# Chapter 12
# Extracellular Carbohydrate-Active Enzymes of *Trichoderma* and Their Role in the Bioconversion of Non-edible Biomass to Biofuel


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## 12.1 Introduction

Constant depletion of fossil fuels, increasing world population and concerns for environments, in particular the impact of climate change on our ecosystem, demand futuristic sustainable technologies. India is presently ranked third in oil consumption. Moreover, growing population size, growth in automobile and other industrial sectors in India, led to increase in energy consumption. The need for environmental friendly and renewable energy resources such as biofuels produced from agricultural-based biomass can decrease our dependence on fossil fuels (Borin et al. 2017). Therefore, efforts for developing alternate energy resources are on high priority. As per the records of US Department of Energy, United States and Brazil contributed to approximately 80% (24,570 million gallons) of the global ethanol production (http://www.afdc.energy.gov) (Borin et al. 2017). The bioprospection of agricultural biomass in particular from non-edible sources can be a better alternative and sustainable approach with minimal environmental concerns in the future (Gaurav et al. 2017). Agricultural biomass which is often a major source for environmental pollution can be of vital importance for biofuel production as an alternate energy resource (Ning et al. 2016; Wan et al. 2001; Chirino-Valle et al. 2016). Limitations of biomass from grain-producing crops demand alternative second-generation biofuels from non-edible agricultural crops (Ayrinhac et al. 2011) and other biomass sources. These carbohydrates from different non-edible biomass can be explored for biofuel using a combination of enzymes (Gaurav et al. 2017). Biofuels are categorized into three generations on the basis of raw material. Initially for first generation

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biofuel, crops plants were explored followed by second generation agricultural by-products and marine resources such as seaweeds and cyanobacteria (Demirbas 2008; Kang et al. 2014; Gaurav et al. 2017).

Biofuels from second-generation agricultural wastes offer several benefits. Biofuels from renewable resources can be exploited as promising and almost carbon-neutral fuel enhancers of octane in unleaded gasoline for cleaner combustion which can reduce environmental pollution. Plant biomass containing lignocellulose in terrestrial ecosystems is one of the most potential raw materials due to its availability, price and high sugar content (Barros-Rios et al. 2016; Zhao et al. 2016). The basic constituents of lignocellulose include cellulose, hemicellulose and lignin (Sindhu et al. 2016) which are interconnected through covalent and non-covalent bonds (Gaurav et al. 2017; Zhang et al. 2017). Cellulose which is a major part of plant biomass has been widely recognized and explored for developing sustainable processes and can help in mitigating the impact of climate change, occurs through consumption of fossil fuels (Gupta and Verma 2015; Zhang et al. 2017). Conversion of lignocellulose-based plant biomass is a major bottleneck in developing sustainable processes for alternate energy resources and other value-addition products (Kuhad et al. 2011; Villares et al. 2017). The breakdown of recalcitrance lignocellulose and chitin containing biomass using chemical pretreatment often results in toxic side effects to the ecosystem (Margeot et al. 2009; Wang et al. 2017) (Fig. 12.1).

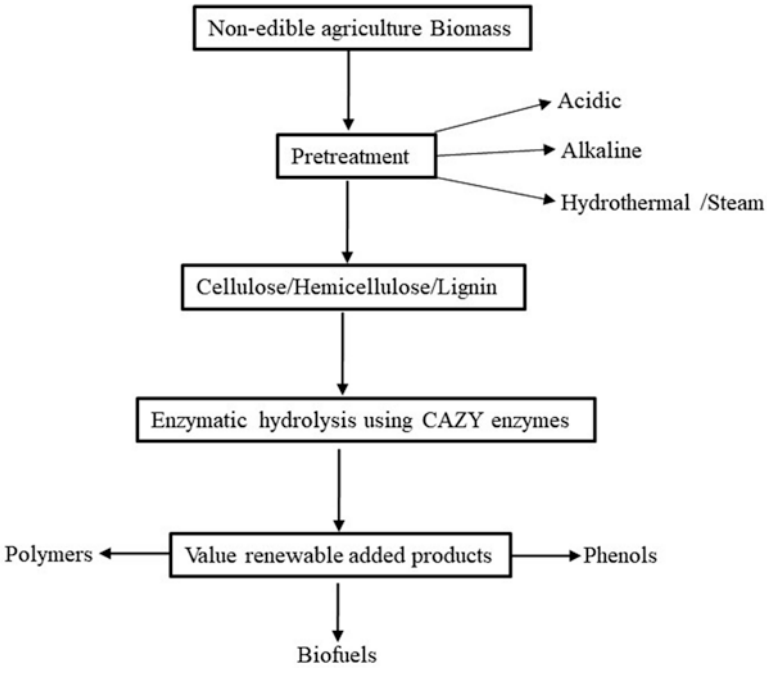


**Fig. 12.1** A schematic overview of the conversion of non-edible agricultural biomass to value-added products using carbohydrate-active enzymes

The conversion of plant biomass into value added products can be achieved through the breakdown of recalcitrant plant biomass via pretreatment and enzymatic hydrolysis (Zhang et al. 2017). Efficient utilization of the lignin, hemicellulose and cellulose can decrease the cost of biofuel production up to 25% (Zhao and Xia 2009, 2010; Zhao et al. 2018a).

