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Abstract

Valorising plant cell-wall, marine and algal polysaccharides is of utmost importance for the development of the circular bioeconomy. This is because polysaccharides are by far the most abundant organic molecules found in nature with complex chemical structures that requires a large set of enzymes for their degradation. Microorganisms produce polysaccharide-specific enzymes that act in synergy when performing hydrolysis. Although discovered since decades enzyme synergy is still poorly understood at the molecular level and thus is difficult to harness and optimize. In the last few years, more attention has been given to improve and characterize enzyme synergy for polysaccharide valorisation. In this review we summarize literature to provide an overview of the different type of synergy involving carbohydrate modifying enzymes and the recent advances in the field exemplified by plant cell-wall degradation.

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Introduction

To reduce our carbon footprint and develop a more sustainable economy, it is crucial and urgent to move toward a circular bioeconomy model meaning using biotechnological tools to process biological residues into products, which can be reused, recycled, or released safely to the environment [1]. This implies tackling the deconstruction of chemically and structurally complex molecules that are naturally resistant to degradation such as lignocellulosic biomass that compose plant cell-walls [2]. Lignocellulosic biomass is mainly composed of cellulose, the most abundant polysaccharide on earth, a β-1,4 linked D-glucose molecule, generating a crystalline structure; hemicellulose, mainly xylans in cereals, which backbone sugar is composed of β-1,4-linked D-xylose units that is decorated with a variety of sugars and acetyl groups; and lignin, an aromatic polymer [3].

Lignocellulolytic microorganisms produce an arsenal of enzymes that efficiently hydrolyse in a concerted manner the different plant cell-wall polysaccharides to access the carbon source [4]. All these enzymes cooperate to degrade or modify the complex polysaccharides by generating a product that becomes the substrate of the next enzyme. When the overall activity of the concerted enzyme action is greater than the sum of individual enzyme activities, the co-operation between enzymes is described as synergistic [5]. Enzyme synergy has been demonstrated not only for plant cell-wall polysaccharide including cellulose, hemicelluloses, pectins, but also for complex marine polysaccharides such as alginate, or chitin [6,7]. It is employed in advanced biorefineries to optimize enzyme cocktails that efficiently hydrolyse plant polysaccharides, thus producing their component monomers [8,9]. Synergy is often described or sought for, but rarely explained at the molecular level although its understanding will improve the efficiency of industrial biomass valorization for sustainable fuels and chemical products. In this review, we provide an overview/update of enzyme synergies, with a focus on recent developments that provide a better

understanding of how enzymes catalyse, in space and time, plant cell-wall polysaccharide deconstruction.

Plant cell wall enzymatic deconstruction

Lignocellulolytic microorganisms have evolved different enzyme systems or strategies to produce a large set of enzymes enabling them to use plant cell-wall polysaccharides as a carbon source. Aerobic microorganisms, of which fungi are seen as the most active lignocellulosic biomass degraders, secrete in the extra-cellular environment a large set of enzymes [8]. Some anaerobic bacteria, in particular *Clostridia*, produce extracellular multi-enzyme complex anchored to the cell surface called the cellulosome [10]. The cellulosome is composed of a set of scaffolding grafting enzymes with different substrate specificities, making this complex highly efficient on lignocellulose. This efficiency was attributed to a high synergy among enzymes enhanced by their proximity to one another termed intermolecular synergy [11,12]. *Bacteroidetes* is one of the main bacterial phylum in soil, ruminant and human microbiota that encodes fine-tuned gene clusters dedicated to polysaccharide metabolism called Polysaccharide Utilization Loci (PULs) [13]. Each PUL contains genes that encode enzymes which orchestrate the detection, sequestration, enzymatic digestion, and transport of dedicated complex polysaccharides [14]. This organization allows efficient internalization of the oligosaccharide rapidly after a first degradation, and their subsequent hydrolysis in the periplasm by diverse enzymes working in synergy [15,16].

