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Insect cellulolytic enzymes: Novel sources for degradation of lignocellulosic biomass

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Abstract: Alternative and renewable fuels derived from lignocellulosic biomass offer the potential to reduce our d ependence on fossil fuels and mitigate global climate change. Cellulose is one of the major structural components in all lignocellulosic wastes and enzymatic depolymerization of cellulose by cellulases is an essential step in bio-ethanol production. Wood-degrading insects are potential source of biochemical catalysts for converting wood lignocellulose into biofuels. Cellulose digestion has been demonstrated in more than 20 insect families representing ten distinct insect orders. Termite guts been have considered as the "world's smallest bioreactors" since they digest a significant proportion of cellulose (74-99%) and hemicellulose (65-87%) components of lignocelluloses they ingest. The lower termites harbor protistan symbionts in hindgut whereas higher termites lack these in the hind gut. Studies on cellulose digestion in termites and other insects with reference to ligno-cellulose degrading enzymes have been well focused in this review. The studies on insect cellulolytic systems can lead to the discovery of a variety of novel biocatalysts and genes that encode them, as well as associated unique mechanisms for efficient biomass conversion into biofuels.

Keywords: Bioethanol , Insect cellulases, Lignocellulosic biomass, Sustainable energy, Termites

INTRODUCTION

The world's energy demand is increasing steadily as is the human population and economic development. The dramatic rate of fossil fuel depletion and the resultant global economic and environmental consequences have spurred the search for alternative renewable energy sources such as biofuels (Lynd *et al.,* 2008). Worldwide interest has been focused on the development of technologies using new and renewable sources of energy like biomass, geothermal, solar power, wind, and hydropower (Gokcol *et al.,* 2009). Lignocellulosic ethanol has been suggested as a desirable biofuel, mostly due to its sustainability, reduced competition as a food resource, net energy production, and reduced input costs related to production of ethanol from corn-derived starch (Lynd *et al.*, 1991; Schmer *et al.*, 2008). One of the limiting steps in the biomass-to -ethanol process is the degradation of cellulose to fermentable sugars (saccharification). This currently relies on the use of bacterial and/or fungal cellulases, which tend to have low activity under biorefinery conditions and are easily inhibited (Fischer *et al.,* 2013). Insects have evolved endogenous and symbiotic enzymes to efficiently use lignocellulosic material as a source of metabolic glucose (Willis *et al.,* 2010). The present review describes the current status of insect cellulases for degradation of lignocellulosic biomass.

Lignocellulose structure: Lignocellulose is a general term referring to a natural complex of the three biopolymers cellulose, hemicellulose and lignin. Cellulose has attracted worldwide attention as a renewable resource that can be converted into bio-based products and bioenergy (Li *et al.,* 2009). Cellulose is a fibrous, insoluble, crystalline polysaccharide. It is a major polysaccharide constituent of plant cell wall, composed of repeating D-glucose units linked by β-1,4 -glucosidic bonds. Cellulose is synthesized in nature as individual molecules which undergo self-assembly at the site of biosynthesis (Brown *et al.,* 2000). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibres. (Lynd *et al.,* 2002). Hemicellulose is composed of shorter β-1, 4-linked polymers of mixed sugars. Mannose is usually the dominant sugar present in hemicelluloses of soft woods fed upon by termites, with lesser amounts of xylose, galactose, rhamnose, arabinose, glucuronic acid, mannuronic acid and galacturonic acid (Saha 2003). Lignin is not a carbohydrate, but a 3-dimensional polymer of phenolic compounds that are linked to each other and to hemicellulose by ester bonds. Lignin is composed of the three 'mono-lignol' monomers *p*-coumaryl alcohol, coniferyl alcohol, and

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sinapyl alcohol combined in different ratios depending on the plant species (Anderson and Akin, 2008).

Role of cellulases: The degradation of lignocelluloses into fermentable or otherwise utilizable sugars is a complex process that requires a diversity of enzymes. Cellulases are a class of hydrolases that perform several enzymatic activities, namely endo-*β*-1, 4-glucanase (also known as endocellulase or carboxymethyl cellulase (CMCase), exo-*β*-1,4-glucanase (exocellulase) and β-glucosidase activity (Fry, 1995). The synergistic action of these enzyme components breaks the microfibril structure down into small linear chains of glucose. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol. Even though cellulolytic enzymes have been commercialized for use in diverse industrial applications, current cellulolytic technologies to degrade lignocellulosic biomass still need improvement to reduce biofuel production costs.

Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Genetic techniques have been used to clone the cellulase coding sequences into bacteria, yeasts, fungi and plants to create new cellulase production systems with possible improvement of enzyme production and activity (Sun and Cheng, 2002).

