Folia Microbiol (2013) 58:163–176
DOI 10.1007/s12223-012-0184-8

Present and potential applications of cellulases in agriculture, biotechnology, and bioenergy

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Received: 26 October 2011 / Accepted: 10 July 2012 / Published online: 26 July 2012

Abstract Cellulase (CEL) presently constitutes a major group of industrial enzyme based on its diverse ranges of utilization. Apart from such current and well-established applications—as in cotton processing, paper recycling, detergent formulation, juice extraction, and animal feed additives—their uses in agricultural biotechnology and bioenergy have been exploited. Supplementation of CELs to accelerate decomposition of plant residues in soil results in improved soil fertility. So far, applying CELs/antagonistic cellulolytic fungi to crops has shown to promote plant growth performance, including enhanced seed germination and protective effects. Their actions are believed mainly to trigger plant defense mechanisms and/or to act as biocontrol agents that mediate disease suppression. However, the exact interaction between the enzymes/fungi and plants has not been clearly elucidated. Under mild conditions, removal of plant cell wall polysaccharides by CELs for protoplast preparation results in reduced protoplast damage and increased viability and yields. CELs have recently shown great potential in enzyme aid extraction of bioactive compounds from plant materials before selective extraction through enhancing release of target molecules, especially those associated with the wall matrix. To date, attempts have been made to formulate CEL preparation for cellulosic-based bioethanol production. The high cost of CELs has created a bottleneck, resulting in an uneconomic production process. The utilization of low-cost carbohydrates, strain improvement, and gene manipulations has been alternatively aimed at reducing the cost of CEL production. In this review, we focus on and discuss current knowledge of CELs and their applications in agriculture, biotechnology, and bioenergy.

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Abbreviations

BG	β-Glucosidase
C	Carbon
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CEL	Cellulase
CHI	Chitinase
EG	Endo-1,4-β-D-glucanase
EXP	Expansin
B1,3 G	Endo-β-1,3-glucanase
B1,6 G	Endo-β-1,6-glucanase
HC	Hemicellulase
K	Potassium
N	Nitrogen
NAGLU	*N*-Acetylglucoaminidase
P	Phosphorus
PT	Pectinase
XET	Xyloglucan endotransglycosylase
XGH	Xyloglucan hydrolase

Introduction

At present, enzymes receive considerable interest because of their excellent substrate specificity, regioselectivity,

stereospecificity, and ability to function at mild conditions, thus offering target products with an environmentally friendly production process (Buchholz and Seibel 2008). The estimated value for enzymes in the global market is about US$2.3 billion/year, a value in which food enzymes constitute the major market share (Singh 2010). On the basis of applications, enzymes are distributed to food (45 %), detergent (34 %), textiles (11 %), leather (3 %), and pulp and paper (1.2 %) industries (Singh 2010). Currently, cellulase (CEL) constitutes the third largest volume of enzyme usage worldwide (Wilson 2009). It has been utilized in cotton processing, paper recycling, detergent production, juice extraction, and animal feed additives (Bhat 2000). The demand for CELs is likely to increase, since plant biomass appears to be a major source of fermentable sugars for second generation bioethanol production (Wilson 2009).

CEL is a collective term referring to enzymes able to hydrolyze cellulose (Bhat and Bhat 1997). Although cellulose is a homopolymer of repeated units of cellobiose, the β-1,4-glycosidic linkages make the structural organization highly ordered and tightly packed (crystallinity), with few amorphous regions. To achieve complete hydrolysis of cellulose, three categories of CELs are required. Firstly, endoglucanases (EG; endo-1,4-β-D-glucanase, EC 3.2.1.4), preferably, attack amorphous regions and randomly cleave the internal bonds of the glycan chains, thus providing reducing or nonreducing ends of cellooligosaccharides for cellobiohydrolases (CBH; or exoglucanase, 1,4-β-D-glucan-cellobiohydrolase, EC 3.2.1.91) to attack. CBH then hydrolyzes those chain ends in the processive manner, yielding cellobiose as the major product. Lastly, β-glucosidase (BG; cellobiase, β-D-glucosideglucanohydrolase, EC 3.2.1.21) further hydrolyzes cellobiose to glucose and also releases glucose from the nonreducing ends of soluble cellooligosaccharides (Fig. 1) (Jørgensen et al. 2007; Lynd et al. 2002).

Unlike soluble substrates that can diffuse the active sites of enzymes, cellulose is insoluble; thus, CELs, on the contrary, have to diffuse, attach, and move the segment of the cellulose polymer to their active sites (Wilson 2011). Most CELs are modular proteins comprising discrete catalytic modules that typically appended one or more carbohydrate-binding modules (CBMs) joined by a flexible linker (Shoseyov et al. 2006). The CBM functions as a cellulose probe, in which the main responsibility is binding the enzyme to the cellulose and increasing the effective concentration of enzymes on the surface of the cellulose (Araki et al. 2010). In addition, some CBMs are known to possess the ability to disrupt crystalline cellulose (Shoseyov et al. 2006). Therefore, the presence of CBMs appears to be important in enhancing the enzymatic activity toward insoluble polysaccharides, as well as crystalline cellulose.

CELs are mostly found in plants, insects, and microorganisms, of which bacteria and fungi appear to be major sources (Watanabe and Tokuda 2010). In nature, bacteria and fungi play a vital role as decomposers that accelerate the decay of plant biomass (Sánchez 2009). The aerobic bacteria typically produce cellulolytic enzymes in a "free" form, in which individuals work synergistically during cellulose hydrolysis. In contrast, several anaerobic bacteria have been found to produce a CEL system as a cell-associated enzyme complex called the cellulosome (Bayer et al. 2007). The cellulosome exists as a discrete, multienzyme complex consisting of several subunits. Based on the cellulosome of *Clostridium thermocellum*, the primary protein called scaffoldin contains nine cohesin modules, which account for binding the dockerin-bearing catalytic subunits into the complex. The scaffolding protein also contains one family 3 CBM responsible for adhesion to cellulose (Ding et al. 2008a). Some reports have shown that the cellulosome is superior for efficient hydrolysis of cellulose to noncomplexed CELs (Ding et al. 2008a; Tachaapaikoon et al. 2011). However, cellulosome production under anaerobic conditions is expensive and time-consuming. Recently, a facultative bacterium *Paenibacillus curdlanolyticus* B-6 was found to produce cellulosome like multienzyme complexes under aerobic conditions (Waeonukul et al. 2009a). A study of this microorganism is currently underway, and it seems likely that strain B-6 uses other mechanisms to assemble the enzyme complex, rather than the cellulosomal mechanism, based on the absence of cohesin and dockerin sequences (Pason et al. 2010; Sakka et al. 2011; Waeonukul et al. 2009b). This ongoing study will provide us with new knowledge on enzyme complex formation among bacterial species in the future.