Enzymes in the CAZy database are categorized into four classes: glycoside hydrolases (GHs), polysaccharide lyases, glycosyl transferases and carbohydrate esterases. The glycosyl hydrolases (GHs) have potential to break the non-edible biomass into oligo- or monomers (Ferreira Filho et al. 2017). Additionally, a family of auxiliary enzymes known as lytic polysaccharide mono-oxygenases (LPMOs) which is a major component of saprophytic fungi like *Trichoderma* and *Aspergillus* catalyses copper-dependent oxidation of C-H bonds in complex polysaccharides (Obeng et al. 2017; Borin et al. 2017; Monclaro and Filho 2017; Cologna et al. 2018) (Fig. 12.2).

In general, the stains of *Trichoderma* are used as biocontrol agents due to their diverse attributes (Sharma and Shanmugam 2012; Sharma et al. 2013, 2016a, b, 2017a, b, 2018a, b). Besides this, enzymes from filamentous fungi such as *T. reesei* are paradigms for industrial application in paper, textile, pulp, food and biofuel processing industries (Kumar et al. 2008; Singhania et al. 2010; Seiboth et al. 2011; Marx et al. 2013; Tiwari et al. 2013) (Table 12.1).

Laccases or phenol oxidases and lytic mono-oxygenases can enhance the activity of lignocellulases and thus lower the enzyme required to break down alkali-pretreated

