Green Production and Biotechnological Applications of Cell Wall Lytic Enzymes

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Abstract: Energy demand is constantly growing, and, nowadays, fossil fuels still play a dominant role in global energy production, despite their negative effects on air pollution and the emission of greenhouse gases, which are the main contributors to global warming. An alternative clean source of energy is represented by the lignocellulose fraction of plant cell walls, the most abundant carbon source on Earth. To obtain biofuels, lignocellulose must be efficiently converted into fermentable sugars. In this regard, the exploitation of cell wall lytic enzymes (CWLEs) produced by lignocellulolytic fungi and bacteria may be considered as an eco-friendly alternative. These organisms evolved to produce a variety of highly specific CWLEs, even if in low amounts. For an industrial use, both the identification of novel CWLEs and the optimization of sustainable CWLE-expressing biofactories are crucial. In this review, we focus on recently reported advances in the heterologous expression of CWLEs from microbial and plant expression systems as well as some of their industrial applications, including the production of biofuels from agricultural feedstock and of value-added compounds from waste materials. Moreover, since heterologous expression of CWLEs may be toxic to plant hosts, genetic strategies aimed in converting such a deleterious effect into a beneficial trait are discussed.

Keywords: Cell wall lytic enzymes; lignocellulose; sustainable biofactory; heterologous expression; microalgae; biofuel; plant immunity

1. Introduction

The plant cell wall is a complex and heterogeneous structure composed of polysaccharides and phenolic compounds assembled in two distinct layers called primary and secondary cell walls [1]. The primary cell wall is mainly composed of cellulose, hemicelluloses, and pectin and is typical of cells under active development and of young tissues. The secondary cell wall is deposited close to the primary one in mature cells and, besides cellulose and hemicellulose, is formed by lignin, while pectin is present only in small amounts. Clusters of secondary cell walls constitute lignocellulose, a heterogeneous material mainly composed by lignin, hemicellulose, and cellulose; in addition, to provide structural support to the plant, it represents a powerful barrier against both biotic and abiotic stresses [2].

In order to open a breach and depolymerize the cell wall polysaccharides into simple sugars, microbes have evolved specialized enzymes, referred to as cell wall lytic enzymes (CWLEs). In general, lignocellulolytic fungi and bacteria secrete different types of CWLEs at the microbe–plant interface in a sequential manner, mainly depending on the substrate they are dealing with. Many microbial species also evolved cellulosomes, which are multi-CWLE complexes constituted by distinct catalytic and binding domains carrying different degrading activities in a single polypeptide chain [3]. The high heterogeneity of CWLEs makes these enzymes highly valuable in different fields ranging from the agriculture to medicine. CWLEs are classified in the CAZy database (http://www.cazy.org/), where glycoside hydrolases, the most numerous class of carbohydrate active enzymes, are represented by more than 160 different families.

Energy demand is constantly growing, also because of the unstoppable growth of the world population, and the extensive use of fossil fuels has negative effects on air pollution and, ultimately, on climate. To avoid this, clean forms of energy are required. Lignocellulose, the most abundant carbon source on Earth, may be a solution since it is composed of complex carbohydrates that, if efficiently hydrolyzed, can be used to produce biofuels. Degradation of lignocellulose may be achieved by different methods including chemical, physical, and biological methods [4]. Efficient degradation of lignocellulose by chemical methods or by combining physico-chemical treatments are expensive and, in some cases, highly polluting. Conversely, the conversion of lignocellulose into fermentable sugars by using CWLEs may be considered as an eco-friendly alternative. Moreover, the broad substrate specificity of many CWLEs makes these enzymes highly versatile and, thus, exploitable in other important fields such as agriculture, food processing, and medicine. However, the industrial use of CWLEs is characterized by a high cost and low efficiency. Under this perspective, both the identification of novel CWLEs and the optimization of CWLE-expressing biofactories are crucial. In this review, recent advances in the heterologous expression of CWLEs as well as some of their possible biotechnological applications will be discussed. In order to support a sustainable biofactory of CWLEs, we will focus on the expression system in plants, since it is characterized by a high productivity/cost ratio and it consumes atmospheric CO_2 through photosynthesis, thus positively impacting global warming.

2. Lignocellulose: Structure and Functions

Lignocellulose is a complex and heterogeneous material constituted by clusters of dried cell walls whose primary role is to confer mechanical resistance and protection against microbial invasions [1]; therefore, lignocellulose has evolved to be highly resistant towards a broad range of different abiotic and biotic stresses. Lignocellulose is composed of lignin (10%–30%), hemicellulose (20%–30%), cellulose (35–45%), and, to a lower extent, by pectin, proteins, extractives, and ash [5] (Figure 1). Cellulose is organized in fibrillary structures that engage hemicellulose through hydrogen bonds; these structures, in turn, are occluded by lignin that confers hydrophobicity and robustness to the entire assembly [6]. The cellulosic microfibril is a homopolymer comprising (20–40) β-1,4-glucan chains that interact with each other through hydrogen bonds; its chemical repeat unit is cellobiose, a disaccharide of β-1,4-D-glucose. On the opposite, hemicellulose is a branched heteropolymer whose composition varies depending on the plant species. The most abundant hemicellulose derived from agricultural feedstock (e.g., straw, corn cob, bran) is represented by xylan, a β-1,4-polymer whose repeat unit is xylobiose, a disaccharide formed by β-1,4- D-xylose. Xylan can be modified by the acetylation of D-xylose units in the C2 or C3 (less frequent) position or by the addition of L-arabinose units (i.e., arabinoxylan) [7]. Lignin is formed by cross-linked aromatic polymers whose composing units (i.e., monolignols) are coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [8]. The relative abundance of each monolignol is not conserved in lignin but varies between the different plant species. In primary cell walls, the "cellulose–hemicellulose–lignin" assembly is surrounded by pectin, an acid polysaccharide mainly composed by α-1,4- D-galacturonic acid units. On the contrary, in secondary cell walls, the presence of pectin is strongly reduced in favor of an increased amount of lignin (Figure 1).
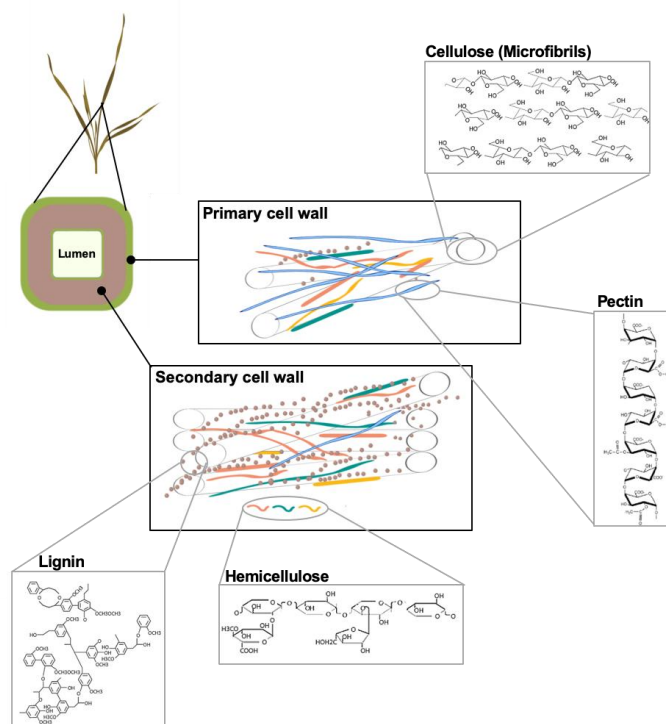
Figure 1. Schematic representation of plant primary and secondary cell walls. In the primary cell wall, cellulose chains are organized in fibrillar structures (white) that engage hemicellulose (orange, yellow, and green) through hydrogen bonds. This assembly is surrounded by a complex matrix of pectin (turquoise). In the secondary cell wall, pectin decreases in favor of a higher content of lignin (brownish dots) that adsorbs to cellulose fibers and confers recalcitrance to enzymatic hydrolysis. Structures of arabinoxylan and substituted homogalacturonan are reported as representative classes of hemicellulose and pectin, respectively.