Like bacteria, aerobic fungi also produce EGs, CBHs, and BGs that work in concert during cellulolysis. The significant characteristic of cellulolytic fungi that attracts our interest over bacteria is the fact that they produce extracellular cellulolytic enzymes in great amounts. For example, several strains of *Trichoderma* are known as hyperproducers of EGs and CBHs, whereas *Aspergillus* species are great at BG and pectinase (PT) secretions (Kumar et al. 2008). In addition, a few anaerobic fungi, such as *Orpinomyces* and *Piromyces*, were reported to produce cellulosome (Doi and Kosugi 2004).

To date, commercial CELs are still derived from fungal sources, although the bacterial CELs show competitiveness in cellulose hydrolyzing efficiency. *Trichoderma reesei* (*Hypocrea jecorina*) is the best-known cellulolytic enzyme producer on the industrial scale, and it has been studied extensively since its discovery during World War II. *T. reesei* is considered a powerful degrader for crystalline cellulose, which is evident by a high degree of CEL secretions (Gusakov 2011; van den Brink and de Vries 2011). The mutant strains are able to secrete large amounts of crude CELs (over 100 g/L) with high specific enzyme activity

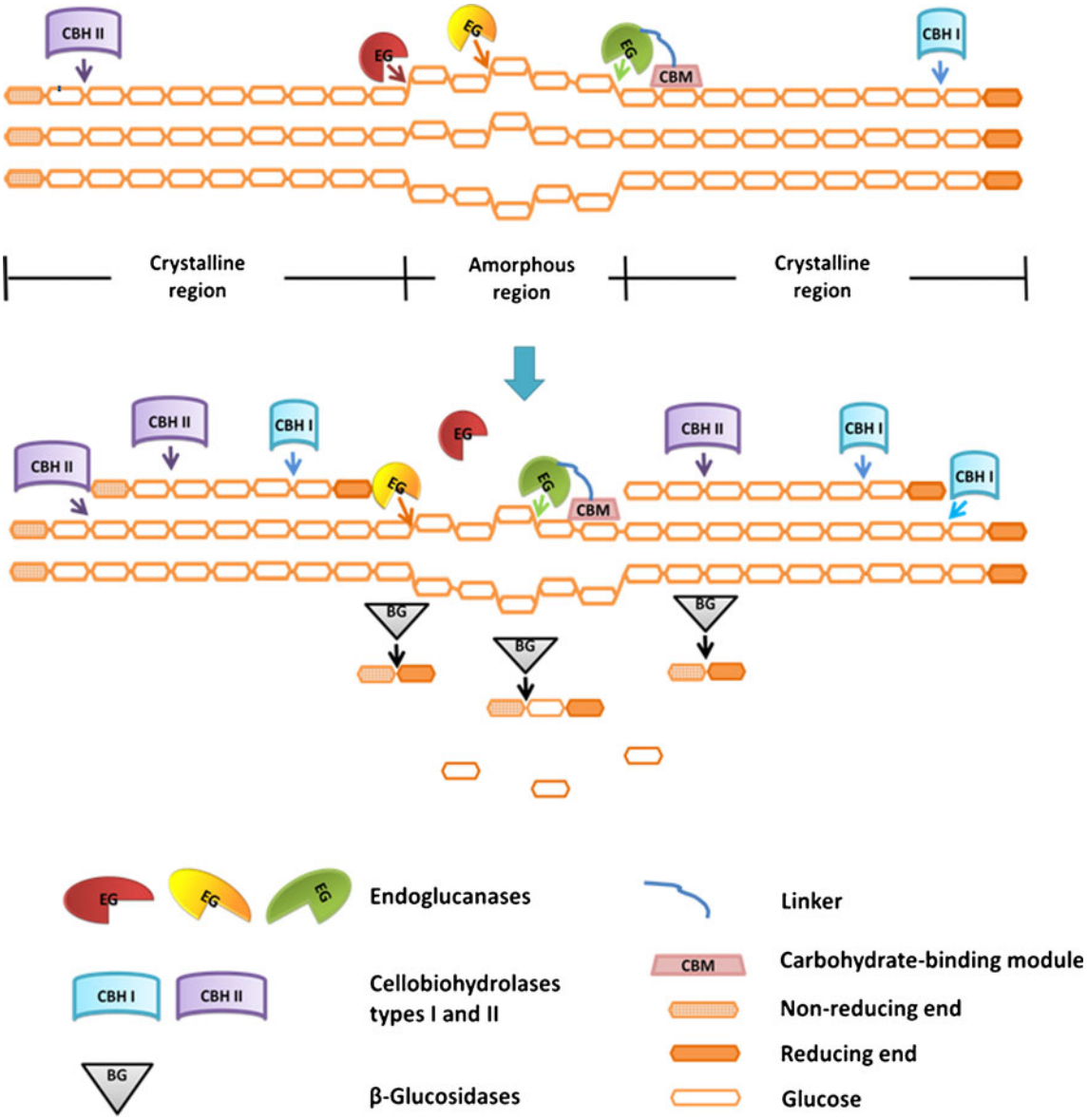

Fig. 1 A simplified model of enzymatic hydrolysis of cellulose. EGs are presumed to first cleave amorphous regions of the cellulose polymer, thus providing reducing or nonreducing ends of cellooligosaccharides for CBHs to attack and processively hydrolyze those chain ends. BGs further hydrolyze the resulting products, cellobiose, to glucose and also release glucose from the nonreducing ends of the higher oligomers (modified from Lynd et al. 2002)