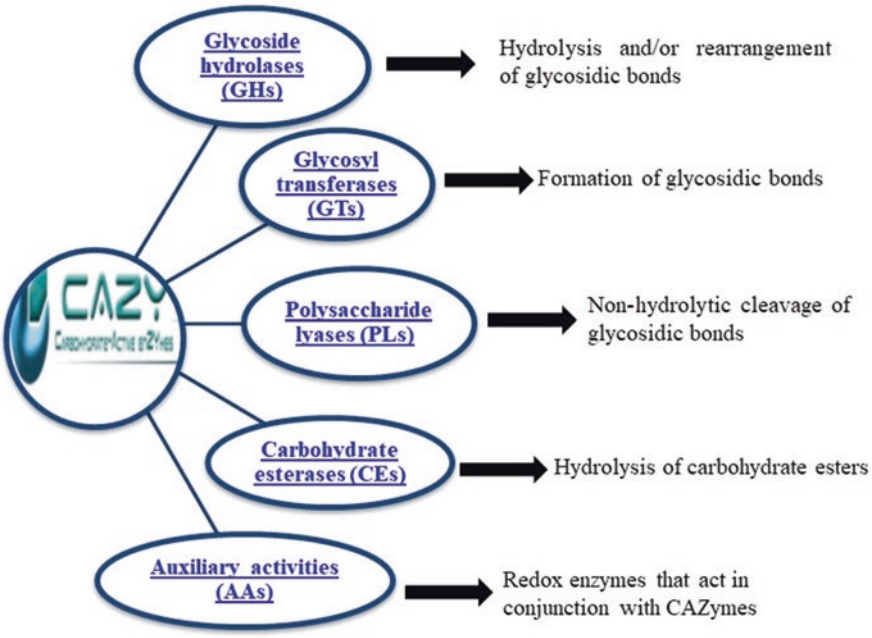


**Fig. 12.2** Overview of carbohydrate-active enyzmes as per cazy database http://www.cazy.org/

**Table 12.1** Recent examples of CAZymes for different applications

| Enzymes/CAZy family | Fungal species | Role | References |
|---|---|---|---|
| Cellulase complex | *T. reesei* SCB18 | High cellulase capacity for biomass saccharification and high β-glucosidase (BGL) activity | Gao et al. (2017) |
| Glycosyl hydrolases such as GH1, GH3, GH18, GH35 and GH55 families of chitinases, glucosidases, galactosidases, and glucanases | *T. hamatum* strains YYH13 and YYH16 | Offers scope for developing β-glucosidase with high cellobiose-hydrolysing efficiency | Cheng et al. (2017) |
| GH95, GH67, GH62, GH54, GH43, GH26, GH11 and GH10 | *T. reesei* RUT-C30 | Hemicellulose degradation | Ferreira Filho et al. (2017) |
| GH7, CBM1; GH5, GH7, GH12, GH45, GH1, GH3 and GH6 families; 23 CBM1 domains; two auxiliary families | *T. harzianum*, *T. reesei* RUT-C30 | Cellulose degradation | Borin et al. (2017); Ferreira Filho et al. (2017) |
| Chitinase | *T. saturnisporum* and other *Trichoderma* species | Protoplast isolation, fungal pathogen management, treatment of chitinous waste | Dahiya et al. (2006); Sharma and Shanmugam (2012); Sharma et al. (2017a, b, c) |
| Glucanases | *T. harzianum* | Fungal pathogen suppression through mycoparasitism | Sharma et al. (2017a, b, c) |
| Cellobiohydrolase I | *T. longibrachiatum* | The action of LPMOS promoted the efficacy of cellobiohydrolase I, endoglucanase and β-glucosidase in pretreated bacterial microcrystalline cellulose | Song et al. (2018) |
| Lytic polysaccharide mono-oxygenase | *T. reesei* | | |
| Lytic polysaccharide monooxygenase, AA9 and CBM1 | *T. reesei* RUT-C30 | Acts on cellulose and β-glucan | Borin et al. (2017) |
| Xylan esterase, CE3, CE5 and CBM1 | *T. reesei* RUT-C30 | Acts on xylan | Borin et al. (2017) |
| Xylanase, GH10 and GH11 | *T. reesei* RUT-C30 | Acts on xylan | Borin et al. (2017) |
| Xylanase | *T. reesei* QM6a | High biotechnological relevance | Ramoni et al. (2017) |

agricultural biomass containing lignocellulose (Ladeira Azar et al. 2018). For examples, xylanase of *A. niger* and *T. reesei* are found to be inhibited by the presence of phenol at 1.5 mg and 0.3 mg per mg of protein, respectively. On the other hand, laccases of *C. cubensis* and *Penicillium pinophilum* are reported active at a concentration of 35 mg of phenol per mg of protein (Ladeira Azar et al. 2018). The glcyosyl hydrolase family plays a vital role in the breakdown of complex plant biomass, whereas the role of the auxiliary activity (AA) family has been discussed in recent studies (Levasseur et al. 2013). Among different CAZymes, β-1,4/(1,3)/(1,6)-type glycosyl hydrolase family breaks down complex plant polysaccharides to oligomers or monomers (Vu and Marletta 2016). Lytic mono-oxygenases belonging to AA9 (formerly GH-61), AA10 (formerly CBM-33) and AA11 enzymes are capable of targeting recalcitrant non-edible carbohydrates such as chitin, cellulose, starch and other polysaccharides containing β-linkages between glucose and substituted glucose units (Ravalason et al. 2012; Vu et al. 2014; Gong et al. 2015; Ning et al. 2016). The genomes of *A. niger* and *T. reesei* share about the same (2.5%) proportion of CAZymes in comparison to total predicted genes; still, the transcriptomic response of *A. niger* is found to be diverse and revealed upregulation of 190 CAZymes which belong to 62 different families, whereas for *T. reesei*, 105 CAZymes belonging to 51 families were upregulated (Borin et al. 2017).

The recent developments in genomic, transcriptomic, metabolomic or proteomic technologies have led to the identification of several CAZymes and other genes of *Trichoderma* which are active during agricultural biomass degradation. Keeping in view the importance of CAZymes in plant biomass degradation for various applications, attempt has been in present chapter to provide an overview of different lytic enzymes of *Trichoderma* strains in white biotechnology for biofuel production.

## 12.2 Biocatalysis of Plant Biomass Using Lignocellulases

Lignocellulose from plant biomass is the major raw material for biofuels, foods and other livestock feeds (Kumar et al. 2008). Studies on fungal lignocelluloses-mediated lysis have revealed several pathways for lignin metabolism (Mansur et al. 2003). The lignocellulose is a promising biomass pretreatment alternative, and fungal lignocellulases are one of the potential enzymes in debasing lignin of plants (dos Santos et al. 2007; Dias et al. 2007; Plácido and Capareda 2015;Martinez et al. 2009). Moreover, the lignocellulases are also explored for the removal of toxic compounds as well as supplementing the pre-existing technologies of sugar hydrolysates after conventional pretreatment (Plácido and Capareda 2015; Bilal et al. 2018). Higher white fungi are known to produce a plethora of lytic enzymes. The lignin-degrading enzyme complex in white fungi is mainly consists of lignin peroxidase, manganese peroxidase and laccase along with other enzymes which include peroxidase, aryl alcohol oxidase, glyoxal oxidase and oxalate. The broad specificity of substrates also makes them vital enzymes which are capable of breaking a wide range of xenobiotics and pollutants having structural similarities to lignin (Hofrichter

2002; Bilal et al. 2018). A combination of co-culture techniques is found to enhance production of these enzymes. For example, 2.6-fold enhancement in laccase activity compared to *C. comatus* monoculture with higher delignification of up to 66.5% and conversion of 82% of polysaccharides into fermentable sugars was recorded (Ma and Ruan 2015).

