests

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Author Contribution The authors contributed to the study's conception and design. Material preparation and data collection were performed by Sunrixon Carmando Yuansah. The first draft of the manuscript was written by Sunrixon

422 Carmando Yuansah. Amran Laga and Pirman reviewed the article. The authors read
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427

428 **Reference**

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