The enzymes involved in hydrolysis are glycoside hydrolases (GH), carbohydrate esterases (CE) polysaccharide lyase (PL) and lytic polysaccharide monooxygenases (LPMOs). All these enzymes are classified in sequence-based families in the CAZy database (http://www.cazy.org) [17]. Many of them are multi-modular, displaying one or several catalytic modules linked to one or more carbohydrate binding modules (CBM) [18]. The main role of the latter is to address the enzyme onto its substrate and contributes to intramolecular co-operation although important in the enzyme

activity they will not be discussed here [11,19–21]. Cellulases are glycoside hydrolases that degrade cellulose which include endo-β-1,4-glucanases, cellobiohydrolases (CBHs) (or exo-β-1,4-glucanases) and β-glucosidases [22]. These enzymes are classified in different CAZy families. It is now also well known that LPMOs, oxidative enzymes, classified as Auxiliary Activities (AA) in CAZy are key players in crystalline cellulose or chitin degradation. They oxidize the C1 or C4 carbon of β-1,4-linked polysaccharides [23]. Hemicellulose is a chemically complex group of polysaccharides composed of different monomeric units with different type of linkages that requires the action of endoglycanases for polysaccharide backbone degradation and exoglycosidases that hydrolyze decoration linked to the backbone [24]. Xylans, the main hemicellulose in cereals and grasses, are composed of β-D-1,4-xylose in the backbone and generally decorated with α-1,2- and/or α-1,3-linked L-arabinosyl residues, α-1,2-linked D-glucuronosyl and 4-*O*-methyl-D-glucuronosyl groups and are frequently modified by acetyl groups that are linked to the main chain D-xylosyl moieties [25]. Therefore, xylan acting enzymes are a group of β-1,4-xylanases that attack xylan backbone at random positions, β-1,4-xylosidases to hydrolyze xylobiose, the product of xylanases, into xylose and α-1,2 and/or α-1,3-L-arabinofuranosidases, carbohydrate esterases, and α-D-glucuronisades, that hydrolyze substituents. All enzymes are classified in different GH and CE enzyme families with different specificities (Fig. 1) [26].

Enzyme Synergy

During decades, cellulose has been the center of attention in biomass conversion strategies because of its deconstruction into glucose as exemplified by 70 years of research on *Trichoderma reesei* enzymes that showed the first examples of enzyme synergy [27]. Hemicellulases were considered as accessory enzymes and were shown later to be important key players not only to valorize pentoses but also to maximize cellulose degradation [28] leading to cellulase-hemicellulase synergism. In order to develop efficient enzymatic cocktail for biorefinery, enzyme synergies have been characterized not only between enzymes from the same organism [29,30] but also between enzymes encoded by different microorganisms [31]. Enzyme synergy can be evaluated by calculating the degree of synergy (DS) which is the ratio of the activity (or product released) of an enzyme mixture to the sum of their individual activities (or product released). Synergy is observed when the DS is greater than one. Two types of synergy have been defined: homeosynergy, when the enzymes hydrolyze the same part of the polysaccharide, i.e., both enzymes of interest hydrolyze either the main chain or the side chain of the polysaccharide, and heterosynergy when enzymes hydrolyze different parts of the polysaccharide, *i.e*., the main chain and the ramifications (Fig. 2). Examples of enzyme homeosynergy and/or heterosynergy have been described for different polysaccharides such as cellulose [32], hemicelluloses [15], pectins [33], alginate [6], or chitin [34]. In this review we will further detail enzyme synergies dedicated to lignocellulose degradation.

Homeosynergy

Cellulose is a homopolymer therefore all cellulases fit into the group of homeosynergy. The cellulase synergy is named after the enzyme mode of action *i.e.* endo-exo synergism (when synergy occurs between endoglucanase and cellobiohydrolase), exo-exo synergism (between

reducing and non-reducing end cellobiohydrolases) and, endo-β-glucosidase synergism (between endoglucanase or CBH and β-glucosidase).