### *12.2.1 Cellulases*

Cellulase, a complex of three enzymes, leads to the complete breakdown of cellulose to glucose units which can be used as fermentable sugar for biofuel production. The cellulose is degraded initially through endoglucanase (EG) (1,4-β- D-glucan-4-glucano-hydrolases) (EC 3.2.1.74) by random action into oligomers which are then targeted by exoglucanase (EC 3.2.1.74 and EC3.2.1.91) into cellobiose and glucose units. The β-glucosidases belonging to EC 3.2.1.21 hydrolyse the cellodextrins, cellobiose into glucose units (Keshwani and Cheng 2009; Jeya et al. 2009). The cellobiohydrolases (CBHs, named as CBH1 and CBH2), β-glucosidases (BGLs) and endoglucanases (EGs) act in a coordinated and complementary fashion to hydrolyse cellulose (Cavaco-Paulo et al. 1997; Gusakov et al. 2007; Jørgensen et al. 2007; Ma et al. 2011). The cocktail of different cellulolytic enzymes play vital role in the hydrolysis of complex plant polysaccharides. For example, a mixture of CBH1, CBH2 and EG1 is found to responsible for up to 80% of cellulose breakdown (Rosgaard et al. 2007). *T. reesei*, an industrial strain, is known to secrete CBH1, CBH2, EG1, EG2, EG3 and EG5 which act in a synergistic manner to completely hydrolyse the lignocellulose (Fang and Xia 2013). CBH1 and CBH2 are reported as major components of cellulase complex and accounts for 50–60% and 10–15% of the secreted protein, respectively (Rosgaard et al. 2007). Compared to CBH1, the specificity of CBH2 is approximately twice for crystalline cellulose (Zhou et al. 2008), and optimum synergism is reported at a 2:1 molar ratio (Zhou et al. 2009).

The other components of cellulase complex in *T. reesei* such as endo-β-1,4-D-glucanases are reported from glycosyl hydrolase families GH5, GH7, GH12 and GH45, whereas cellobiohydrolases are reported from families GH6 and GH7. The GH7 family contains endo-β-1,4-D-glucanases of CEL7B, previously known as EGL1 and CBHs (CEL7A, named as CBH1). The family GH5 cellulases is mostly explored from fungi strains (Li and Walton 2017), and three candidates of this family have been reported from *T. reesei*. The enzymes of GH7 family are distributed commonly. The orthologues of CEL7A cellulases are prevalent in the secretome of fungi-degrading biomass. The members of GH6 family comprise cellulase which acts exclusively from the non-reducing end of cellulose chain. The synergistic action of CEL7A and CEL6A is considered to play a key role in biomass degradation. The members of GH12 are typically low molecular weight (25 kDa) and do not contain cellulose-binding domain (CBM1) and glycosylation site. Due to their small size, GH12 can diffuse deeper into cellulosic material, and hence preferred for their role in laundry industry. On the other hand, members of GH45 cellulases are in

general small and have a wide substrate range compared to families GH5 and GH7. The members of GH45 enzymes share interesting structural similarities to plant expansins. Further intensive research efforts with genetic engineering strategies for single-enzyme cellulase components have increased the scope of *T. reesei* strain's improvement (Pryor and Nahar 2015; Qian et al. 2016, 2017; Wang and Xia 2011; Zhang et al. 2010).

### *12.2.2 β-Glucosidase*

A heterogeneous family containing exo-glycosyl hydrolases catalyses the cleavage of β-glycosidic bonds in disaccharide or glucose-substituted molecules (Bhatia et al. 2002; Chandra et al. 2013; Cheng et al. 2017; Leah et al. 1995; Zagrobelny et al. 2008). According to the classification of CAZy (http://www.cazy.org) (Henrissat 1991; Cantarel et al. 2009), β-glucosidases are classified into two families: 1 and 3 of glycosyl hydrolases (Jeng et al. 2011). These enzymes enhance the action of cellulose-degrading enzymes by releasing phenolic compounds and hence are an attractive choice for renewable bioenergy. β-glucosidases hydrolyse the oligosaccharides and cellobiose oligomeric units obtained after the endoglucanases and cellobiohydrolases activities into monomeric glucose (Chandra et al. 2013).