(Wilson 2009). Moreover, the readily available genome sequence of this fungus provides genetic information that can be tailored for enzyme cocktails for specific uses (Wilson 2009). Preparations of commercial CELs based on the *T. reesei* system have been developed from time to time by such private companies as Novozymes and Genencor. To date, CEL preparations are particularly formulated for cellulosic ethanol production. Novozymes recently launched a product with the trade name Cellic CTec3 (http://www.novozymes.com); meanwhile, Genencor promotes Accellerase Trio (http://www.genencor.com) for biomass saccharification. A promising research finding of Novozymes is that supplementing glycoside hydrolase family 61 from *Trichoderma terrestris* enhances CEL activity of *T. reesei* significantly (Harris et al. 2010). Other fungal strains, such as *Humicola insolens* and *Aspergillus niger*, are utilized for commercial enzyme production with different purposes (Table 1). For example, CELs from *H. insolens* are generally used in textile and detergent applications, presumably due to the presence of specific CELs and the ability of the enzymes to function at mild alkaline conditions and at elevated temperatures (Martin 1997; Sukumaran et al. 2005), whereas *A. niger* is a major source for BG and accessory enzymes, such as α-L-arabinofuranosidase production, owing to a high degree of expression and high specific

Table 1 Potent microbial sources for commercial cellulases (Bhat 2000; Gusakov 2011; Howard et al. 2003; Wilson 2009)

Substrate	Enzyme class	Microorganism
Cellulose	Endoglucanase	*T. reesei*, *T. viride*, *H. insolen*, *A. niger*
	Cellobiohydrolase	*T. reesei*
	β-Glucosidase	*A. niger*

enzyme activity (Dan et al. 2000; Howard et al. 2003). For deep cellulose saccharification, some fungi belonging to genera *Penicillium*, *Acremonium*, and *Chrysosporium* have shown promise and appear to be competitive with *T. reesei* in some aspects, such as protein production level and cellulose-hydrolyzing performance per unit of activity or milligram of protein. More data was accumulated and intensively reviewed by Gusakov (2011).

Cellulases in agriculture, biotechnology, and bioenergy

Plants synthesize polysaccharides to form complex cell walls in which cellulose and hemicellulose are the dominant structural components. Plant CELs, hemicellulases (HCs), and PTs are associated with the growth and development of living plants, whereas exogenous CELs from microbes, particularly plant pathogens, are likely produced in order to facilitate the breach of the plant cell walls and to utilize them as sources of nutrients. Here, the promising applications of these enzymes in plant agricultural technology, biotechnology, and bioenergy are discussed as follows (Table 2).

Recovery of soil nutrients by accelerating straw decomposition: soil nitrogen availability, microbiota, and pH

Fertility of soil is one of the most important characteristics for gardeners and farmers to consider prior to planting. Fertile soils should be rich in basic nutrients, such as nitrogen (N), phosphorus (P), potassium (K), and trace elements required for plants' growth. Globally, rice, corn, wheat, and barley are major and economical crops. After harvesting, crop residues, especially rice and wheat straw, are left in fields in considerable amounts. Farmers usually dispose of these wastes in preparation for subsequent planting by open air burning, which leads to air pollution, and losses of nutrients, organic matter, humus, and useful microorganisms present on soil surfaces. Utilization of the straw in the form of composts or straw-incorporated soils appears to be a better solution for waste management and improved soil quality (Beedy et al. 2010; Nezomba et al. 2010). It has been found that plant debris in soil creates a long-term positive effect on plant growth promotion, but for a few weeks after straw incorporation, the soil N appears to be more deficient according to N immobilization by microorganisms present on the residues (Shindo and Nishio 2005). Therefore, increasing the level of plant debris decomposition is a promising means of recovery and increases soil N availability and other nutrients from plant organic matter, as well as shortens the time required for soil preparation for the next cultivation. Han and He (2010a) studied the effects of CEL application to soils incorporated with or without straw on N and P release. It was found that addition of CELs to rice and wheat straw-amended soils increased the release of N and P content, whereas there were no significant differences in N and P concentrations in the control (straw-amended soil). Therefore, available soil nutrients are mainly dependent on the action of CELs that degrade cellulose in straw-amended soils.

Soil microbiota help improve fertility and stability of soil, and their growth is likely associated with soil nutrients, particularly N. Henriksen and Breland (1999) found that when the concentrations of available N (organic N from straw and soil inorganic N) were below 1.2 % of straw dry matter, it significantly reduced the rate of carbon (C) mineralization from straw residues and the growth of total soil microbial biomass, especially fungal growths. They also roughly divided the active microbial biomass into two groups: the first was a bacteria-dominated community that grew rapidly on simple substrates, whereas the latter was a slower-growing functional community dominated by fungi that utilized the cell wall polymer at a rate dependent on N availability. In terms of immediate effects, application of CELs to straw amendment greatly increased the amount of microorganisms. The number of fungi increased significantly, but there was no significant effect on the amount of bacteria or actinomycetes (Han and He 2010a). In the same work, the authors suggest the role of exogenous CELs in peeling off cellulose and lignin for fungal activity, since those components are known to promote fungal growth (Fujii et al. 2010; Pu et al. 2001). In addition, one possible explanation is that CELs degrade cellulose structures, thus releasing into the soil organic and inorganic N associated with cellulosic material. As a result, the sufficiency of available N supports fungi to synthesize lignocellulolytic enzymes to utilize growth substrates (Novotný et al. 2009). Unlike fungi, bacteria and actinomycetes likely require an easily mineralizable fraction of organic matters in soils (Gryndler et al. 2003; ŘezáČová et al. 2007).

Soil pH is another parameter to be considered, since it directly affects the solubility of several nutrients required for proper plant growth and development. Although the pH value is a function of cationic and anionic ions, it also

Table 2 Potential applications of CELs and related enzymes and/or microorganisms in agriculture, biotechnology, and bioenergy