Insect cellulases: Insects have evolved very effective strategies to use lignocellulosic substrates as sources of energy (Willis *et al.,* 2010) which makes them an optimal resource to prospect for novel cellulolytic enzymes. Throughout evolution, insects have established symbiotic interactions with different microorganisms to carry out key hydrolytic activities (Moran, 2006) for degradation of cellulose to simple sugars in the insect midgut and thus facilitating, insect phytophagy (Douglas, 2009). The isolation of midgut microorganisms carrying out cellulolytic activities constitutes an important step towards the implementation of second-generation biofuels, which use non-food crops or feedstock crops as substrate for bioethanol production, overcoming the problems related to first-generation biofuels in terms of sustainability (Naik *et al.,* 2010).

Wood-degrading organisms are important for their roles in carbon turnover in the environment and as potential sources of biochemical catalysts for efforts aimed at converting wood (lignocellulose) into biofuels. Cellulose digestion has been demonstrated in more than 20 insect families representing 10 distinct insect orders including Thysanura, Plecoptera, Dictyoptera, Orthoptera, Isoptera, Coleoptera, Trichoptera, Hymenoptera, Phasmida and Diptera (Sun and Scharf, 2010).

Termites play an important role in the turnover and mineralization of complex biopolymers, such as wood and other cellulose and hemicelluloses containing materials (Wenzel *et al.,* 2002). Termite guts been have been considered as the "world's smallest bioreactors" (Brune 1998). Termites are said to dissimilate a significant proportion of cellulose (74-99%) and hemicellulose (65-87%) components of lignocelluloses they ingest (Ohkuma, 2003).The first endogenous insect cellulase was discovered in 1998 in termite *Reticulitermes speratus*, which retains its ability to feed on wood even when gut was destroyed. The *Reticulitermes speratus* cellulase (RsEG) encodes an endo-β (1,4)-glucanase (Watnabe *et al.*, 1998).

The compartmentalization of the cellulose digestion by endogenous and exogenous cellulolytic enzymes in diverse regions of the digestive tract has been extensively studied to optimize cellulose digestion in termites (Nakashima *et al*., 2002; Tokuda and Watanabe 2007; Zhou *et al*., 2007). Traditionally, exogenous cellulolytic enzymes are localized to the insect hindgut, while endogenous enzymes localized to the foregut, midgut and salivary glands. Nakashima *et al.* (2002) reported that lower termite *C. formosanus* use both endogeneous endogluconases and protozoan hydrolases, but these enzymes do not act synergistically. Thus it has two independent cellulose-digesting systems: one in the midgut where cellulose digestion is accomplished by endogenous cellulases and the other in the hindgut which makes use of other cellulases possibly from symbiotic flagellates. However, the presence of a single unified cellulose digestion system, whereby endogenous and symbiotic cellulases work sequentially and collaboratively across the entire digestive tract of lower termite *Reticulitermes flavipes* (Kollar) was reported by Zhou *et al*. (2007). The higher termites lack protozoan fauna but have abundant prokaryotic flora in the hindgut. The presence of cellulases in the bacterial insoluble fraction of hindgut of wood feeding higher termites *Nasutitermes takasagoensis* have been reported by Tokuda and Watanabe (2007). The hindguts of these xylophagous flagellate-free termites *Nasutitermes takasagoensis* and *Nasutitermes walkeri* contained up to 59% cellulase activity against crystalline cellulose when compared with the midgut.

In termites, cellobiohydrolase, multidomain cellulases, hemicellulases as well as lignin degrading enzyme are not involved in the initial cellulose digestion occurring in the midgut. The absence of these enzymes may be due to thoroughness of mastication of the insects to expose the cellulose microfibrils from wood structure. The absence of cellobiohydrolase and multidomain enzymes is compensated by the presence of symbiotic cellobiohydrolase (Watanabe and Tokuda, 2010).