The β-glucosidases of *T. reesei* are categorized into GH1 and GH3. The members belonging to family GH1 are exclusively intracellular in nature, whereas GH3 β-glucosidases are predominantly extracellular (Guo et al. 2016). CEL3A previously categorized as BGL1 is responsible for majority of the β-glucosidase activity. The 'exo/endo' concept revealed that CEL7A is also able to act in endo-manner; therefore, it is not a true exocellulase (Stahlberg et al. 1993; Kurasin and Valjamae 2011). However, neither the EGs nor the CBHs from fungi can cause massive cellulose decomposition (Payne et al. 2015). The lytic polysaccharide mono-oxygenases which were identified previously as endoglucanases belonging to GH61 (Sharma et al. 2018b) are now known as auxiliary family and cleave β-glucan in an oxidative fashion. The members of the family GH61 are also reported for their weak endoglucanase activity. The genome of *T. reesei* (http://www.genome.jgipsf.org/Trire2/Trire2.home.html) is reported to contain at least 10, β-glucosidases-encoded genes which include cel1A, cel1B, cel3A, cel3B, cel3C, cel3D, cel3E, cel3F, cel3G and cel3H. The gene encoding cel3A (bgl1) was found to be major extracellular β-glucosidase, whereas cel1A (bgl2) (Saloheimo et al. 2002a, b) and cel1B (Zhou et al. 2012) were reported to be intracellular. Additionally, cel3B, cel3E, cel3F, cel3G and cel3H are assumed to be extracellular, and cel3C, cel3D and cel3H are depicted as intracellular (Guo et al. 2016). Different knockouts, amino acid substitution and mutation of the BglR transcription factor in the PC-3-7 strain have been used to reveal the function of β-glucosidases (Fowler and Brown 1992; Zhou et al. 2012; Nitta et al. 2012; Xu et al. 2014; de Porciuncula et al. 2013; Shida et al. 2015; Li et al. 2016).

### *12.2.3 Xylanases*

With a backbone of β-(1-4)-linked xylose units, polysaccharide xylan are structurally diverse and complex polysaccharides and predominantly composed of hemicelluloses which are linked to cellulose microfibrils (Scheller and Ulvskov 2010). The side chains are connected through C2 and C3 positions of D-xylosyl units (Puls and Schuseil 1993), and these chains can be substituted with acetyl, 4-methyl-D-glucuronosyl or L-arabinosyl units (Wong et al. 1988; Dodd and Cann 2009). Endo-β-1,4-xylanases or β-1,4-D-xylan xylanohydrolases (EC 3.2.1.8) are one of the important lytic components which can target the glycoside bonds in xylan backbone internally (Biely 1985; Polizeli et al. 2005; Mangan et al. 2017). Members of xylanase family belong to glycoside hydrolase (GH) families 5–12, 16, 26, 30, 43, 44, 51 and 62. Enzymes classified in 16, 51 and 62 families contain two catalytic domains compared to 5–11 and 43 families which have a true catalytic domain with endo-1,4-β-xylanase activity. The 9, 12, 26, 30 and 44 families may possess residual or secondary xylanase activity.

In recent classifications based on hydrophobic cluster analysis of catalytic domains and amino acid sequence similarities, xylanases are classified as GH10 and 11 and have a retaining type of mechanism. The information on catalytic properties of families 5, 7, 8 and 43 are very limited. The members of GH families 5, 7, 8, 10, 11 and 43 are different in their structure, mode of action, physicochemical properties and substrate specificities (Collins et al. 2005). The members of GH 10 family include high-molecular-mass proteins with cellulose-binding and catalytic domains and are connected through linker peptides. The estimated pI is 8–9.5 with $(\alpha/\beta)_8$ fold TIM barrel structure. On the other side, the GH11 family with low molecular mass and pI are further divided into two, alkaline and acidic (Buchert et al. 1995; Juturu and Wu 2012). The GH11 members exclusively catalyse endo-β-1,4–mediated cleavage (EC 3.2.1.8) in xylan and hence are also known as true xylanases. The high catalytic efficiencies of these enzymes due to small size, vast temperature and pH optima provide them an edge for their exploitation in various biotechnological applications (Paes et al. 2012).

Xylanases of *Trichoderma* are one of the widely explored enzymes, and Rut C-30 strain of *T. reesei* is well explored for commercial applications of xylanase and cellulase production (Gerber et al. 1997). The xylanases produced by *T. harzianum*, *T. lignorum*, *T. koningii*, *T. longibrachiatum*, *T. pseudokoningii* and *T. viride* also have been investigated (Silveira et al. 1999; Chen et al. 2009). Xylanases from a psychrotrophic *Trichoderma* strain have been characterized (Zhou et al. 2011) and genes encoding them have been cloned from *Trichoderma* species and expressed in heterologous hosts such as *E. coli* (Min et al. 2002), *S. cerevisiae* (Ahmed et al. 2005) and *P. pastoris* (He et al. 2009). In the *T. reesei* genome, three xylanases belonging to the GH11 family have been identified, and two of these were reported in the early 1990s, whereas the third GH11 xylanase XYN5 was identified in a recent study (Martinez et al. 2008; Dos Santos Castro et al. 2014; Peciulyte et al. 2014; Saloheimo and Pakula 2012).

### *12.2.4 Lytic Polysaccharide Mono-oxygenases (LPMOs)*

The recently discovered enzyme class, the LPMOs, stimulates the hydrolysis of plant biomass and enhances the efficacy of glycosyl hydrolases (Hu et al. 2014). Unlike cellulases which target glycosidic bonds by hydrolysis, LPMOs are copper dependent and catalyse the breakdown of polysaccharides through oxidation at C1 or C4 glucose units in the presence of external electron donors.