Pectin is characterized by four regions with different structures and compositions: homogalacturonan, xylogalacturonan, and type I–II rhamnogalacturonan. Importantly, non-substituted D-galacturonic acid residues can bind di-cationic ions (mainly Ca++) through the carboxylic groups of two residues from adjacent chains, increasing the viscosity of the matrix that, in turn, hinders the accessibility of CWLEs to the inner layers, such as those represented by the hemicellulose–cellulose assembly [9]. Despite the low abundance in lignocellulose (less than 5%), pectin may affect the accessibility of CWLEs to cellulose, as shown by the increased saccharification of leaf and stem material obtained from transgenic wheat plants with altered pectin structure [9]. Moreover, pectin has a prominent role in alerting plants for the presence of pathogens [10] (see Sections 4.2 and 5.3).

Besides being the most abundant carbon source on Earth [11], lignocellulose exploitation in energy production is impeded by its complex structure and composition.

This review will focus mainly on CWLEs with degrading activity towards cellulose, hemicellulose, and pectin; further notions on CWLEs with degrading activity towards lignin, such as laccases and polyphenol-oxidases, are described in other recent reviews [12–14].

3. Degradation of Lignocellulose by CWLEs

Arrays of CWLEs are secreted by microbes to efficiently hydrolyze the variety of polysaccharides in plant cell walls into simple sugars [15]. Ligninases, hemicellulases, and pectinases, together with cellulolytic enzymes, are required to efficiently hydrolyze the lignocellulosic biomass. Hemicellulose and pectin are branched polysaccharides with highly heterogeneous compositions; therefore, the usage of enzymatic blends with different substrate specificities is mandatory for their efficient deconstruction. Substrate specificity is defined by the presence of specific and conserved

domains or modules, such as the glycosyl-hydrolytic (GH) domain and the carbohydrate binding module (CBM). In Table 1 the most common families of CWLEs are reported in accordance with the CAZy database. Bioinformatic analyses of conserved domains may be helpful to predict the substrate specificity of CWLEs with unknown functions based on sequence homology [16]. It is worth noting that the CBM is considered as an auxiliary module that is mainly conserved in cellobiohydrolases and multienzyme complexes (i.e., cellulosomes) [3]. On the other hand, some CWLEs are multifunctional enzymes that contain catalytic domains belonging to different GH families, as in the case of XynA from *Thermotoga neapolitana* [17] and CelB from *Caldicellulosiruptor saccharolyticus* [18].

Table 1. Distribution of conserved domains in major cell wall lytic enzymes (CWLE) families. Auxiliary activity (AA), polysaccharide lyase (PL), and glycoside-hydrolase (GH) domains are indicated. Numeration of conserved domains is in accordance with the CAZy Database (www.cazy.org).

SUBSTRATE	CWLE	AA
	Lytic Polysaccharide Mono-Oxygenase	9, 10, 15, 16.
	Cellobiose dehydrogenase	3.
		GH
CELLULOSE	endo-1,4-β-glucanase	5, 6, 7, 8, 9, 10, 12, 44, 45, 48, 51, 74, 124.
	Exoglucanase	1, 5, 9.
	Cellobiohydrolase	6, 7, 9, 48.
	β-glucosidase	1, 3, 5, 9, 30, 116.
HEMICELLULOSE		
	endo-1,4-β-xylanase	5, 8, 11, 30, 43, 51, 98.
Xylan	β-xylosidase	1, 2, 3, 30, 39, 43, 51, 52, 54, 116, 120.
Mannan	endo-1,4-β-mannanase	26, 45, 113, 134.
	β-mannosidase	1, 2, 5, 164.
Galactan	endo-1,4-β-galactanase	53.
	β-galactosidase	1, 2, 35, 42, 59, 147, 165.
Xyloglucan	Endoxyloglucanase	5, 9, 12, 16, 44, 45.
	Exoxyloglucanase	3, 74.
Arabinose branched	L-α-Arabinofuranosidase	2, 3, 43, 51, 54, 62.
PECTIN		
	endo-1,4-α-polygalacturonase	28.
Homogalacturonan	Exopolygalacturonase	28.
		PL
	Pectate lyase	1, 2, 3, 9, 10.

3.1. Cellulases and Lytic Polysaccharide Mono-Oxygenases

Degradation of cellulose is carried out by glycoside hydrolases and oxidoreductases [19]. These enzymes synergistically cooperate to degrade amorphous and crystalline regions of cellulose, respectively (Figure 2a). Glycoside hydrolases include endo- and exoglucanases, cellobiohydrolases, and β-glucosidases with cellobiase activity. Endo-1,4-β-glucanases mainly cleave the hairy region of cellulose, releasing fragments with different lengths. Concomitantly, the exo-acting cellobiohydrolases depolymerize such fragments into cellobiose, which is converted into glucose by β-glucosidases [20]. Cellobiohydrolases are processive enzymes and are divided in two main subgroups depending on their capability of starting the degradation from the non-reducing or the reducing end of the cellulose chain. In general, cellobiohydrolases are characterized by a GH domain

linked to a CBM that enhances the binding of the substrate and confers high specificity to the catalysis (Table 1). Importantly, endoglucanase and cellobiohydrolase are sensitive to product-inhibition mechanisms since they are both inhibited by cellobiose [21]. Indeed, lack of β-glucosidase activity in commercial enzymatic blends is one of the major bottlenecks at the industrial level since it negatively impacts the efficiency of cellulose degradation [22]. It is worth mentioning that not all the β-glucosidases are characterized by cellobiase activity (i.e., capable of degrading cellobiose into two D-glucose units; therefore, β-glucosidases are divided into three main subgroups depending on their substrate specificity, namely, aryl-β-glucosidases, pure cellobiases, and broad-specificity β-glucosidases.
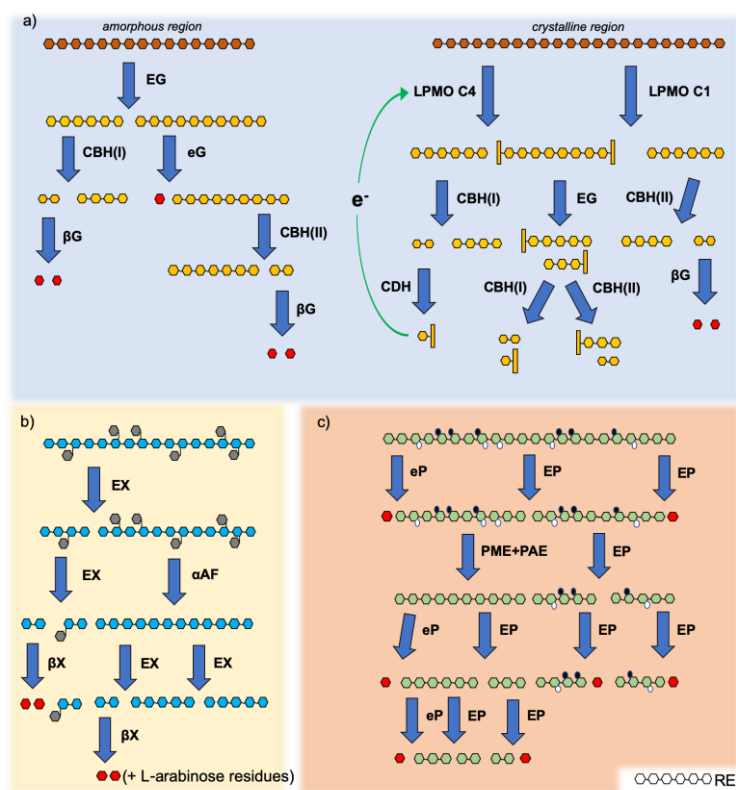