Application	Enzyme/microorganism	Function	Outcome/advantage	Limitation/disadvantage	Reference
Soil fertility	CELs, proteases	Accelerated decomposition of plant residue incorporated with soil	Recovery and increases soil available N, microbial activity, and organic matter-buffered soil pH	Increase in nutrients and final soil pH depending on plant residues used and soil properties Cost-extensive	Han and He (2010a, b), Henriksen and Breman (1999), Novotný et al. (2009), Xu et al. (2006b)
Growth promoting agents					
Cell growth	CELs and relevant enzymes, particularly XETs, XGXs, and PTs	Synergistic interaction with: Expansin proteins during cell extensibility, by degradation or modification of the plant cell wall polysaccharides	Enhanced cell loosening and enlargement during cell growth and developmental process	Vulnerability of biotic infection, susceptibility of diseases, and reduction of pathogenic resistance	Brummel and Harpster (2001), Catalá et al. (2000), Cosgrove (2005), Ding et al. (2008b), Payasi et al. (2009)
Seed germination and protective effects	CELs, B1,3Gs, B1,6Gs, CHIs, peroxidases *Trichoderma* spp., *T. harzianum*, *T. kiningii*, *T. koningiopsis*, *T. longibranchiatum*	Seed colonization Degradation of fungal cell walls Solubilization of some active compounds acting as fungicides or elicitors from plant cell walls Root colonization	Enhanced seed germination and shortened nursery period Improved self-defense mechanisms Enhanced nutrient solubilization and improved root environment	Variation in conditions used Requiring field experiments/conditions	Cotes et al. (1996), Inbar et al. (1994), Moreno et al. (2009)
Biocontrol of pathogens and diseases	CELs, *Pythium oligandrum*, *Trichoderma longibrachiatum*	Disruption and degradation of fungal cell walls Induction of abnormal morphology of pathogens Mycoparasitism	Facilitated host cell penetration Improved plant growth Self-biofertilizer Environmentally friendly process	Environmental factors particularly other soil microbes probably involving in defense response in situ Genomic and proteomic analyses required for unraveling systematic mechanisms upon antagonism	Benhamou et al. (1999), Haran et al. (1996), Picard et al. (2000)
Bioactive compound extraction	CELs, HCs, PTs, BGs	Breakdown of plant cell walls	Increased liberation of desired bioactive molecules, yield, and preserved antioxidant activity Facilitated release of cell wall-associated biomolecules Improved degradation of rigid and thick cell walls Reduced chemicals (i.e., acids) used in cell wall degradation and severe condition Reduction in waste management	Requiring suitable organic solvents to extract target molecules Loss of pigment and antioxidant activity due to side-enzymatic activity	Barzana et al. (2002), Ishida and Chapman (2009), Kapasakalidis et al. (2009), Kim et al. (2005), Sun et al. (2005)
Plant protoplast production	CELs, HCs, PTs, BGs	Removal of plant cell polysaccharides	Reduced protoplast damage by chemicals used		Lim and Lian (2001)

Table 2 (continued)

Application	Enzyme/microorganism	Function	Outcome/advantage	Limitation/disadvantage	Reference
			Increased protoplast viability and yield	Optimized enzyme formulation required for different plant tissues used	Takebe et al. (1968), Tamura et al. (2002)
			Increased subsequent cell division and plant regeneration		
Bioethanol production	CELs, HCs, PTs, BGs	Degradation of plant cell wall	Increased release of fermentable sugars	Requiring pretreatment to alter structure	Beukes and Pletschke (2010,), Himmel et al. (2007), Jeya et al. (2010)
			Reduced sugar loss and toxic compound formation	Requiring lignin removal High cost of enzymes Optimized enzyme formulation required for different plant residues	Taylor II et al. (2008), Yu et al. (2007)

appears to be related to N availability and microbiota presence. A number of plants, especially food crops, have their own optimum pH for growth, typically near neutral, and for macronutrient (N, P, K, Ca, Mg, and S) utilization, around pH 5.5 to 7.5. Incorporation of plant biomass into soil has been shown to influence the acidity and alkalinity of soil, in which the direction of pH change likely depends on the types, compositions, and ash alkalinity/acidity of plant residues, as well as soil characteristics (Xu et al. 2006b). For example, Yan and Schubert (2000) found that application of wheat and faba bean increased soil pH. For wheat, alkalinity mostly depended on the presence of simple organic anions, such as malate, whereas that for faba bean was likely related to organic anions and macromolecules like pectic substances. Xu and Coventry (2003) showed that addition of plant biomass to soil led to increasing, decreasing, and unchanged pH according to time. The initial increase in soil pH is presumably due to the release of ash alkalinity from plant residues and ammonification of organic N (from proteins or peptides), yielding NH_3. Later, the pH decreases—owing to the nitrification of mineralized N, which causes a decrease in pH owing to H^+ production—and the final steady value of soil pH possibly results from the balance of those reactions (Xu and Coventry 2003). Recently, one study showed the effect of CEL addition to straw-amended soils on soil pH change. The amendment of straws increased soil pH from 7.32 to 8.16 for rice straw and 7.99 for wheat straw after incubation for 45 days; however, with the supplementation of CELs, the soil pH declined to neutral pH. It is possible that CELs themselves accelerate the decomposition of plant biomass, thus rapidly releasing organic acids (acidity) from the residues that may neutralize the soil pH to neutral, the suitable pH level for plant growth (Han and He 2010a; Xu et al. 2006b). On the other hand, since organic N and acids are available N and C sources for microorganisms, the release of N and organic acids from the residues by the actions of CELs may promote microbial growth and, consequently, may stimulate microbial activity, which could result in the subsequent change of soil pH (Xu et al. 2006a). Additionally, this mechanism is presumably accompanied by the presence of proteases in soils (Han and He 2010b). Some nutrients, such as P, copper, iron, and zinc, are limited in availability when soil pH increases (Gupta et al. 2008). Supplementation of CELs in rice- and wheat straw-amended soil appears to accelerate soil acidification to neutral pH, thus facilitating solubilization and recovery of those available nutrients for plant growth. Accordingly, accelerating straw degradation in soils by CEL supplementation likely leads to (1) an increase in soil availability N, (2) an increase in microbial activity, (3) increased organic matters and/or humus, and (4) buffered soil pH.

Plant growth promotion: cell growth, and seed germination and protection

CELs and nonpathogenic fungi, especially *Trichoderma* spp., appear to be involved in diverse biological events that may be related to plant growth, including cell expansion, and seed germination and protection. However, their roles are not clearly understood, though they have been studied extensively (Bhat 2000; Vinale et al. 2008).

To achieve proper growth, plants need to weaken their cell walls using hydrolytic enzymes, such as CELs and PTs; also, proteins are reported to play a vital role in cell wall extensibility. Expansins (EXPs) are small extracellular plant proteins involved in cell wall enlargement and in developmental processes that require wall loosening (Cosgrove 2005). Although EXPs themselves have no hydrolytic activities towards polysaccharides, the proteins are able to weaken the mechanical strength of cellulose (Li and Cosgrove 2001). Therefore, the main action of EXPs is not considered as cell wall degradation but a disruption of noncovalent-bonding between cellulose microfibrils and matrix polymers, thereby leading to cell wall loosening. In addition, the CBMs of EXPs are also believed to collapse the crystalline structure of cellulose (Cosgrove 2005).