Endo- Exo-cellulases synergy

Since, Reese et al., first documented synergism in 1950 [35] a model of endo-exo synergy was proposed [36,37]. Exo-acting cellobiohydrolases (CBH) start the hydrolysis from cellulose chain ends in a processive manner whereas endo-acting cellulases hydrolyze cellulose at random positions and create new ends from which the exo-acting cellobiohydrolases (CBH) can release cellobiose from either the reducing (GH7 and GH48) or nonreducing (GH6) ends. This model was completed by Kostylev et al., (2014) which results suggested that the endocellulase from *Thermobifida fusca Tf*Cel9A was more active on a uniform (undamaged) cellulose surface and generated eroded surface while the exocellulase *Tf*Cel48A would preferentially hydrolyze the more accessible substrate and replenish the uniform surface required by the endocellulase [38]. This finding illustrates that the substrate site generation is still a question and that it is not yet clear how the free dispersed enzyme behaves on a solid substrate. Very recently, thanks to atomic force microscopy (AFM), the synergistic endo-exo synergy was observed at single-molecule resolution. Zajki-Zechmeister and colleagues showed that only when *Trichoderma reesei Tr*Cel7A exocellulase and *Tr*Cel7B endocellulase hydrolyze cellulose fibril, the dispersed enzymes move to the same direction of the cellulose fibril and cluster on cellulose breaches. This phenomenon produces a molecular proximity between enzymes and an efficient multilayer-processive mode of degradation occurs [39]. The cooperativity induces the exocellulase to move 100-fold faster than when acting alone, leading to efficient cellulose hydrolysis. In this case *In fine* free enzymes on the substrate mimic the spatial confinement observed in cellulosome assemblies.

This observation is somehow similar to what high speed AFM showed earlier in exo-exo synergy with *Tr*Cel7A and *Tr*Cel6A exocellulases [40]. Although enzymes hydrolyzed the substrate from reducing- and non-reducing end respectively, when one molecule was stopped, enzymes were

clustered in “traffic jams” meaning that the enzymes were productively bound to the cellulose surface and peeled of the crystalline cellulose [40]. The hypothesis was that the shorter active site of *Tr*Cel6A could open and generate nicks on the cellulose substrate like an endocellulase and that these nicks could be the starting and end point of the more processive *Tr*Cel7A like in endo-exo synergy. Recent characterization of cellulase demonstrated the important role of the substrate binding strength for interfacial enzymes irrespective to the enzyme structure or GH family [41]. In the case of synergy, the trade-off between binding and activity has also to be considered.

endo- β-glycosidase synergy: Synergy between Cellulases and β-glucosidase or Xylanase and β-xylosidase

The full cellulose and/or hemicellulose saccharification of lignocellulosic biomass into fermentable sugars requires the action of β-glycosidases, which hydrolyse oligosaccharides into monosaccharides. In the case of cellulose, β-glucosidase hydrolyses cellobiose, the product of CBHs, exocellulases, into glucose units. CBH and β-glucosidase act in synergy to maximize the hydrolysis rate. Indeed, CBHs can be inhibited by their product, cellobiose, which causes a lower conversion rate of cellulose polymers into cellobiose. β-Glucosidases produce β-D-glucose from cellobiose and this monosaccharide is a lower inhibitor on CHBs compared to cellobiose [42–44]. Therefore, to improve cellulose saccharification and increase synergy with cellulases, several studies focused on the engineering of β-glucosidases to make the enzymes more active and/or more tolerant to glucose [45–47]. Lee *et al*., (2012) engineered a β-glucosidase (*Tr*Bgl2, GH1) using rational design and identified L167W mutation showing better enzyme activity and displayed an improved synergism with cellulases compared to wild-type [48]. This mutation is located at the entrance of the active site and increases the affinity (K_M) of the enzyme to cellobiose. It is suggested that this mutation offers a better coordination of the substrate in the active site which leads to improve affinity and enzymatic efficiency. Similarly, the *Trichoderma harzianum Th*Bgl

β-glucosidase from GH1 was engineered and the resulted double mutant L167W/P172L narrowed the entrance of the active-site and prevented product inhibition [45]. Mixed with cellulases, this mutant enhanced significantly biomass conversion to glucose. By mixing different enzymes, synergy experiments are not always performed under optimal conditions for each enzyme therefore improving stability or activity in defined conditions can improve enzyme synergy. Cao et al., discovered high glucose tolerant β-glucosidases (Bgl6 and Bgl15) and engineered these enzymes for improved stability. The resulting engineered Bgl6 β-glucosidase mutant displays better stability and activity while maintaining a high glucose tolerance and significantly improves sugar cane bagasse hydrolysis when added to commercial cellulases [49]. Mutations at position 167 in Bgl15, close to the active site, showed that increasing hydrophobicity of this region by introducing a valine or alanine improved the catalytic performance of the enzymes and their glucose tolerance [47]. Altogether, results indicated that improving activity, glucose tolerance and stability of β-glucosidases boosted synergy with cellulases and maximized the total saccharification of cellulose polymers.