Figure 2. Schematic representation of cell wall polysaccharides and their degradation by CWLEs. (**a**) Degradation of (left) amorphous and (right) crystalline cellulose. Intermediate (yellow) and end-products (red) from each enzymatic reaction are shown. Oxidized glucose (i.e., gluconic acid) is represented as a rectangle. Cellobiose oxidation by cellobiose dehydrogenase (CDH) restores electrons (e-) to the lytic polysaccharide monooxygenase (LPMO). (**b**) Degradation of arabinoxylan, here reported as representative hemicellulose polysaccharide. Arabinose residues are in grey. Intermediate (turquoise) and end-products (red) from each enzymatic reaction are shown. (**c**) Degradation of substituted homogalacturonan, here reported as representative pectin polysaccharide. Intermediate (green) and end-products (red) of each reaction are reported. Acetyl and methyl groups are indicated as white and black circles, respectively. As a reference, the reducing end (RE) is indicated on the oligosaccharide in the white rectangle. αAF: α-arabinofuranosidase, βG: β-glucosidase, βX: β-xylosidase, CBH(I): nonreducing-end cellobiohydrolase, CBH(II): reducing-end cellobiohydrolase, CDH: cellobiose dehydrogenase, EG: endoglucanase, eG: exoglucanase, EP: endopolygalacturonase, eP: exopolygalacturonase, EX: endoxylanase, LPMO C1: C1-oxidizing LPMO, LPMO C4: C4-oxidizing LPMO, PAE: pectin-acetylesterase, and PME: pectin-methylesterase.

The crystalline region of cellulose is the substrate of cellulolytic oxidases (Figure 2a), such as lytic polysaccharide monooxygenase (LPMO). LPMO breaks cellulose chains by oxidative cleavages, thus improving the action of endoglucanases and cellobiohydrolases [23]. Depending on the type of LPMO, oxygen can be introduced at the C1 (producing gluconic acid) or at the C4 position (producing

4-ketoglucose) or at both C positions of the cleavage site as in the case of LPMO10A from *Thermobifida fusca* and LPMO9A from *Thermoascus aurantiacus* [24,25]. In order to maintain enzyme activity, the copper-containing active site of LPMO must be reduced after each cycle of reaction (e.g., microbial cellobiose dehydrogenase (CDH) can restore electrons to LPMO by oxidizing cellobiose units) [26]. Recently, Brenelli and collaborators [27] demonstrated that phenolic molecules released from lignin by the action of laccases work as electron donors of LPMOs as well, indicating that lignin decomposition can boost cellulose oxidative cleavage. Moreover, lignin, if not efficiently removed, adsorbs to the CBMs of endoglucanases and cellobiohydrolases in an irreversible manner and reducing their loading in the enzymatic mixture [28]. However, further studies are required for isolating novel polyphenol-oxidases and laccases that may synergistically cooperate with LPMOs and cellulases to efficiently convert cellulose into fermentable sugars [14].

3.2. Hemicellulases

Hemicellulose is abundant in lignocellulose, accounting for 20%–30% of total dry weight depending on the plant species. Both the heterogeneous composition and the branched structure of hemicellulose limit the access of cellulolytic enzymes to cellulose; therefore hemicellulose depolymerization is needed to efficiently degrade cellulose [29] (Figure 2b). Moreover, inefficient enzymatic degradation of hemicelluloses generates inhibitory by-products towards both CWLEs and fermentative processes, further reducing the conversion of lignocellulose into valuable products [30]. For example, oligomers derived from the partial breakdown of xylan, a hemicellulose abundant in secondary cell walls, inhibit the activity of cellobiohydrolase, a key enzyme for the degradation of cellulose [31,32]. Importantly, several commercial cellulolytic powders, commonly employed in the pretreatment of raw lignocellulosic material for use in biofuel paths, are enriched in hemicellulases with different substrate specificities [33]. Amongst the many hemicellulolytic enzymes employed in the degradation of lignocellulosic substrates at the industrial scale, it is worth mentioning

- xylanolytic enzymes: endo-1,4-β-xylanase degrades xylan and arabinoxylan in oligomers with different degrees of polymerization and xylobiose that, in turn, is degraded by β-xylosidase in two D-xylose units;
- mannanolytic enzymes: endo-1,4-β-mannanase degrades mannan and galactomannan in oligomers with different degrees of polymerization and mannobiose that, in turn, is degraded by β-mannosidase in two D-mannose units;
- galactanolytic enzymes: endo-1,4-β-galactanase degrades galactan and type I arabinogalactan in oligomers with different degrees of polymerization and galactobiose that, in turn, is degraded by β-galactosidase in two D-galactose units;
- α-L-arabinofuranosidase hydrolyses terminal, nonreducing α-L-arabinofuranoside residues in α-L-arabinosides, which can be found in arabinoxylan and arabinogalactan;
- xyloglucanolytic enzymes: xyloglucanases are divided into two subgroups depending on their ability to cleave xyloglucan through an endo- or an exo-mode of action. Xyloglucan is mainly distributed in the primary cell walls of dicotyledonous plants [34].

3.3. Pectinases

Pectin is structurally one of the most complex families of polysaccharides constituting 35% of primary walls in dicots and nongraminaceous monocots and 5%–10% in grass [35]. Pectin is the less-abundant polysaccharide in lignocellulosic biomass, accounting for 5% of total dry weight, but it represents a major component in agricultural scraps such as peels and pomace. During the plant–pathogen interaction, homogalacturonan, a linear polymer of α-1,4-D-galacturonic acid, is the first polysaccharide target of degradation by invading microbes. Pectinolytic enzymes include endo-polygalacturonases, exo-polygalacturonases, and pectate liase (Figure 2c) [36]. Endopolygalacturonases cleave homogalacturonan in oligomers with different degrees of polymerization by releasing digalacturonic and galacturonic acid units from their reducing ends. Concomitantly, exo-polygalactuonases release galacturonic acid from the nonreducing end of

galacturonyl-oligomers released by the action of the endopolygalacturonases [37]. Pectate lyase degrades homogalacturonan to produce oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their nonreducing ends by transeliminative cleavage. In order to slow down the hydrolysis of homogalacturonan by pectinolytic enzymes, plants (i) can produce proteinaceous inhibitors against microbial endopolygalacturonases [38] and (ii) can modify pectin to different extents, for instance by adding methyl- and acetyl- groups in C6 and C2–C3 positions, respectively, of D-galacturonic acid residues. On the other hand, microbes have evolved endopolygalacturonases that elude recognition by plant proteinaceous inhibitors [39] and pectin methyl- and acetyl-esterases that remove modifications from the homogalacturonan chain, thus supporting the action of pectinolytic enzymes.