Although Cosgrove and Durachko (1994) showed that application of CELs and PTs to the native cell wall of cucumber enhanced cell wall extensibility, several cell wall-degrading enzymes, including CELs, PTs, and xyloglucan endotransglycosylase/hydrolase (XET/XGH), are, to date, reported to coexpress with EXPs (Payasi et al. 2009), suggesting the occurrence of a series of controlled biochemical events acts upon expansive growth. The correlation between those enzymes and EXPs likely occurs during wall loosening and growth. For example, to achieve expansive growth, EXPs are assumed to loosen the tight association of glucan–xyloglycan chains in the cell wall by disrupting the hydrogen bonds between those two polymers, thus allowing accessibility for CELs to attack (Brummell and Harpster 2001). PTs are believed to promote wall extension by removing pectins that limit accessibility of EXPs to cellulose–xyloglucan (Wei et al. 2010), while XETs may be involved in xyloglucan modification in the cell wall hemicelluloses (Cosgrove 2005). Additionally, some phytohormones, such as auxin, were reported to characteristically regulate coexpression of CELs, XETs, and EXPs during cell extension (Catalá et al. 2000; Sharova 2007).

It is notable that although loosening the cell walls is a key for plant growth, it may, in turn, make the plant prone to infection by virulent invaders. Some pathogenic bacteria produce auxin analogs in order to induce expression of EXPs to weaken plant cell walls (Navarro et al. 2006), and may, simultaneously, secret extracellular enzymes, such as CELs and PTs, to degrade host tissues (Laine et al. 2000). The loosening cell walls may be susceptible to hydrolytic action, thus enhancing pathogenic colonizing ability and nutrient utilization (Balestrini et al. 2005; Laine et al. 2000). Ding et al. (2008b) reported that overexpression of the auxin-responsive gene *GH3-8* increased disease resistance by reducing auxin accumulation. However, the phenotype of plant traits showed abnormal growth, owing to the suppression of EXPs via auxin signaling.

Hydrolytic activities are necessary for protective effects during seed germination. Cotes et al. (1996) studied the correlation between hydrolytic activities of *Trichoderma*-colonized seeds and protection against pathogens. The pregerminated seeds dressed with *Trichoderma kiningii* TH-11 for 24 h showed the highest degree of seed colonization and 100 % protection level towards *Pythium splendens* with the increase of CEL, β-1,3-glucanase (B1,3 G) and chitinase (CHI) activities found in seed tissue. B1,3 G and CHI activities were suggested as defense mechanisms, since they degraded the cell walls of *P. splendens* based on the liberation of reducing sugars from the mycelium of *Pythium*, whereas CEL activity was believed to originate from the fungi, presumably necessary for colonizing the seeds (Cotes et al. 1996). The hypothesis on CEL-aided colonization was supported by the fact that the application of commercial CELs to pregerminated seeds increased the level of colonization of the inferior colonizing strain, *Trichoderma longibranchiatum* TH-13, with an improved protective effect (Cotes et al. 1996).

Besides facilitating *Trichoderma* seed colonization, the seeds pretreated with CELs alone also exhibited increased protection. It is possible that CELs might have direct effects on *P. splendens*, perhaps by liberating some toxic chemicals from the seeds that inhibited germination of *P. splendens*, or by releasing some glucans that might act as plant endogenous elicitors (Cotes et al. 1996). According to the protective effects mentioned above, the application of *Trichoderma* spp. in seed priming, and at early stages of growth, also showed an enhanced rate of germination and development during the nursery period, thereby shortening required nursery time, which is important for economic reasons (Inbar et al. 1994; Moreno et al. 2009).

Biocontrol of pathogens and diseases

Fungi appear to be dominant plant pathogenic microorganisms that cause a significant loss of crops worldwide. Serious fungal diseases include blackleg in canola (*Brassica napus*), chestnut blight fungus, Dutch elm disease, and damping-off (Sankaran et al. 2010). However, some fungal strains are known to be nonpathogenic or hypovirulent (Sneh 1998). According to the hazardous impact of pesticides and chemicals remaining in the ecosystem, research on the applications of hydrolytic enzymes and nonpathogenic fungi for

biocontrol of plant pathogens and diseases has received considerable attention.

CELs appear to play an essential role in antagonisms of pathogenic oomyctetes, as their cell walls contain cellulose as a major component (Bartnicki-Garcia 1968). Early studies showed improvement of antifungal activity of *Trichoderma longibrachiatum* towards *Phythium ultimum* by enhancing CEL activity. The hypercellulolytic transformants were able to significantly reduce *Phytium* damping-off on cucumber, suggesting that CEL activity might be involved in biocontrol of *P. ultimum* by *T. longibrachiatum* (Haran et al. 1996).

A better understanding of the roles of CELs utilized by biocontrol agents has been further clarified by the work of Picard et al. (2000). Insight into mechanisms of CELs as an antifungal activity is emphasized in mycoparasitic processes against *Phytophthora parasitica* by *Pythium oligandrum* using dual culture tests (Fig. 2). The production of CELs and their cellulolytic activities appeared to play a key role in host cell penetration and enlarged wall appositions, as evident by severe damage of the host cellulosic walls at potential penetration sites (Picard et al. 2000). The successful host-CEL-aided penetration led to subsequent biochemical events, including alteration of cytoplasm and active multiplication of the antagonist in the host hyphae, resulting in host cell breakdown (Benhamou et al. 1999; Picard et al. 2000). Therefore, *P. oligandrum* and its CEL system may be developed as a biocontrol agent. However, field experiments may be required to predict the behavior of the strain and its biological controls before commercial use. Information on CEL-facilitated mycoparasitism processes induced by *Pythium* antagonists may be further elucidated in detail by genomic and proteomic techniques.

Improvement of extraction of plant-bioactive compounds

There is great interest in extraction of bioactive compounds from plants according to their beneficial and health-promoting effects. Plant-bioactive compounds can be considered as extranutritional or inherent non-nutrient constituents of plants (Kris-Etherton et al. 2002), and are also known as secondary metabolites (Porto et al. 2009). Their molecular structures vary with different functions. Phenolic compounds, including flavanoids, represent the majority of the bioactive substances present in all plants. A number of phenolic compounds possess antioxidant activities, and some have shown positive effects on thrombosis and tumorigenesis. Other phytochemicals, such as alkaloids, anthraquinones, and peptides, were reported to have pharmacological properties, including immunemodulator, antiviral, anti-inflammatory, antioxidant, and cytostatic activities (Porto et al. 2009).