### *12.2.5 Laccases*

Laccases are also known as phenol oxidases or benzenediol: oxygen oxidoreductase (EC 1.10.3.2) belongs to the multicopper oxidase (MCO) family and represents a group of metalloenzymes. These enzymes are used in various biotechnological applications. The search for strains producing such laccases has gained increased attention in recent times. In general, laccases are monomeric glycoproteins of 60–70 kDa in size, and carbohydrates approximately contribute to 30% of their molecular weight (Cázares-García et al. 2013). Laccases oxidize compounds containing a variety of phenolic, diamines and aromatic amines (Abd El Monssef et al. 2016). In lignocelluloses containing biomass, laccases play an important role in developing a clean biocatalytic process and improve cellulose recovery from feedstocks containing lignocellulose (Avanthi and Banerjee 2016). Additionally, the affinity of laccases for different aromatic compounds make them a promising and attractive tool for de-colouration and detoxification of different synthetic dyes and phenolic pollutants. These chemicals are often a source of water contamination and thus can cause problems to public health and our environment (Anbia and Ghaffari 2011). A combination of laccases and cellulases enhances delignification and thus increases the efficiency of developing enzymatic processes for biofuels and other value-added product generations such as coal solubilization (Chakroun et al. 2010). The extracellular laccase of *T. virens* is reported for their role in mycoparasitism against the sclerotia of plant pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Catalano et al. 2011; Cázares-García et al. 2013).

Fungi of basidiomycetes and ascomycetes division are known to degrade lignin, xenobiotics, chemicals used for guaiacol synthesis and vanillin metabolites at industrial scales (Dekker et al. 2002; Halaburgi et al. 2011; Younes and Sayadi 2011). The wood-rotting fungi such as *Trametes* spp., *Cerrena maxima*, *Lentinus tigrinus*, *Coriolopsis polyzona* and *Pleurotus eryngii* are prominent laccase producers (Saloheimo and Niku-Paavola 1991; Morozova et al. 2007; Madhavi and Lele 2009). In general, fungal laccases are known to possess high redox -potential and broad substrate specificity compared to laccases of bacterial origin. The pH optima of fungal laccases is reported at acidic pH, whereas for bacterial laccases, like oxidases, it operates close to neutral-alkaline pH (Kolomytseva et al. 2017). Laccases can target phenolic constituents of lignin and have compatibility to work at industrial

pH, in solvents and at especially high temperatures and therefore are potential source for wood delignification for bioethanol production (Shanmugam et al. 2018). The laccase-encoding genes from other fungi such as *Pycnoporus sanguineus* and *Phlebia radiata* have been cloned and expressed using the Pcbh1 promoter and the Tcbh1 terminator of *T. reesei* (Zhao et al. 2018b).

Among ascomycetes, *Trichoderma* species have been extensively explored for cellulase production (Tsao and Chiang 1983). *Trichoderma* strains with laccase activity are more efficient in breaking natural substrates than strains without these enzymes (Assavanig et al. 1992). The laccases from ascomycetes have characteristic features which are not present in basidiomycetes laccases. The presence of additional L1–L4 signature domains (Kumar et al. 2003) helps their differentiation from other multicopper oxidases. The laccase activity has been reported in strains of *T. atroviride*, *T. reesei*, *T. viride*, *T. longibrachiatum* and *T. virens* (Assavanig et al. 1992; Krastanov et al. 2007; Gochev and Krastanov 2007; Catalano et al. 2011; Cázares-García et al. 2013). In addition, the conidia of *T. atroviride*, *T. viride* and *T. harzianum* are also reported for laccase activity (Holker et al. 2002; Pokorny et al. 2005). Studies on purification and characterization of laccases of extracellular nature have been conducted in *T. harzianum* (Sadhasivam et al. 2009), *T. atroviride* (Chakroun et al. 2010) and *T. reesei* (Levasseur et al. 2010) strains. The infection in *Pleurotus ostreatus* cultures with *T. viride* spores is also reported to induce higher laccase activity (Divya et al. 2013).

## 12.3 Distribution and Identification of CAZy Genes in *Trichoderma* Genome

The comprehensive information on carbohydrate-active enzymes glycoside hydrolases, carbohydrate esterases, polysaccharide lyases and glycosyltransferases which contribute to the breakdown and modification of glycosidic bonds can be gained from CAZy database. A number of enzymes and active transcripts involved in plant biomass degradation have been identified using genomics, transcriptomics or proteomics approaches. The majority of these transcripts have been identified as glycosyl hydrolases and carbohydrate esterases (Fig. 12.3).