4. Production of CWLEs in Microbial and Plant Expression Systems

Amongst the various organisms producing CWLEs, lignocellulolytic fungi and bacteria are the most relevant. These organisms are highly specialized in degrading the different components of lignocellulose since they use monosaccharides derived from the plant cell wall to survive and proliferate; thus, they represent a precious source of CWLEs with different substrate specificities and enzymatic characteristics. In general, these organisms evolved to produce a variety of CWLEs in sufficient amounts for their own subsistence. An overall distinction of such organisms could be done based on their lifestyle, for instance, specialized (i) in degrading the cell wall from living plants, as in the case of plant pathogens, or (ii) in degrading dead plant matter, as in the case of saprophytes. However, the exploitation of wild-type microbes as biofactories to obtain CWLEs has a main limitation represented by the low level of enzyme production. This strongly impacts the cost of CWLEs obtained from wild-type hosts, making the entire process of enzymatic degradation less sustainable. Heterologous expression of CWLEs using *Escherichia coli*, yeasts, and plants may be a valid alternative. In the last decades, these organisms succeeded in producing high levels of CWLEs; hereafter, advantages and disadvantages of expressing CWLEs in these different hosts are discussed.

4.1. Heterologous Expression of CWLEs in Fungi and Bacteria

To date, different bacteria and yeast species have been used as expression hosts for CWLE production. These microorganisms differ significantly in their cell wall structure and subcellular compartments, thus affecting both protein secretion and expression efficiency.

Over the past years, expression of both single CWLE and enzyme mixtures has been performed in different bacteria species (i.e., *Bacillus* [40] and *Clostridium* species [41] as well as *E. coli* [42,43]). Constitutive promoters were exploited to maximize the expression of many cellulases [44–47], avoiding the additional cost from the use of chemical compounds as transcriptional inducers. However, side effects occurred in some cases, as the saturation of transmembrane transport mechanisms had inhibitory effects on cell growth and viability [46–48]. To reduce such toxic effects, weakening the promoter strength through mutagenesis was of help [46,48]. Alternatively, cellulases were expressed under the control of a growth phase specific inducible promoter [47,49]. Moreover, to overcome inefficient expression caused by the different codon usages of the various bacterial species, synthetic genes were codon-optimized according to the codon usage of the expression host [45,50]. Although these molecular tools have led to significant progress, efficient heterologous expression of secreted CWLEs from bacterial hosts is still challenging. The low levels of soluble proteins and the poor secretion ability represent the main limitations of Gram-negative bacterial hosts. Although a number of secretion pathways have been elucidated in the model organism *E. coli* [51], the translocation step across a double membrane system negatively impacts the yield of secreted CWLEs [52]. Different strategies have succeeded in increasing the secretion efficiency of heterologous proteins from *E. coli*. For example, the solubility of some cellulases was enhanced (i) by fusing their GH domain to the CBM from another (highly soluble) cellulase [53], (ii) by replacing the native signal peptide with those from efficiently secreted proteins [44,47,54], or (iii) by expressing synthetic sequences optimized with the codon-usage of the expression host [55]. Other approaches allowed the

purification of active cellulase and hemicellulase from inclusion bodies by using denaturing agents such as urea and β-mercaptoethanol coupled to a subsequent refolding step [56] or by reducing the temperature of expression in order to ensure correct protein folding [57]. Alternatively, different species, especially those belonging to Gram-positive bacteria (e.g., *B. subtilis* or *L. lactis*), were selected as expression hosts since they secrete the recombinant proteins more efficiently than Gram-negative bacteria [58,59].

In eukaryotic organisms, yeasts are eligible hosts for overcoming the solubility and secretion limits that affect heterologous expression in the bacterial system. Several yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, and *Yarrowia lipolytica* are characterized by high expression yields and the ability to perform eukaryotic post-translational modifications (i.e., disulfide bond formation and N-glycosylation), which are often present in CWLEs, therefore enhancing their stability and activity.

CWLEs not efficiently produced in bacterial hosts can be secreted in active forms from yeast, as in the case of the endo-pgA from *Aspergillus niger* that failed to acquire proper folding upon expression in *E. coli*; conversely, the active form of endo-pgA was successfully secreted in high amounts by *Saccharomyces cerevisiae* [60].

Many CWLE-encoding genes have been successfully expressed as secreted proteins in *S. cerevisiae*, representing the host of choice for expressing a variety of cellulases [61,62], hemicellulases [63,64], and pectinases [60]. Another well-described and widely applied yeast for the heterologous expression of CWLEs is the methylotrophic yeast *P. pastoris* [65]. Amongst the many advantages of this expression system, the low secretion level of native CWLEs [66] allowed pure enzymes to be obtained from the culture filtrates without requiring further expensive purification procedures [67]. Recombinant cellulases [68–71] and pectinases [72–74] were expressed in high yields by *P. pastoris*. Differently from *S. cerevisiae*, *P. pastoris* does not heavily glycosylate the secreted proteins, thus avoiding hyperglycosylation and, therefore, protein instability.

Different tools have been developed in order to improve the expression levels of CWLEs in yeast. These include the use of synthetic [75] and constitutive promoters, such as the P_{TEF1} and P_{PGK1} promoters from *S. cerevisiae* [61,76]. Codon-usage optimization is another approach that has succeeded in increasing the expression yields of cellulases and hemicellulases from *Pichia pastoris* [68,77]. Lastly, selection of multicopy transformants (i.e., mutants carrying multiple construct copies integrated in their genome) has allowed high expression yields to be reached for several cellulolytic enzymes [76,77].

Considering that plant biomass is efficiently degraded by the synergistic action of cellulases, hemicellulases, pectinases, and ligninases, an interesting perspective from the heterologous expression of CWLEs is the possibility of expressing multiple enzymes in the same recombinant strain. Co-expression of multiple genes can be obtained by cloning multiple genes in a single plasmid [78] or by combining different strains expressing different enzymes [79–81]. Optimization of these systems may generate microbial cell factories capable of expressing complete sets of glycoside hydrolases, thus reducing the production cost of recombinant CWLEs.