β-Xylosidases are a group of enzymes that removes xylose units from xylo-oligosaccharides, and these enzymes are mainly found in GH3, 39,43 and 52 families and act in synergy with endoxylanases [50,51]. Indeed, xylanases produce xylo-oligosaccharides (XOS), mainly xylobiose and xylotriose, and studies have shown that adding β-xylosidase clearly improved the activity of endoxylanases [52–54]. β-Xylosidase, usually high xylose tolerant enzymes, hydrolyses XOS into xylose units and decreases the inhibition effect of XOS on xylanase activity [51]. This synergy leads to acceleration of the deconstruction of hemicellulose polymers [55].

Synergy between oxidative LPMOs and cellulases or xylanases

LPMOs are cooper-containing enzymes classified in CAZy database into auxiliary activities families (AA9, 10, 11, 13, 14, 15, 16 and 17) [56]. They oxidatively cleave the β-1,4-glycosidic linkage of polysaccharides. Several studies show that LPMOs and cellulases synergistically

191 depolymerize crystalline cellulose and it was reported that this oxidative-hydrolytic synergism
192 improves the saccharification yield of recalcitrant biomass [32,57,58]. For instance, the
193 exoglucanase *Tr*Cel6A and the LPMO *Ta*AA9A (*Thermoascus aurantiacus*) maximized the
194 degradation of Avicel, and bacterial cellulose, with a synergy index equal to 2 and 2.5, respectively
195 [32].

196 A large part of the described LPMOs acts on crystalline cellulose and cause local disruption of the
197 ordered cellulose structure [57,59]. LPMOs are adsorbed into crystalline cellulose and induce
198 fibrillation by impacting the cellulose fiber architecture. Then LPMOs catalyze the oxidation of
199 glucose units which leads to creation of new reducing and non-reducing oxidized termini [60–62].
200 In such manner, LPMOs generate new binding and acting sites for processive cellulases. A single-
201 molecule study by AFM demonstrated the positive effect of LPMOs activities on *Tr*Cel7A
202 exocellulase [63]. The treatment with LPMOs increased the fibrillation of crystalline cellulose
203 which boosted CBH activity and the release of soluble products. In addition, LPMOs also
204 increased the dynamics of cellulose-cellulase interactions. The adsorption – desorption events of
205 cellulases is improved and their mobility on cellulose surface is enhanced in the presence of
206 LPMOs. This finding demonstrates the positive effects of LPMOs on cellulases adsorption and
207 activity [63].
208 In order to improve LPMOs – cellulases synergy, the AA10 LPMO form *Hahella chejuensis*
209 (*Hc*LPMO10) was engineered and one mutation improved catalytic activity due to an improved
210 binding capacity of the protein toward cellulosic polymers [64]. Combined with cellulases, this
211 mutant boosted the hydrolysis level of microcrystalline cellulose. More recently, Srivastava et al.,
212 focused on the ability of multimodular LPMO (AA9) to bind recalcitrant biomass and the synergy
213 between LPMO and cellulases. Authors demonstrate the positive effects of LPMO-cellulose
214 binding on LPMO-cellulase synergy [65]. Altogether, these results suggest the crucial role of

LPMOs on the deconstruction of cellulose and improving their adsorption leads to increase their activities and their synergy with cellulases during cellulose hydrolysis.