In industrial strain *T. reesei*, a limited number of carbohydrate active enzymes (CAZymes) have been characterized, whereas genome sequencing revealed presence of several candidates genes which may have been transferred horizontally from bacteria (Häkkinen et al. 2012). Phylogenetic analysis of different CAZy genes has identified around 201 glycoside hydrolase-encoding genes, 22 carbohydrate-encoding esterase genes and 5 polysaccharide lyase genes. Among glycosyl hydrolases, β-glucosidases of GH3, α-galactosidases of GH27 and chitinases of GH18 have been reported in abundance (Häkkinen et al. 2012). In the genome of *T. reesei*, 61 CAZy families were predicted which exclude family CE10. The complete list of CAZy families in *T. reesei* can be obtained from a study

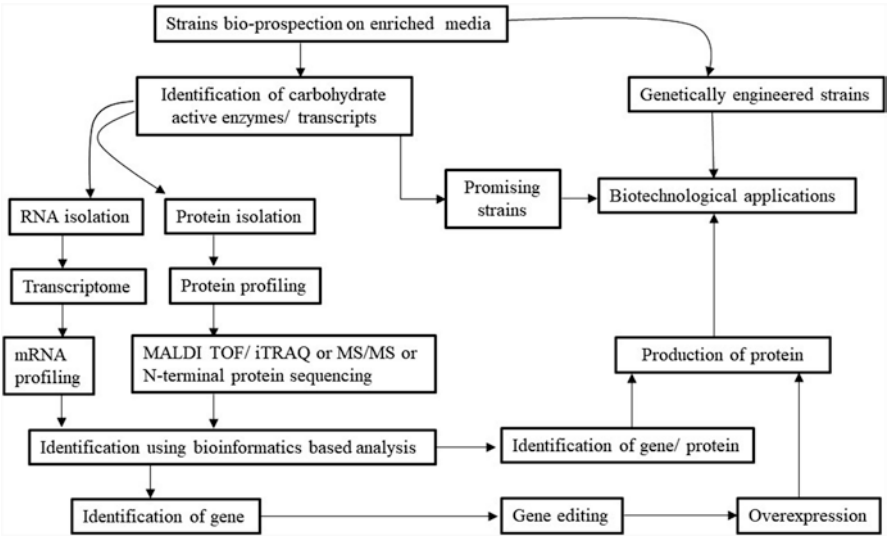


**Fig. 12.3** An overview of mining carbohydrate-active candidate transcripts/proteins for biomass conversion

conducted by Häkkinen et al. (2012). A comparison overview of *T. reesei* CAZY enzymes with other fungi revealed that cluster containing AGLIII and other four candidate α-galactosidases are restricted to *T. reesei*. The cluster for β-glucuronidase genes of families GH79, GH18 and GH92 also revealed expansion in *T. reesei*, whereas families GH43 and Gh61 showed reduction (Häkkinen et al. 2012). The CBHI/CEL7A and CBHII/CEL6A acts in exo-fashion on cellobiohydrolases whereas five endo-acting cellulases such as EGII/CEL5A, EGI/ CEL7B, EGIII/ CEL12A, EGV/CEL45A and EGIV/CEL61A are also reported form *T. reesei* strain (Penttilä et al. 1986; Saloheimo et al. 1994; Saloheimo et al. 1997; Okada et al. 1998). Additionally three putative endoglucanases (CEL74A, CEL61B and CEL5B) were reported (Foreman et al. 2003). In the genome of *T. reesei*, two β-glucosidases (BGLI/CEL3A and BGLII/ CEL1A) (Barnett et al. 1991; Fowler and Brown 1992; Takashima et al. 1999; Saloheimo et al. 2002a, b) and five β-glucosidases (CEL3B, CEL3D, CEL1B, CEL3C, CEL3E) also have been reported (Foreman et al. 2003). A protein named as swollenin (SWOI) involved in the biomass degradation by disrupting cellulose crystalline structure without the release of sugars has been also reported (Häkkinen et al. 2012). On the other hand, a number of other enzymes such as xylanases (XYNI, XYNII, XYNIII and XYNIV), mannanase (MANI) (Stalbrand et al. 1995), acetyl xylan esterase (Foreman et al. 2003; Margolles-Clark et al. 1996a), α-glucuronidase (GLRI) (Margolles-Clark et al. 1996a), arabinofuranosidases (ABFII and ABFIII) (Margolles-Clark et al. 1996b; Foreman et al. 2003; Herpoël-Gimbert et al. 2008), α-galactosidases (AGLI, AGLII and AGLIII) (Margolles-Clark et al. 1996b; Zeilinger et al. 1993) and β-xylosidase (BXLI) (Margolles-Clark et al. 1996c, d) has also been reported from *T. reesei* and other

filamentoru fungi (Tenkanen et al. 1992; Torronen et al. 1992; Xu et al. 1998; Knob et al. 2010). These proteins are known to play a vital role in breaking xylan-derived oligosaccharides. Also, several novel candidate lignocellulose-degrading genes have been identified from *T. reesei* genome (Martinez et al. 2008).