4.2. Heterologous Expression of CWLEs in Plants

Plant expression of microbial CWLEs is a major challenge that biotechnologists are currently facing. Plants are desirable expression hosts since they are characterized by low production costs and high productivity. Moreover, they consume atmospheric CO_2 through photosynthesis, which positively impacts global warming and points to the plant expression system as a valuable green alternative. The *in muro* targeting of CWLEs may enhance the hydrolysis of cell wall polysaccharides, allowing an efficient conversion of fermentable sugars into biofuel-related compounds [82]. However, expression of CWLEs using plants as a heterologous system may impart unwanted and undesired side effects. CWLEs are produced by microbial pathogens to open a breach in the cell wall, concomitantly supporting the infection process [83]. Moreover, the degradation of cell wall polysaccharides provides sugars for sustaining the heterotrophic growth of phytopathogens inside the plant tissue [84]. In order to counteract the various CWLEs secreted by the pathogen, plants

synthesize different specialized defense proteins that hinder their hydrolytic activity [38,85–88]. Moreover, plants have evolved a complex system of cell surface receptors that promptly perceive CWLEs by sensing the products of their activity (i.e., cell wall derived fragments, formerly known as damage-associated molecular patterns (DAMPs) [89–91]), or to perceive the CWLEs themselves [92,93] as microbe-associated molecular patterns (MAMPs) by specific recognition mechanisms [94]. Such recognition events mainly occur at the apoplast/outer membrane interface, where plant pattern recognition receptors (PRRs) are localized [95]. Upon perception, plants activate defense responses in qualitative and quantitative manners; in general, a higher concentration of MAMPs/DAMPs will result in the activation of more intense defense responses. The amplitude of such responses varies also depending on the type of molecule/epitope triggering the plant defense; to date, oligosaccharides from plant cell walls with a proven nature of DAMPs (i.e., oligosaccharins) include oligogalacturonides [10], cellodextrins [91,96], and xyloglucan fragments [97]. Although the defense responses protect plants against microbial infections, hyperactivation of immunity negatively impacts plant development. Therefore, the uncontrolled *in planta* expression of CWLEs may result in impaired growth, reduced productivity, and lethality [90,98,99]. In order to circumvent these undesired effects, different CWLEs expression strategies may be adopted, such as (i) compartmentalized expression/accumulation, (ii) inducible gene expression, (iii) inducible enzymatic activity, and (iv) use of plant hosts that are not sensitive to CWLE activity. Compartmentalized expression was attempted to constitutively accumulate CWLEs into different organelles such as chloroplasts [100,101], lytic vacuoles [102,103], and cytoplasm [104,105], thus avoiding interaction with cell wall polysaccharides as well as the activation of PRRs that, in turn, may trigger immune responses (Figure 3a). In general, chloroplast-localized expression of CWLEs allows a high yield of recombinant protein to be obtained, even if, in some cases, chloroplast expression of cellulases has resulted in stunted growth and a pale-green phenotype [101]. The chloroplast expression system is not indicated for expressing fungal CWLEs that require glycosylation for proper activity and stability [106] and, more importantly, needs several rounds of selection for reaching homoplasmy (i.e., stable expression condition in which the plastomes of all chloroplasts are recombinant [107]). Alternatively, recombinant proteins may be targeted to vacuoles upon passage through the endoplasmic reticulum (ER) and the Golgi apparatus by fusing specific C-terminal propeptide sequences to the protein of interest [108,109]. Delivery of recombinant proteins to lytic vacuoles (LVs) has already been attempted in the past, although this compartment was expected to be hydrolytic. Certain CWLEs such as cellobiohydrolase and endoglucanase accumulate at high yields in LVs [110]. However, vacuole sorting of CWLEs was strictly dependent on the development of LVs in different plant tissues, and protein accumulation was strongly reduced in both young and senescent leaves [111]. Interestingly, compartmentalized expression of CWLEs in crop plants concerns a very limited number of cellulases [102,104,105,112–114]. Brunecky and colleagues [115] succeeded in the apoplast accumulation of a truncated version of cellulase by expressing only the GH domain and excluding the CBM. Stable plant expression of CWLEs such as xylanases and β-glucosidases were easier to obtain than that of cellulases, hinting that expression of cellulolytic enzymes *in planta* has intrinsic limitations. In accordance with this observation, it has been shown that cellulase activity generates fragments, such as cellodextrins and cellobiose, which act as powerful DAMPs capable of inducing plant defense responses [91,96]. Therefore, an uncontrolled activity of cellulolytic enzymes could lead to hyperactivation of immune responses and, consequently, largely affect plant growth and development. This scenario is avoided naturally by plants, since they are endowed with an enzymatic system that can inactivate, through specific modifications, the elicitor activity of different types of oligosaccharins [87,88]. This plant characteristic should be further investigated and exploited to optimize CWLE production in this host.

Inducible gene expression of CWLEs was achieved by using synthetic promoters such as those induced by chemical compounds (e.g., ethanol [116] and β-estradiol (i.e., XVE-based expression vector [117])) or by using endogenous promoters such as those induced by pathogens [118] and senescence [119] (Figure 3b). The strategy of using endogenous promoters is characterized by two major disadvantages: (i) the leakiness of the promoter and (ii) the developmental stage (e.g.,

senescence) at which the promoter is more active that and strongly reduce the expression potential. On the other hand, the use of synthetic promoters such as those induced by ethanol and β-estradiol makes the inducible systems not viable for field applications.
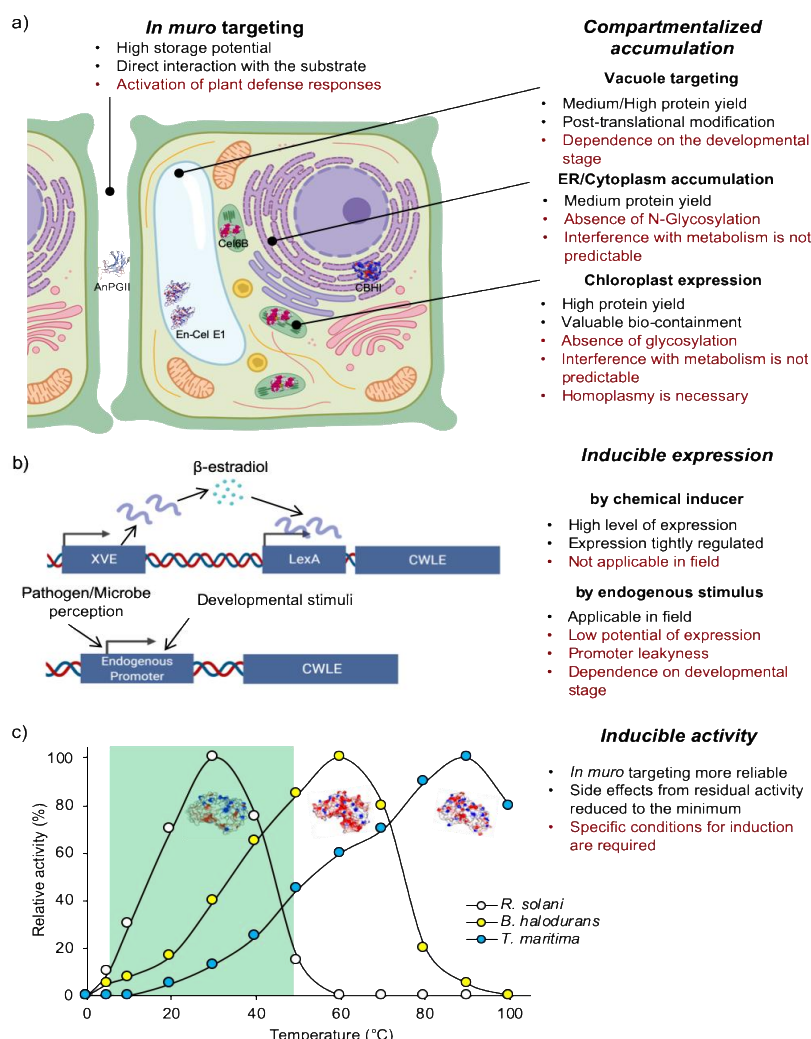

Figure 3. Plant production of microbial CWLEs. Production of CWLEs as obtained by (**a**) constitutive expression and compartmentalization or (**b**) inducible gene expression. Advantages and disadvantages of each strategy are indicated in black and red, respectively. In (a), the represented CWLEs were successfully produced upon compartmentalized expression. (**c**) Theoretical optimal temperature of XynA from the mesophilic fungus *R. solani*, from the thermophilic bacterium *B halodurans*, and from the hyperthermophilic bacterium *T. maritima,* here reported as a representative set of CWL isoenzymes. Activity of XynA from *T. maritima* is strongly reduced in the temperature range of plant growth (green box) and increases the chance of preserving the productivity of the transgenic plant. AnPGII: endo-1,4-α-polyglacturonase II from *A. niger* (98); Cel6B: endo-1,4-β-glucanase from *T. fusca*, (100); En-Cel E1: endo-1,4-β-cellulase from *A. cellulolyticus*, (102); and CBHI: cellobiohydrolase I from *T. reseei*, (104).