In 2014, Agger et al, reported a new LPMO (*Nc*LPMO9C) form *Neurospora crassa* acting on xyloglucan and glucomannan, two hemicellulosic compounds [66,67]. This finding opened the door to the study of LPMOs acting on hemicellulose and their potential synergism with hemicellulases during the degradation of hemicellulosic compounds [68]. Recently, a LPMO acting on xylan (*Pc*AA14B from *Pycnoporus coccineus*) was tested with a xylanase GH11-M4 (GH11) from *Aspergillus niger* [68] and significantly increased the saccharification level releasing high levels of xylobiose and xylotriose from beechwood cellulosic fibers. Understanding the synergic interaction at the molecular level between LPMOs and their interplay with other hemicellulases is still rare. In the same publication, *Pc*AA14B was assayed with the xylobiohydrolase *Tt*Xyn30A from *Thermothelomyces thermophilaa* acting on xylan polymers. Despite low activity on different pretreated biomasses, *Pc*AA14B and *Tt*Xyn30A synergy index reached up to 5.70 on H_2O/acetone pretreated beechwood substrates suggesting that *Pc*AA14B degrades the recalcitrant hemicellulose chain adsorbed on crystalline cellulose and constantly creates substrates sites available for *Tt*Xyn30A. The degree of synergism is inversely proportional to the recalcitrant hemicellulose content, probably due to less accessibility of *Pc*AA14B to adsorbed xylan polymers.

Overall LPMOs act in synergy with cellulases and hemicellulases and this can be further boosted by laccases that release phenolic molecules able to donate electrons to LPMOs [70]. However, the structural determinants, physical and kinetics parameters involved in hemicellulosic LPMOs synergy remain unexplored.

Heterosynergy

Synergy between Cellulases and Hemicellulases

239 The use of hemicellulases with cellulases has been shown to improve the hydrolysis of
240 lignocellulosic biomass, because they remove hemicellulose polymers that interact with cellulose
241 making cellulose microfibers more accessible to cellulase attack while hydrolyzing xylo-
242 oligosaccharides shown to inhibit cellulases [28]. Synergy between *Tr*Cel7A and xylanases or
243 mannanases, suppressed the inhibitory effect of soluble and adsorbed hemicellulosic compounds
244 on cellulases activity [71]. Cellulases-hemicellulases synergism was observed during the
245 hydrolysis of several lignocellulosic biomass such as hardwood and softwood, corn stover and
246 wheat straw [72–74]. Nevertheless, resulting global hydrolysis can be controversial as it was
247 observed with cellobiohydrolyses, xylanases and α-L-arabinofuranosidases. Although xylanase
248 and α-L-arabinofuranosidase together enhanced the hydrolysis of hemicellulose their unsubstituted
249 oligosaccharide products inhibit the activity of cellobiohydrolyses [63]. It is suggested that
250 arabinose substituents on xylooligosaccharides in absence of arabinofuranosidase could prevent
251 their accommodation hemicellulosic compounds in the cellobiohydrolase active site thus
252 displaying no inhibition.
253
254 ***Synergy between xylanases and accessory enzymes***
255 The complete degradation of xylan requires several enzyme activities (Fig. 1), the main ones being
256 xylanases and xylosidases, as described above. However, accessory enzymes are important players
257 to liberate substitutions and make the polymer more accessible to depolymerizing enzymes. α-L-
258 Arabinofuranosidase (Abf) is a debranching class of enzymes that removes arabinose substitutions
259 from arabinoxylan backbone and enhances the hydrolysis of this polymer by synergetic action with
260 xylanases, they are classified in GH 3, 5, 43, 51, 54, 62 and 159 [64–66]. Xylanases, in GH10 and
261 GH11 families, often display a low tolerance to substitutions [79]. Thus, removal of arabinose
262 substitutions leads to an optimal activity on arabinoxylan and synergy between xylanases and α-
263 L-arabinofuranosidases [80]. Synergy index of 1.30, 1.39 and 1.24 were reported when XynA1

(GH10 endoxylanase) was tested with AbfA (GH51 arabinofuranosidase) on oat spelt xylan, Birchwood xylan and Beechwood xylan, respectively [52].

α-D-Glucuronidases remove glucuronic acids substitutions from glucuronoarabinoxylan and make the polymer more accessible to depolymerizing enzymes [81]. Indeed, the large part of characterized α-glucuronidases belongs to GH67 and removes glucuronic acids from short xylooligosaccharides [82]. More recently a new family of α-glucuronidases was discovered (GH115) and biochemical characterization indicates that these enzymes act on long chain of arabinoglucoronoxylan [83–85]. This family was tested in mixture with the endoxylanase Xyn10C from *Clostridium thermocellum* during the hydrolysis of softwood arabinoglucoronoxylan and it was shown that GH115 has a positive effect on xylanase activity, the amount of oligosaccharides released by the xylanase increases dramatically thanks to GH115 [81].