In nature, bioactive compounds generally occur in insoluble, suspended, or colloidal forms associated with cell wall components (Kim et al. 2005), thus requiring the complete breakdown of cell walls to enhance the release of those molecules. Conventional extraction methods typically begin with the use of chemicals, particularly acids, with harsh conditions to disrupt the cell walls, thereby leading to the loss of phytochemicals in terms of yields and activity. Additionally, in industrial practice, the use of chemical pretreatment liberates a vast amount of effluent that requires treatment before draining into the environment. Concerning environmentally friendly procedures and consumer demand for "green-labeled" products, cell wall-hydrolyzing enzymes, including CELs, HCs, and PTs, have shown promise as an alternative means of improving extraction methods

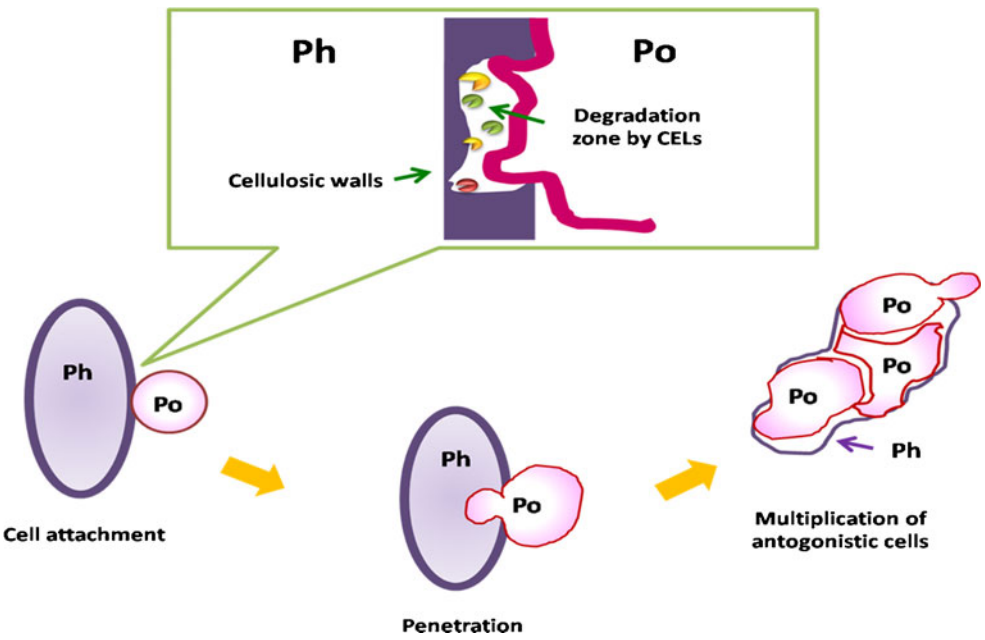

Fig. 2 A simplified diagram demonstrating the potential roles of CELs in the biological control of *Phytophthora parasitica* (Ph) by *Pythium oligandrum* (Po). After cell attachment, *P. oligandrum* appears to secrete a large amount of CELs, as evident by the lysis zones of the host cellulose-enriched cell walls. Cellulolytic activity is believed to be a key factor in the mycoparasitic process, which may facilitate host cell penetration. Active multiplication of antagonistic cells in the host hyphae may lead to host cell breakdown (Picard et al. 2000)

according to their capability to hydrolyze main cell wall polysaccharides. The enzymes may first facilitate cell wall degradation; suitable extraction methods are then employed to obtain desired compounds (Fig. 3). Because of their hydrophobic nature and limited solubility in water, extraction techniques such as CO_2-supercritical fluid, microwave-assisted, and ultrasound-assisted extractions have been introduced (Garcia-Salas et al. 2010). However, in the food industry, organic solvent-based extraction using nontoxic and environmentally friendly solvents have been commonly used to obtain target molecules (Ishida and Chapman 2009), presumably due to economic reasons and ease of controlling manufacturing processes.

Kim et al. (2005) showed improved release of phenolic compounds from apple peel by use of *Thermobifida fusca* CELs. Their study demonstrated the importance of synergism between endo- and exoglucanase activities combined in enzyme preparation for phenolic recovery, and the CBM was suggested to be essential for cellulolysis in apple peel. The addition of fungal enzyme preparations, namely, Pectinase 62 L or 690 L alone, or the combination of Pectinase 62 L and Cellulase CO13P, were able to solubilize bergamot peel at high loadings with the release of aglycone (hesperetin), a flavone that offers health benefits including antioxidant, anti-inflammatory, and anticarcinogenic effects, and prevention of bone loss (Nielsen et al. 2006). Kapasakalidis et al. (2009) utilized press residues from the manufacture of blackcurrant juice as a source of anthocyanins and antioxidants, and the mixture of CELs and HCs with a low BG activity and various side activities from *Trichoderma* spp. was used for enzyme-mediated extraction. The enzyme treatment significantly improved the degradation of cell wall polysaccharides and increased availability of phenols for subsequent methanolic extraction, with high antioxidant activity. Barzana et al. (2002) developed an extraction method for marigold colorants from fresh flowers, known as simultaneous enzymatic solvent extraction. An enzyme preparation, including PTs, CELs, and HC, which acted in organic solvents with low humidity, was selected. It was found that without enzymes, only 44 % of the carotenoids could be retrieved, whereas recovery in excess of 85 % was obtained when the enzymes are incubated with the flower before extraction. Additionally, for such difficult-hydrolysable plant materials as coffee bean, Kasai et al. (2006) proposed the sequential step using CEL-aided treatment for the cell wall digestion. The stepwise procedure was alkali boiling (0.1 mol/L Na_2CO_3 buffer, pH 10, and 0.1 mol/L NaOH), CEL digestion, autoclaving with 0.1 mol/L NaOH, and CEL redigestion. Cell walls became very thin and residue was easily broken into small pieces. Moreover, this method gained >95 and >96 % total digestion yields for green and roasted coffee beans, likely useful for high extraction of a coffee brew or use of the residue of the roasted coffee.