In the last instance, an inducible activity of CWLEs can be achieved by expressing CWLEs with pH- and temperature-dependent activities such as those enzymes from acidophilic/halophilic and hyper-thermophilic bacteria (HCWLEs), respectively (Figure 3c). Plant expression of CWLEs with inducible activity (i.e., optimal activity at pH and temperature values extremely different from those of the plant) can be used to further reduce (dangerous) residual activities in vivo, thus preserving plant health and productivity. Notably, accumulation of HCWLEs was achieved in the apoplast of transgenic *Arabidopsis thaliana* plants without adverse effects on plant growth [120,121]. When

compared to their mesophilic enzymatic counterparts, HCWLEs have several advantages, which are mainly dependent on the temperature at which HCWLEs are active [122]. At industrial levels, high temperatures promote the relaxation of cell wall structures, thus facilitating CWLE substrate binding and preserving the reaction mixture from the contamination of mesophilic and thermophilic microbes [123]. Importantly, high temperatures inactivate plant CWLE-proteinaceous inhibitors, which are localized in the cell wall as a defense mechanism [38,124,125], thus preventing them from interfering with enzymatic degradation. Moreover, the robust protein structure, a common feature of many HCWLEs, allows their use even in the presence of anionic surfactants, extreme pH conditions, and harsh chemicals [126]. In turn, industrial practices can exploit these severe conditions in order to deconstruct lignocellulose, favoring downstream enzymatic reactions [127].

Another attractive strategy resides in the use of microalgae as a platform for the expression of CWLEs. Microalgae are promising expression hosts since they are characterized by a relatively fast growth cycle, and their cultivation is less expensive compared to that of other microorganisms (e.g., bacteria and yeasts) [128]. Moreover, differently from higher plants, microalgae do not require arable lands for their cultivation, thus avoiding the loss of areas that may be employed in the agri-food sector. Contrary to plant cells, some species of unicellular green algae, such as *Chlamydomonas reinhardtii,* possess a cell wall mainly constituted by proteins [129]; thus, the lack of polysaccharides in their cell wall circumvents the deleterious effects of expressing CWLEs in plants. Although *C. reinhardtii* secretes two different endo-β-1,4-glucanases upon cellulose perception [130], these endogenous enzymes are not sufficient alone to efficiently degrade hydrolysis-recalcitrant substrates such as lignocellulose. Thus, an interesting perspective is to express a set of secreted CWLEs (including pectinases, hemicellulases, and cellulases) in a unique algal culture, similar to yeast systems that have yielded strains able to grow on cellulose [81]. Alternatively, the expression of CWLEs can be carried out in the microalgal chloroplast in order to maximize the yield of recombinant proteins by circumventing gene-silencing events that affect nuclear expression [131].

5. Bio-Applications of CWLEs

The use of plant-expressed CWLEs can be exploited in different industrial and agricultural processes in order to reduce costs and obtain more environmentally and human-safe products (Figure 4a). The latest advances in the biotechnological application of CWLEs in the production of second- and third-generation biofuels, medical and nutraceutical fields, food processing, and enhancement of plant resistance to pathogens are discussed.
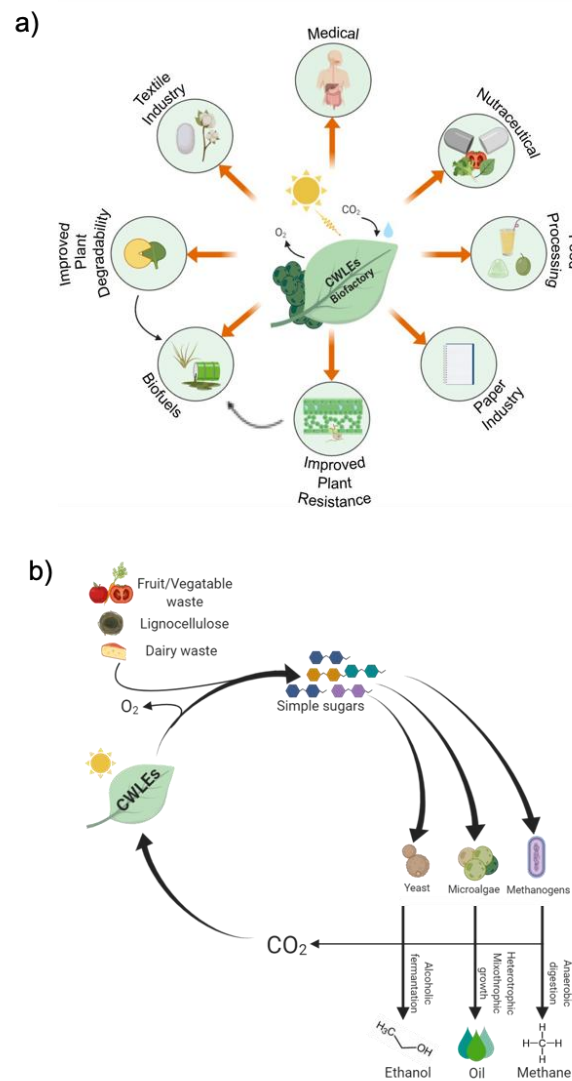
Figure 4. Biotechnological applications of CWLEs. (**a**) Possible applications of plant-expressed CWLEs in agriculture, industrial, and medical fields; both an increased degradability and biomass productivity positively impact the release of fermentable sugars from plant biomass. (**b**) Use of plant-expressed CWLEs for the production of second- and third-generation biofuels; sugars released from waste materials upon CWLE-treatment are used as feed for yeasts, microalgae, and methanogenic bacteria. CO_2 emitted from these metabolic processes may be converted into CWLEs and O_2 by the plant biofactory.

5.1. Use of CWLEs for the Production of Biofuels from Agricultural Feedstock

Chemical or enzymatic treatments are employed to convert lignocellulosic biomass to fermentable sugars that, in turn, are used to produce biofuels. Chemical methods are harmful to the environment, clashing with the rationale of using lignocellulose as a clean fuel source. On the other hand, lignocellulose treatment with microbial CWLEs is limited by the costs and low efficiency [132,133]. Four major factors contribute to decreasing the enzymatic degradation efficiency of lignocellulose: (i) incomplete knowledge of the reactions and of the enzymes at the basis of such a process, (ii) the hydrophobic nature of lignin and cellulose that hinders the accessibility of glycoside hydrolases to the substrate, (iii) the broad heterogeneity of hemicellulose and pectin that requires many specialized CWLEs for their efficient depolymerization [34], and (iv) the property of lignin and of certain oligosaccharides to act as CWLE inhibitors.