Screening of *T. harzianum* isolate for CAZymes via RNA-Seq and bioinformatics approach revealed around 259 transcripts related to glycoside hydrolases, 101 transcripts for glycosyl transferases, 6 for polysaccharide lyases, 22 for carbohydrate esterases, 42 for auxiliary activities (AAs) and 46 for carbohydrate-binding proteins when cellulose was used as substrate. The highest number of genes has been reported from GH18, GH3, GH16, GH2 and GH5 families. For hemicellulases, 24 glycosyl hydrolases belonging to families GH10, GH11, GH26, GH43, GH54, GH62, GH67 and GH95 were identified. The maximum enzymes were reported from GH43 and GH95 families, whereas the lowest number was identified from GH67, GH62, GH54, GH26 and GH10 families (Ferreira Filho et al. 2017).

## 12.4 Strain Improvements

Strains of *T. reesei* have been the topic of investigation for its cellulases. Higher enzyme production cost is one of the key hurdles involved in commercial applications of these enzymes for biofuel production. Screening for high level of cellulase-producing strains is an efficient strategy to address this issue. Due to high porduction cost of enzymes, efforts are required to enhance the production, intrinsic activity and reinforcing the existing biomass degrading enzymes with auxiliary proteins (Wilson 2009; Horn et al. 2012; Peterson and Nevalainen 2012; Hu et al. 2015; Müller et al. 2015; Payne et al. 2015). A number of tools such as genetic engineering advance genetic transformation based on use of marker or marker-free selections, or RNA interference has been discussed by Bischof and Seiboth (2014). *T. reesei* Rut-C30 and *T. reesei* D-7 mutants developed by the use of basic chemicals such as ethyl methyl sulfonate (EMS), and other methods such as plasma irradiation are already used for high cellulase production. The filter paper activity and corn starch hydrolysate higher cellulase production in *T. reesei* strain D-7. Mutant-based study has been successful in obtaining potential cellulase-producing mutants (Zhang et al. 2017). In the last decades, efforts on strain improvement using traditional mutagenesis and screening methods have resulted in *T. reesei* strains RUT-C30 capable of producing up to 30 g/l of extracellular cellulases (Eveleigh and Montenecourt 1979; Eveleigh 1982) and even producing as high as 100 g/l of extracellular protein (Cherry and Fidantsef 2003). The commercial formulation for enhanced cellulase production such as Novozymes and Dupont are also obtained through mutations in *T. reesei*. In recent studies, the advancements of molecular tools in gene/genome engineering using specific insertion or deletion or mutation of nucleotides have been explored to meet the growing demands of different

biomolecules including enzymes. The discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (cas) 9 genes (CRISPR/cas9) system has democratized the genome engineering in a flexible manner either at a single- or multi-loci-based genome-wide modification. The CRISPR/cas9 system nowadays has emerged as a powerful tool for strain improvement in filamentous fungi such as *T. reesei* (Liu et al. 2015; Donohoue et al. 2018).

## 12.5 Conclusion and Future Prospects

The exploration of microbe's innate capacity to convert complex polysaccharides into biofuels with octane value is one of the predominant research areas presently. The filamentous fungi such as *T. reesei* have been widely extensively for cellulase and hemicellulase production. The genetic manipulation of *T. reesei* using mutagenesis has led to improved strains with higher cellulase production. Advancements in biotechnological tools have significantly contributed in developing alternate and efficient technologies. Enzymatic treatment offers advantage over chemical and physical methods being environmentally friendly. In several studies, either single or a combination of physical and chemical methods of mutations such as UV irradiation, ethyl methanesulfonate and N-Methyl-N′-nitro-N-nitrosoguanidine had been deployed in *Trichoderma*, *Aspergillus* and other fungi. The commercial formulation developed by the enzyme industry in companies such as Novozymes and Dupont was obtained through mutations for enhanced cellulase production in *T. reesei*.

Enzyme-mediated delignification has been used for enhancing enzyme production using rational, semi-rational and directed evolution-based molecular and protein engineering strategies. In rational approach, modification through the use of site direct mutagenesis for lignolytic enzymes such as laccases has been used successfully. Alternatively, a mixture of two filamentous fungi such as *T.reesei* and *A. niger* has been found better for cellulase production. Despite the challenge associated with the expression of active recombinant proteins in heterologous system, paucity of signal peptides and expression system, genetic engineering through the use of codon optimization and substitution with unnatural amino acids in recombinant proteins is emerging field and can provide us enzyme systems with better catalytic property and enhanced self-life. However, the concern for low or lack of production of potent hemicellulases and β-glucosidases in *T. reesei* secretome needs alternative potential strategies which could either replace or supplement *T. reesei* enzyme system. Therefore, efforts are required for exploring microbial enzymes for biofuel production from agricultural biomass.

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