The cellulolytic bacteria have been isolated from the larval gut of the logicorn beetle *Saperda vestita*, and the dark beetle *Ips pini* and *Dendroctonus frontalis* (Delalibera *et al.*, 2005). The bacterial isolates were active against carboxymethyl cellulose, whereas only the isolate of *S.vestita* showed activity against filter paper. While cellulases from microorganisms are important for cellulose degradation in termites (Nakashima *et al*., 2002) and Coleoptera larvae (Kukor and Martin, 1986), there are numerous examples in lower termites (Tokuda and Watanabe, 2007) and other insect groups (Scrivener *et al*., 1989; Treves and Martin, 1994) in which endogenous insect cellulases are sufficient for effective cellulose degradation and survival on plant biomass. Tokuda and Watanabe (2007) reported that the endogenous cellulase secreted from the midgut tissue were the sole source of cellulases in these termites. Treves and Martin (1994) conducted antibiotic feeding studies on the firebrat, *Thermobia domestica* (Zygentoma, Lepismatidae) to determine if the insect's gut cellulases were of insect or microbial origin and concluded that the gut cellulases of firebrats are of insect origin. The studies concluded that symbiont-independent cellulose digestion is a primitive trait in insects and that symbiont-mediated cellulose digestion is a derived condition. Scrivener *et al.* (1989) studied the symbiont-independent cellulose digestion in *Panesthia cribrata* Saussure, an Australian wood-eating cockroach. The endogenous cellulase is secreted in the epithelium of the anterior ventriculus and is found predominantly (98%) in the foregut and the anterior ventriculus of insect.

The availability of enzymes that degrade unprocessed forms of cellulose, such as microcrystalline cellulose (MCC), in the most proximal regions of the digestive tract (i.e. the salivary glands) would be advantageous for efficient hydrolysis of cellulose from ingested plant material (Tokuda *et al.*, 2005). Conversely, activity against a more readily accessible cellulose form, such as Carboxymethyl cellulose (CMC), would be expected in later stages of digestion and absorption in the mid gut. Plant-derived cellulose is structurally more similar to MCC than CMC, thus enzymatic attack on ingested cellulosic material would be more efficient with an initial synergistic action by a cellulolytic enzyme complex (i.e. high activity against MCC), when followed by EG hydrolysis of the more bio available cellulosic by-products in the gut (i.e. higher activity against CMC). Oppert *et al.* (2010) carried out quantitative determinations of the cellulolytic activity in gut or head-derived fluids from 68 phytophagous or xylophagous insect species belonging to eight different taxonomic orders. Apart from the wood-feeding termites, the highest CMC gut fluid activities were found in Dictyoptera, Coleoptera, Isoptera, and Orthoptera, while highest MCC gut fluid activities were found in Coleoptera, Hymenoptera, Lepidoptera, and Orthoptera. In most cases, gut fluid activities were greater with CMC compared to MCC substrate, except in Diptera, Hymenoptera, and Lepidoptera. In contrast, cellulolytic activity levels in most head fluids were greater on the MCC substrate. A phylogenetic relationship may exist for the origin of cellulolytic enzymes in insects, and that cellulase activity levels correlate with taxonomic classification, probably reflecting differences in plant host or feeding strategies.

Su *et al.* (2013) studied the cellulolytic activities in the gut fluids of 54 insect species that belong to 7 different taxonomic orders using 2 different substrates, carboxymethyl cellulose (CMC) (approximating endo-β-1,4-glucanase) and filter paper (FP) (total cellulolytic activities). The highest CMC gut fluid activities were found in Coleoptera and Orthoptera, while FP analysis indicated that higher gut fluid activities were found in several species of Coleoptera and Lepidoptera. In most cases, gut fluid activities were higher with CMC than with FP substrate, except for individual Lepidoptera species. Willis *et al.* (2010) identified and characterized the cellulolytic enzyme activity in digestive fluids of *D. carolina*. The cellulolytic enzymes were localized to foregut and midgut regions of the *D. carolina* digestive tract. The studies revealed the presence of cellulolytic activity in the digestive system of *D. carolina* and suggested that cellulases of endogenous origin were present in this organism. Considering that this grasshopper species is a pest of grasses, including switchgrass which is one of the bioethanol feedstock, characterization of insect cellulolytic systems may help in developing processes for plant biomass biodegradation for bioethanol production.

Geib *et al.* (2010) reported enzymes of lignocellulose degradation in a wood-feeding beetle, the Asian longhorn beetle. He carried out zymogram analysis to identify and characterize cellulases and hemicellulases active against cellulose and hemicellulose substrates. This beetle feeds on a range of tree species and uses them as sole food sources and thus using insect gut for lignocellulase can potentially yield new enzymes for processing lignocellulolytic material into cellulosic bioethanol. Huang *et al*. (2010) reviewed the physiochemical properties of the scarab beetle gut at larval stage, the diversity and digestive roles that symbiotic microflora play in the scarab gut, and they further discussed the potential for applying these digestive processes in artificial bioreactors. Exploring

another specific cellulose-consuming insect from the order Diptera (crane fly), which is a leaf shredding aquatic insect that lives in forested ecosystems, Rogers and Doran-Peterson (2010) have reported the analysis of cellulolytic and hemicellulolytic enzyme activity within this insect gut (larval stage). They also identified and characterized novel cellulolytic bacterial species isolated from its gut system.