Enzymatic hydrolysis of lignocellulosic biomass can be improved by using selected mixtures of CWLEs and optimized reaction conditions. Under this perspective, the choice of proper enzymatic blend is a critical step, since the number of CWLEs is constantly increasing and part of them are characterized by novel and unknown functions.

Nowadays, despite the many efforts related to the isolation and characterization of novel CWLEs, chemical and physical pretreatments of lignocellulosic biomass are required before proceeding with enzymatic treatment. According to this view, latest-generation enzyme-based products, such as those belonging to the Cellic CTech® series (Novozymes A/S, Bagsværd, Denmark), require chemically treated lignocellulose (i.e., by diluted acid treatment) for efficient hydrolysis; such products are mainly composed of cellulases and LPMOs (application sheets of Cellic® CTec2, HTec2-Enzymes, and Cellic® CTec3 for hydrolysis of lignocellulosic materials), while ligninases, hemicellulases, and pectinases are present in lower amounts [27]. A promising application of CWLEs is in the sector of renewable fuels, namely second- and third-generation biofuels. Soluble sugars obtained from agricultural feedstock (e.g., bagasse, straw, corn cob, grass, bran) as well as the same residual biomass (insoluble) upon CWLE treatment can be used as feed for sustaining the fermentative process of yeasts in order to produce ethanol [134]. Under this perspective, cellulose is the most energetic substrate since it is exclusively composed of glucose, the monosaccharide with the highest yield in ethanol conversion. In recent years, bioethanol production has also been extended to photosynthetic microorganisms such as cyanobacteria and microalgae [135]; however, although photo-fermentation is promising for bioethanol production, only little information is available from industrial applications. Enzymatically treated lignocellulose can be used to produce other forms of biofuels, such as those obtained from oleaginous microalgae, namely, third-generation biofuels. Hydrolysates from lignocellulosic materials can be used as feed for sustaining the mixotrophic growth of oleaginous microalgae such as *Botryococcus braunii, Monoraphidium neglectum,* and those belonging to *Chlorella* spp. [136–138]. Under mixotrophy and specific growth conditions (e.g., nitrogen starvation), these microalgae may accumulate lipids up to 60% of their dry weight [136,139]. Interestingly, different *Chlorella* species such as *C. prototechoides*, *C. vulgaris*, and *C. sorokiniana* may grow heterotrophically, accumulating up to 500% and 270% more biomass and lipids, respectively, than those observed under photoautotrophic conditions [140]. Subsequently, these lipids can be converted in biodiesel by a transesterification process. Compared to the major biofuels produced worldwide (i.e., bio-ethanol from sugar cane and veg-diesel from oil crops), the third-generation biofuels are considered as a promising option, since microalgae are highly productive and provide a solution to the paradigm "food vs. fuel" [141].

Alternatively, agricultural feedstock can be used as feed for promoting the anaerobic digestion of methanogenic bacteria [142]. Pretreatment of lignocellulosic biomass with CWLEs results in a higher biogas production yield by methanogenic bacteria, thus pointing to the increasing market of different enzyme-based products with cellulolytic activities. These products are obtained by culturing fungi and bacteria with cellulolytic activities and are mainly composed of endo-glucanases, cellobiohydrolases, and hemicellulases such as xylanases and galactanases, while they lack ligninases [33]. Therefore, use of chemical pretreatments capable of eliminating lignin and other phenolic molecules (i.e., alkaline and acid treatments) from lignocellulosic material is desirable because of the absence of laccases and peroxidases in such blends [132]. Moreover, lignin irreversibly binds to the CBM of CWLEs, thus poisoning the enzyme. It is worth noting that the low productivity of these cellulolytic microbes impacts the cost of their CWLE-based products. According to this scenario, the exploitation of plant-expressed CWLEs in the context of renewable energy may be highly competitive (Figure 4b).

5.2. Use of Plant-Expressed CWLEs in Medical and Nutraceutical Fields and in Food Processing

Expression of CWLEs in crop plants and microalgae has two major advantages: the low production costs and their GRAS (Generally Recognized As Safe) designation, which allows exploitation of such CWLEs for the production of nutraceuticals and bio-active compounds for human use; however, this does not mean that plant-expressed CWLEs are totally safe for human

health. Importantly, CWLEs may be expressed in a glycosylated form, as in the case of the recombinant proteins targeted to the extracellular environment, causing allergic reactions in humans. In order to circumvent the possibility of such an undesired effect, recombinant CWLEs with future uses in humans can be expressed in the chloroplast or targeted to the cytoplasm where glycosylation events do not occur.

Amongst the many applications of CWLEs, biomedicine and nutraceuticals are two emerging fields where plant-expressed CWLEs find many applications. In general, the most employed CWLEs in this field are fungal cellulases and β-glucosidases. Cellulases from *Trichoderma reesei* are included in enzymatic blends that favor the digestion of cellulose-rich fibrous substances, such as fruits and vegetables, cereals, legumes, bran, nuts, seeds, and soy, in order to help people suffering from slow digestion and particular metabolic disorders. Moreover, cellulases may be used in combination with chitinases and lysozymes to constitute a mixture capable of degrading chitin (i.e., a long-chain polymer of N-acetylglucosamine mainly found in crustacean and insect exoskeleton) into chitosan (i.e., a β-1,4-linked polysaccharide constituted by D-glucosamine and N-acetyl-D-glucosamine) [143]. Chitosan and its derivatives find applications as components in surgical sutures, bone rebuilding, hemostatic dressing, and skin reconstruction. Moreover, chitosan is used in combination with metals as anticoagulants, antibacterials, and anticancer and antidiabetic agents [144,145]. Chitosan is also the main component of many nutraceuticals because of its capability of acting as an immune-stimulating factor in the human immune system [146,147]. The broad substrate specificity of several β-glucosidases can be exploited for converting plant metabolites into biologically active compounds. It is the case of flavanone glycosides from citrus extracts that are converted into flavanone aglycones by the β-glucosidase from *Pyrococcus furiosus* [148]. Upon hydrolysis, flavanone glycosides such as naringin, narirutin, naringenin and hesperidin are converted into aglycones, with strong anticancer activities, by a single-step reaction [149]. Importantly, these metabolites can be obtained by agricultural scraps such as grapefruit and orange-peel extracts, indicating that the achievement of high-value molecules from waste materials is feasible. Moreover, CWLEs can promote the release of many molecules with antioxidant and coloring properties (i.e., carotenoids) from raw plant materials that, in turn, are used as natural food colorants. β-glucosidases find applications also in food processing since most of them are characterized by lactase activity (i.e., capability of degrading lactose in glucose and galactose), pointing to their exploitation in the processing of dairy products for lactose-intolerant people.