Xylan acetylations play a crucial role in the interaction between cellulose and hemicellulose compounds and it modulates the adsorption of xylan on cellulose surface [86]. Acetylation of xylan inhibits the activity of endoxylanases. It alters the hydrophobicity of xylans and impacts the binding efficiency of hydrolytic enzymes [87–89]. Carbohydrate esterases are a class of enzymes that remove acetyl groups from xylan and make this polymer more accessible to depolymerizing xylanases [90]. These enzymes enhance the activity of xylanases and promote the hydrolysis of xylan polymers [91]. All these observations highlight the relationship between enzyme specificity and synergy. Feruloyl esterases, part of CE1 family, are important key players by removing ferulic acid and many examples of synergy were evidenced with xylanases and cellulases [92,93].

Synergy between debranching enzymes is less explored and studied compared to synergy involving main chain acting enzymes. You et al., investigated the synergy between arabinofuranosidases and glucuronidases and confirmed the positive effect of arabinofuranosidases on the activity of one glucuronidase. This effect is probably due to the architecture of the active site of the glucuronidase which is, in some cases, not able to

accommodate substituted arabinoglucuronoxylan [94]. Recently, the same positive effect was observed between glucuronidase and carbohydate esterase. The carbohydrate esterase boosts the activity of glucuronidase by more than 50% on beechwood glucuronoxylan [95].

Overall, facing the complexity of plant cell-wall and, to a wider extent, to polysaccharides from various origins nature has evolved efficient enzymes and systems for their degradation. Nethertheless, the complete deconstruction of polysaccharides is still not mastered and understanding how enzymes operate in synergy to degrade such molecules is highly important. Within the last few years, many PULs have been characterized thus unraveling complete enzymatic cascades where synergy and enzyme co-operation is important [96] constituting an interesting source to find new enzyme synergies [16]. More recently AFM experiments showed that enzymes tackle the cellulose by forming enzyme clusters, where enzyme proximity enhances synergy and seems to be a key stone for the degradation. In this respect developing designer cellulosomes [97] controlling spatial organization [98] or characterizing multimodular enzymes that display high levels of synergy [99,100] could lead to a better understanding of enzyme synergy.

Concluding remarks

- Plant Polysaccharide hydrolysis requires enzyme synergy for efficient valorization. Although synergy has been widely described, the molecular determinants that influence enzyme synergy are not yet mastered.
- Applying visualization technics to follow enzyme synergy on insoluble substrates and or in conditions that mimic the substrate complexity and concentration could be highly relevant. Furthermore, exploring systems to engineer enzyme spatial organization appears to be an interesting approach.
- Enzyme synergy does not always exist between two enzymes and is difficult to predict. However, enzyme engineering can allow increasing synergy when molecular determinants are known. This is why the combination of structural and functional characterization of enzymes with approaches that consider the polysaccharide complexity, will enable progress in this field.

Abbreviations

GH, Glycoside Hydrolase; CE, Carbohydrate Esterase; PL, Polysaccharide Lyase; LPMO, Lytic Polysaccharide Monooxygenases; CAZy database, Carbohydrate-Active Enzymes database; CBM, Carbohydrate-Binding Module; CBH, cellobiohydrolase; AA, Auxiliary Activities; DS, Degree of Synergy; PUL, Polysaccharide Utilization Locus; AFM, Atomic Force Microscopy.

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Figure Caption

Figure 1: Schematic representation of enzyme activities degrading cellulose and xylan. On the left, enzymes acting on cellulose polymers and β-glucosidase acting on cellobiose. On the right, schematic representation of xylan polymers with several ramifications, enzymes acting on xylan and β-xylosidase hydrolyzing a xylobiose.