Some studies, however, have shown negative effects when enzyme preparations were integrated with the extraction process. Sun et al. (2005) reported that PT preparation from *A. niger* significantly degraded rutin (a major antioxidant of asparagus) and decreased the antioxidant activity of asparagus juice. The side activities present in commercial enzyme preparation appear to be the major cause of the loss of pigments and antioxidant activity. For example, BG was able to hydrolyze glycoside of anthocyanins, resulting in pigment loss of strawberry juice (Versari et al. 1997). Glucuronidase activity could cleave quercetin glucuronide, liberating the quercetin aglycon, while esterase activity was able to hydrolyze the tartaric ester of caftaric acid, yielding free caffeic acid (Sun et al. 2005). Therefore, selection of enzyme preparations should be considered for successful

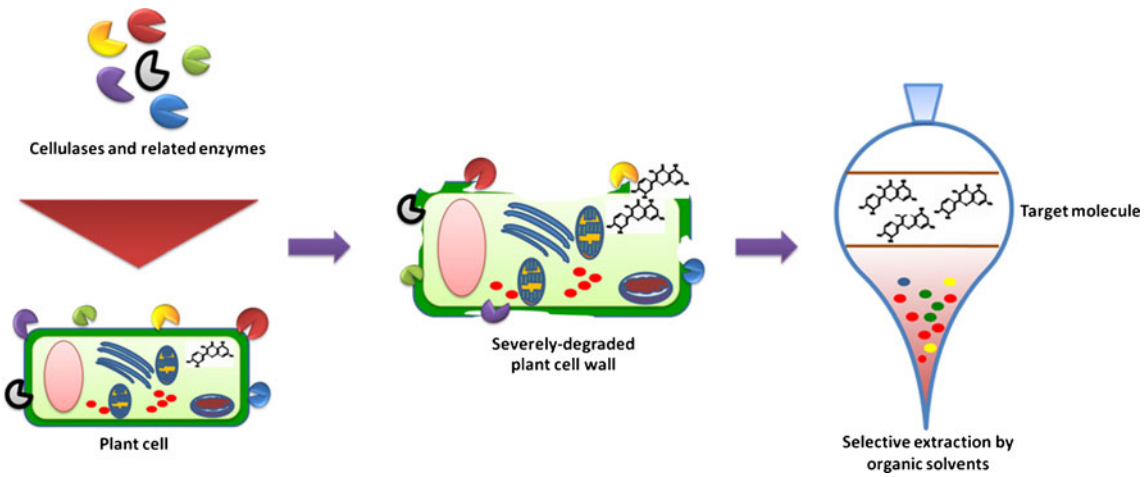

Fig. 3 CEL-assisted extraction of bioactive compounds. CELs and related enzymes may first facilitate cell wall degradation to enhance the release of bioactive compounds from plant cells, and the compound-containing medium may be further separated by selective extraction using organic solvents to obtain the target molecule

processing. In addition, although enzyme treatment allowed the production of extracts rich in phenolic compounds with increased antioxidant activity, the reaction parameters do affect the phenol content and, consequently, influence antioxidant capacity values (Kapasakalidis et al. 2009).

Plant protoplast production

To improve plant traits through plant biotechnology or gene manipulation, protoplasts become a useful material for breeding, cell fusion, and transformation. DNA uptake into protoplasts appears to be very important for transforming plants that cannot use other gene delivery methods, especially *Agrobacterium*-mediated transformation. The use of protoplasts offers benefits in terms of secretion of target products to culture medium and easy-to-handle downstream process (Aoyagi 2011). Protoplasts are living cells that lack cell walls. Preparation of protoplasts through isolation from plant tissues is a critical step, since improper techniques, as well as chemicals used, might damage isolated protoplasts and reduce their viability. Takebe et al. (1968) established an isolation technique to obtain protoplasts from tobacco (*Nicotiana tabacum*) leaves using hydrolytic enzymes. PT was firstly added to separate cells, followed by addition of CELs to remove cell walls, thus releasing spherical protoplasts. The technique has been further developed by various research groups. For example, Tamura et al. (2002) proposed and implemented the use of cellulosomes together with pectatelyase of *Clostridium cellulovorans* to release protoplasts from the cultured tobacco cells and *Arabidopsis thaliana*, with consequent high activity of protoplast formation.

To date, many manufacturers have launched ready-to-use enzyme mixtures as a commercial product for protoplast isolation, such as Macerozyme R-10 and CEL 'Onozuka' RS. However, as different plant tissues require different conditions to obtain protoplasts, the isolation procedure should be optimized for each tissue. Lim and Lian (2001) successfully isolated protoplasts from cotyledon, hypocotyls, and mesophyll tissues from *Capsicum annuum*, *C. baccatum*, and *C. chacoense* using a combination of cellulysin (1 %), macerozyme (0.25 %), and 0.65 mol/L glucitol. Sugar alcohols, such as mannitol and glucitol, are generally used as osmoticum. Moreover, the addition of antioxidants in the enzyme solution was reported to prevent browning of protoplasts. Successful protoplast isolation procedures with high viability are likely to increase subsequent cell division and plant regeneration.

Bioethanol from agricultural wastes

Due to declining reserves of fossil/petroleum-based fuels and increasing demands for energy, a great number of attempts are being made to find alternative resources for bioenergy production. Bioethanol has received attention worldwide, since it is a clean and renewable energy (Gray et al. 2006). First generation ethanol, also known as starch- and sugar-based ethanol, is a mature technology, with industrialized procedures. Starch will be first saccharified, typically by amylolytic enzymes, to simple sugars (saccharification), and the sugars are subsequently fermented by yeast strains (fermentation) to yield ethanol. However, to utilize ethanol as a substituted energy, especially in transportation sectors, a large volume of ethanol is required. Therefore, the use of starch and sugars as substrates for ethanol production seems to be insufficient because these materials can be used as food and are necessary for human needs, leading to competitive prices (Knocke and Vogt 2009). Plant biomass, including cornstover (hull, cob), rice straw, and sugarcane bagasse, is considered as waste and is usually removed by fire. Today, it is becoming an increasingly attractive resource for ethanol production according to its abundance, availability, and low cost, and the fact that it is nonfood. Utilizing these materials, rather than burning them, not only reduces air pollution but also provides the opportunity for rural people to earn more income. Plant biomass is comprised of cellulose, hemicelluloses (mainly xylan), and lignin as structural components (Fig. 4). Accordingly, structural heterogeneity and complexity make the lignocellulosic ethanol production processes more complicated (Himmel et al. 2007). Based on yeast fermentation, the target polymer that needs to be saccharified is cellulose, as conventional yeast strains prefer to take up glucose. However, the native yeasts themselves lack the capability to hydrolyze the β-1,4-glycosidic linkages of the cellulose fibers to gain their fermentable sugars (Banerjee et al. 2010). Although chemistry approaches, such as acid hydrolysis, can break down lignocelluloses, a major loss of sugars occurs with the presence of inhibitors, leading to low ethanol yield and low productivity. Therefore, enzymatic hydrolysis using CELs is an alternative means to hydrolyze cellulose, while other components can be first removed by relevant enzymes or by mild physico/chemical pretreatments to peel off cellulose in order for CEL to attack (Fig. 4) (Beukes and Pletschke 2010, 2011). Jeya et al. (2010) reported that hydrolysis of poplar wood biomass was achieved by the use of CEL mixtures, including EGs, CBHs, and BG activities, from a fungus *Agaricus arvensis*, releasing a total reducing sugar level of 29 g/L (293 mg/g substrate) at an enzyme concentration of 65 FPU/g substrate. Gottschalk et al. (2010) used an enzyme blend composed of *T. reesei* (a major source of CELs) and *A. awamori* (a major source of BGs, xylanases, and ferulic acid esterase) with different ratios (at 25:75, 50:50, and 75:25) to hydrolyze sugarcane bagasse. It was found that after 72 h of incubation, all enzyme