CWLEs are a powerful resource for food biotechnology because of their applicability in a broad range of processes. Fruit and vegetable juice clarification, viscosity reduction of nectars, modification of organoleptic properties, carotenoid extraction, olive oil extraction, and quality improvement of bakery products are the processes in which CWLEs are commonly exploited. The cloudiness of fruit and vegetable juices is a result of floating polysaccharide materials such as cellulose, hemicellulose, lignin, pectin, and starch. The presence of these materials in juice negatively affects the quality of the product and lowers user demand. Cellulases, hemicellulases, and pectinases are employed to degrade these polysaccharides, thus clarifying the juice and increasing the quality of such products [150,151]. During extraction of olive oil, continuous mixing is necessary since it allows the oil droplets to fuse to each other, increasing the oil yield. During mixing, the use of cellulases alone or in combination with pectinases enhances the extraction yield as well as the oil quality. Moreover, such treatment modifies the organoleptic properties of olive oil since it promotes the release of several phenolic compounds [152]. In addition, cellulases in combination with other CWLEs, such as xylanase, find application in the bakery industry since their use improves the loaf volume and bread quality [153,154].

5.3. Use of CWLEs to Improve Plant Resistance Against Pathogens

Plant expression of CWLEs can trigger the host defense, rendering their endogenous accumulation as self-deleterious for plants. Under this perspective, plant expression of CWLEs resembles a double-edged sword: on one side, activation of defense responses may confer increased resistance against pathogens but, on the other side, may strongly affect plant fitness and productivity,

likely because of the well-known growth–defense trade-off [155,156]. A valid strategy to overcome this issue may reside in a balanced activation of defense responses, capable of guaranteeing protection against microbes by concomitantly avoiding exaggerated and deleterious immune reactions.

Notably, plant expression of CWLEs capable of triggering defense responses can be used as an eco-friendly alternative to pesticides, preventing the spreading of disease in plants and lowering the costs for managing pests. CWLEs themselves and/or their activity can trigger the activation of the first layer of plant immunity, generally known as pattern-triggered immunity (PTI) [157]. PTI is driven by sensing of both nonself and altered-self molecules (i.e., PAMPs and DAMPs, respectively), which are recognized by PRRs [157,158]. The recognition of PAMPs/DAMPs leads to the activation of different kinds of intracellular defense responses, such as reactive oxygen species (ROS) production, transcriptional reprogramming of defense-related genes, and callose deposition [159]. Also, activation of defense machinery leads to a "primed" state of the plant, which is prone to respond faster and harder to a hypothetical pathogen attack. For example, pre-treatment with bacterial flagellin or with oligogalacturonides (OGs) makes plant more resistant to the necrotrophic fungus *Botrytis cinerea* [10,160]. However, treatment with PAMPs/DAMPs is not easily obtainable in the field. On the contrary, immunity-induced protection against pathogens can be achieved in plants by expressing specific CWLEs capable of stimulating plant defenses.

Inducible expression of a chimeric protein, constituted by the polygalacturonase from *Fusarium phillophilum* (FpPG) and the polygalacturonase-inhibiting protein 2 (PvPGIP2) from *P. vulgaris*, confers resistance to transgenic *A. thaliana* plants against pathogens characterized by different lifestyles such as *B. cinerea, Pectobacterium carotovorum*, and *Pseudomonas syringae* [90]. In particular, the chimeric protein, named as the OG-machine, releases elicitor-active OGs and allows the activation of a broad range of defense responses on command. By expressing the gene encoding the OG-machine under the control of a truncated version of the *PR1* promoter (mainly induced by pathogens), a plant with potential field application was successfully obtained (patent ID: US10385347B2 [161]). Interestingly, transgenic plant crops expressing PvPGIP2 alone [162–164] showed a lower level of protection compared to OG-machine plants, pointing to the activity of a CWLE (i.e., the FpPG) as pivotal for achieving a high level of protection against a broad range of pathogens.

CWLEs and their activity are mostly known as activators of plant immunity, since most of them are exploited by phytopathogens as "molecular" weapons to break plant cell wall components [83]. Recently, identification of proteins with a novel "counteracting activity" towards CWLEs opened a new perspective for improving plant resistance. Transgenic microbes from *Thricoderma* species (commonly used as biocontrol agents) expressing a glucose oxidase from *Aspergillus niger* (goxA) showed an improved ability both to inhibit phytopathogens and to induce systemic resistance in plants [165]. Interestingly, five berberine-bridge enzyme-like proteins (BBEls) from *Arabidopsis thaliana* are capable of oxidizing oligosaccharides released from the plant cell wall by microbial CWLEs [87,88]. Amongst them, BBEls encoded by the genes *AT4G20830* and *AT4G20860*, named as OG-oxidase 1 (OGOX1) and Cellodextrin-oxidase (CELLOX), specifically oxidized OGs and cellodextrins (CDs), respectively. Once oxidized, both OGs and CDs lose the nature of DAMPs by concomitantly acquiring an increased recalcitrance to enzymatic hydrolysis. The reduced degradability of oxidized oligosaccharides affects their capability to be efficiently metabolized by the fungus *B. cinerea*, leading to the loss of essential carbon sources for the invading microbe. Accordingly, transgenic plants constitutively expressing OGOX1 and CELLOX grew without morphological defects and were more resistant to *B. cinerea*. Thus, oxidation of the reaction products released from the activity of CWLEs strongly reduced the pathogenicity of the invading microbe, pointing to CWLEs as pivotal pathogenic factors.

6. Conclusions

An ideal bio-factory of CWLEs should be characterized by a high yield of recombinant protein, sustainable manufacturing, and a reduced production time and cost. In order to select the most

appropriate production platform, the type of CWLE to express and its biotechnological purpose represent additional factors to be considered. Although bacteria and yeasts can produce high yields of recombinant proteins in very short time, expensive fermentation procedures are required to obtain high-level expressions, negatively impacting the sustainability of the entire process. Furthermore, CWLEs from lignocellulolytic fungi can be characterized by the presence of disulfide bonds and glycosylations; therefore, the expression in bacteria and yeasts could result in misfolded or hyperglycosylated enzymes, respectively. Importantly, the use of CWLEs from microbial biofactories in food processing, nutraceutical manufacturing, and the medical field implies expensive purification procedures and strict quality controls, since bacterial contaminants, as well as yeast glycosylation, may cause allergic reactions in humans. On the other hand, plant biofactories are both a cheaper and more eco-friendly alternative, and, noteworthy, they can guarantee proper post-translational modifications to the eukaryotic proteins. Moreover, they are GRAS organisms, allowing the obtainment of high-value products at reduced production costs. Although the plant production of CWLEs may affect the fitness of the expression host, mainly because of their intrinsic phytopathogenic nature, such deleterious effects can be avoided by the different expression strategies here reported. However, plants need arable land for their growth and longer times to produce significant amounts of recombinant proteins. Under this perspective, microalgae may represent the best compromise since their cultivation does not subtract arable lands from the agri-food sector, and they are characterized by faster growth rates compared to plants. It is worth noting that genetic manipulation of microalgae has been less investigated; therefore, further optimization is still required for improving the expression stability and protein yield of microalgal-based biofactories. In conclusion, the most appropriate CWLE expression host should be determined on a case-by-case basis, taking into account both the characteristics of the specific CWLE to express and its application field.

Structure Modelling and Figure Preparation: Figures 1, 3a, 3b, and 4 were generated using Biorender (https://biorender.com/). In Figure 3c, the 3D structure of XynA from *Bacillus halodurans* (PDB number 2UWF) and from *Thermotoga maritima* (PDB number 1VBR) are reported with that from *Rizhoctonia solani* (UniProtKB A0A0K6FTH) as obtained by structure modeling using SWISS 2D model (https://swissmodel.expasy.org/). Electrostatic surface potentials were generated with CCP4MG software (negative and positive residues are in red and blue, respectively).

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