The advancement of genomics and proteomics research tools are expected to allow new insights into the mechanisms for wood deconstruction by cellulose-feeding insects, as well as facilitate the discovery of new cellulolytic enzymes from a wide range of cellulolytic system (Sun and Scharf, 2010). There have been numerous reports on cellulolytic activity in insects including identification and cloning of insect cellulases (Lee *et al*., 2004, 2005; Wei *et al*., 2006; Kim *et al*., 2008). Lee *et al.* (2004) cloned a cellulase [beta-1,4-endoglucanase (EGase), EC 3.2.1.4] cDNA (Ag-EGase I) belonging to glycoside hydrolase family (GHF) 45 from the mulberry longicorn beetle, *Apriona germari* and also reported the gene structure, expression and enzyme activity of Ag-EGase I and an additional cellulase (Ag-EGase II). Besides this, Wei *et al.* (2006) cloned a novel endogenous beta-1,4-endoglucanase (Ag-EGase III) gene belonging to the glycoside hydrolase family (GHF) from *A. germari*. Further, Kim *et al.* (2008) cloned and characterized a novel endogenous beta-1,4 -endoglucanase (EG) gene belonging to the glycosyl hydrolase family 9 (GHF 9) that was distributed throughout the digestive tract of the cricket, *Teleogryllus emma,* suggesting a functional role of endogenous TeEG-I in a sequential cellulose digestion process. The comprehensive proteome analysis of digestome of lower termite *Coptotermes gestroi* revealed the complete collection of hydrolytic enzymes including cellulases, hemicellulases and pectinases (Franco Cairo *et al.*, 2011). Tarter *et al.* (2009) carried a dual host-symbiont transcriptome sequencing in a single termite species. The sequence database generated represents an important new genomic resource for use in further studies of collaborative host -symbiont termite digestion as well as development of coevolved host and symbiont-derived biocatalysts for use in industrial biomass-to-bioethanol applications. The study revealed that (i) phenoloxidase activities are prominent in the *Reticulitermes. flavipes* gut and are not symbiont derived, (ii) expands the known number of host and symbiont glycosyl hydrolase families in *Reticulitermes*, and (iii) supports previous models of lignin degradation and host-symbiont collaboration in cellulose/hemicellulose digestion in the termite gut. The metagenomic analysis of the bacterial community resident in the hindgut paunch of a wood-feeding 'higher' *Nasutitermes* species (which do not contain cellulose-fermenting protozoa) showed the presence of a large, diverse set of bacterial genes for cellulose and xylan hydrolysis. Many of these genes were expressed *in vivo* or had cellulase activity *in vitro*, and further analysis implicate the role of spirochete and fibrobacter species in gut lignocellulose degradation (Warnecke *et al.,* 2007). The termite digestomics research is to define collaborative lignocellulose digestion; i.e., to define how termite and symbiont systems complement one another to achieve efficient lignocellulose digestion. From termite digestomics research, a clearer picture of collaborative lignocellulose digestion has emerged now thus suggesting collaboration among termite-derived genes expressed in the salivary gland/foregut and midgut, and symbiont genes expressed in the hindgut (Tartar *et al.*, 2009, Scharf and Tartar, 2008). There is now evidence implicating: (i) lignases, GHF1 β-glucosidases, GHF9 endoglucanases, and GHF43 β-xylosidases in the foregut/salivary gland; (ii) apparent feruloyl esterases in the midgut; and (iii) a rich diversity of at least 17 symbiont derived GHFs in the hindgut (i.e., GHF 2, 3, 5, 7, 10, 11, 16, 20, 26, 30, 42, 45, 47, 53, 77 and 92). Of the various symbiont GHFs, family 7 exoglucanases are undeniably the most diverse. Developing a holistic understanding of insect digestomics will not only provide a quantum leap in understanding the fascinating phenomenon of insect/ symbiont co-evolution, but will also meet the ever-important need for refinement in industrial lignocellulose processing.

Conclusion

Inefficiency of industrial lignocellulose depolymerization is a major limitation in plant biomass utilization as a renewable energy source. High production costs and low activity of available enzymatic mixtures from microbes has, therefore, spurred the need for cellulase prospecting and improvement through genetic engineering. The insects and their symbiotic gut fauna, have evolved specialized enzymes that cooperate in lignocellulose processing. Endogenous insect lignocellulases work synergistically with symbiont-derived enzymes, and can confer extremely high efficiency in lignocellulose processing. Exploring insect cellulolytic systems will lead to the discovery of a variety of novel biocatalysts and genes that encode them, as well as associated unique mechanisms for efficient lignocellulosic biomass conversion into bioethanol.

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