Figure 2: Synergic interactions of cellulosic and xylanolytic enzymes. Green boxes indicate cellulosic enzymes and pink boxes indicate xylanolytic enzymes. Only GH, AA and CE families discussed in the review are mentioned. GH : Glycoside Hydrolases, AA : Auxiliary Activities, CE: Carbohydrate esterases

669 Figure 1

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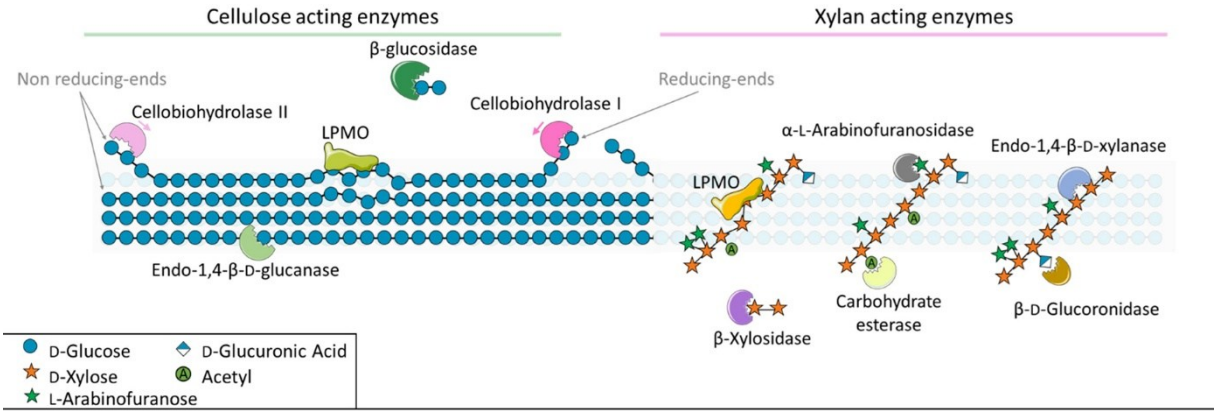

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674 Figure 2

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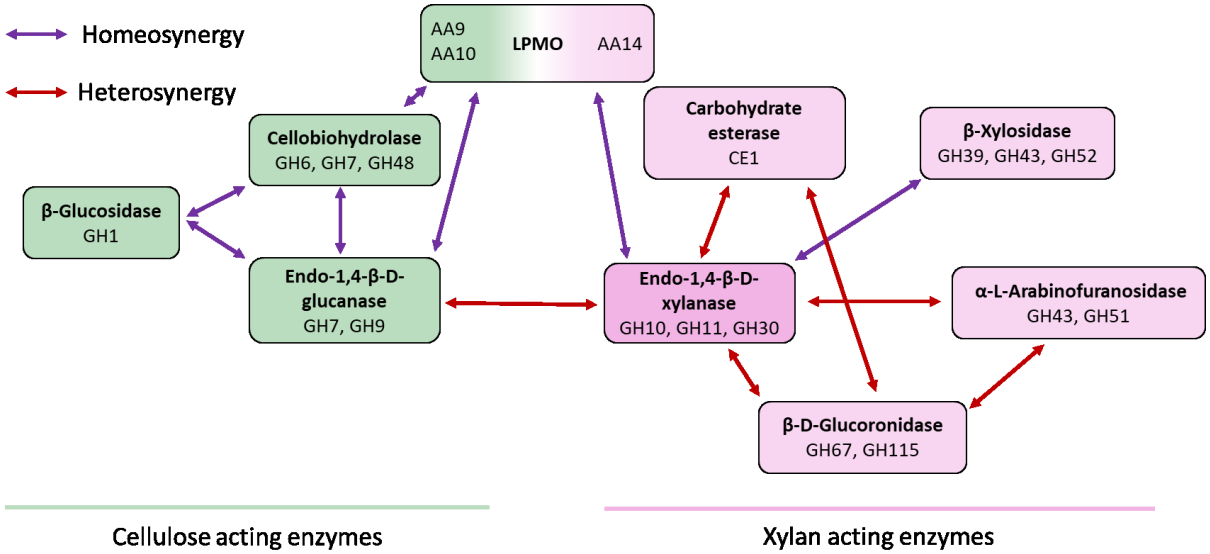

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