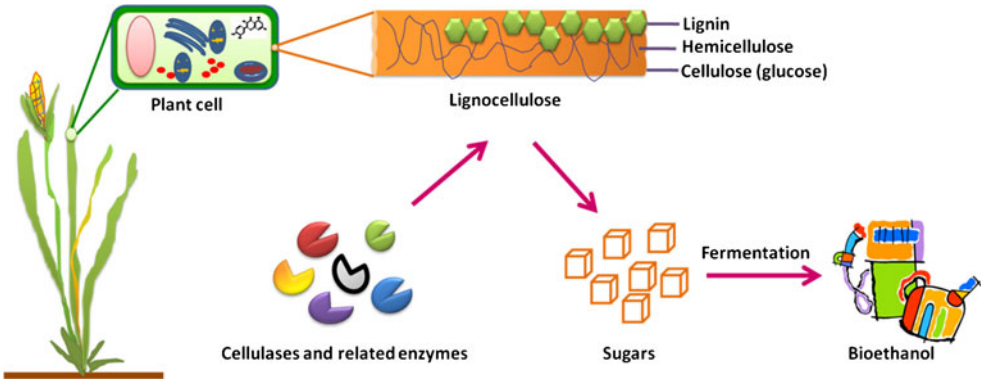

Fig. 4 Plant biomass to bioethanol. Cellulose is the most abundant polysaccharide in plant cell walls and appears as the potential source for fermentable sugar. After mild pretreatment, CELs (mainly EGs and CBHs) may facilitate breakdown of cellulose to cellooligomers, which may be subsequently hydrolyzed by BGs to glucose units. The glucose monomer can be further utilized by ethanologens to yield ethanol

blend ratios yielded comparable glucose concentrations, corresponding to 80 % cellulose hydrolysis yield, without any cellobiose accumulation, and xylan hydrolysis was almost degraded within 6 h. The results suggest that overcoming the recalcitrance of plant structure required a variety of enzymes with different modes of actions. Since lignin has been reported as a barrier for enzymatic hydrolysis, Zhang (2006) proposed the use of the lignin-degrading fungus *Phanerochaete chrysosporium* in the biomass-to-ethanol scheme involving separated fermentation of pentoses and hexoses.

As initially evaluated by the US National Renewable Energy Laboratory (NREL), CEL costs comprised 20 % of total ethanol production costs, leading to high selling prices. Thus, reduction of enzyme cost is an important issue. Since plant biomass is rich in polysaccharides, utilizing it as a source of carbon is a possible way to minimize the costs of enzyme production. Phitsuwan et al. (2010) used corn hulls as a growth substrate in a submerged culture of *Tepidimicrobium xylanilyticum* BT14, and a desired amount of plant cell wall-degrading enzymes was produced. Solid-state fermentation using rice bran, soybean hull, and wheat bran/straw has been highlighted for on-site lignocellulosic ethanol process (Brijwani et al. 2010; Lever et al. 2010). In addition, utilizing plant biomass likely triggers the synthesis of carbohydrate-active enzymes rather than pure substrates (Waeonukul et al. 2008).

Another approach to lowering production cost involves maximizing protein secretion. Mutagenesis of cellulolytic fungal strains to increase CEL expression by various means, such as microwave and ultraviolet mutagenesis, was reported (Li et al. 2010). Recently, interest has been paid to transgenic plants able to express foreign CELs. Yu et al. (2007) developed plastid transformation vectors carrying two *T. fusca* thermostable CELs, namely, Cel6A and Cel6B, and expressed them in nicotine-free tobacco. It was found that both expressed Cel6A, and Cel6B was able to degrade crystalline cellulose; thus, the transgenic plant is possibly an inexpensive source of active CELs. On the other hand, in order to accelerate polysaccharide hydrolysis, thermophilic enzymes, which are active over 50 °C, have also been expressed, offering advantage over the fact that the cell walls would not be damaged at normal growing and storage temperatures but could subsequently be activated by heating (Montalvo-Rodriguez et al. 2000; Taylor II et al. 2008); thus, they are possibly suitable for the saccharification step in ethanol production from lignocellulose. However, remaining enzymatic activity after the pretreatment process should be considered.

Concluding remarks

It is obvious that CELs are involved in various plant agricultural technology, as well as process developments. Demand for these enzymes is growing, particularly in the ethanol industry. However, at the present time, the cost of enzymes is still relatively high compared to the agricultural products themselves. Therefore, research and development should pay attention to low-cost enzyme production with optimized processes. Utilization of CELs in the form of microbial cells may offer benefits for plants in terms of plant protection and biological controls. However, the positive effects may not be as predictable as chemical use (fungiside) and may result in complications after long-term cultivation. As CELs appear to be a key lytic enzyme upon mycoparasitism against pathogenic oomycetes, genetic manipulation may be employed to construct hypercellulolytic antagonistic strains and evaluate their biological control activity and reliability. Our expectation is that the obtained knowledge will shorten the period of plantation, improve product yield and quality, reduce environmental damage, and improve the quality of life for farmers.

Acknowledgments Paripok Phitsuwan was supported by a grant from the Royal Golden Jubilee Ph.D. Program of the Thai Research Fund. We thank the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission for financial support.

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