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REVIEW

Potential of termite-based biomass pre-treatment strategies for use in bioethanol production

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Abstract When considering the current state of the biorefinery industry, it is readily apparent that industrial cellulose and hemicellulose digestion processes are relatively advanced, whereas enzymatic pre-treatment strategies for biomass delignification and cellulose solubilization are not well developed. The need for efficient biomass pre-treatment strategies presents a significant opportunity for researchers studying lignocellulose digestion in termites and other insects. With an emphasis on industrial biomass pre-treatment, this review provides an overview of: (i) industrial biorefining operations (feedstocks, processing, and economics); (ii) recent findings from termite research that have revealed candidate enzymes; and (iii) research needs and opportunities for consideration by entomologists working in this area. With respect to research findings, recently identified candidate lignases (laccases, catalases, peroxidases, esterases), other potentially important detoxification enzymes (cytochrome P450, superoxide dismutase), and phenolic acid esterases (carboxylesterases) that may assist in hemicellulose solubilization are overviewed. Regarding research needs and opportunities, several approaches for identification of candidate pre-treatment enzymes from upstream, symbiont-free gut regions are also described.

Key words bioenergy, bioethanol, biorefining, Isoptera, lignocellulose, renewable energy

Termites, lignocellulose, and review objectives

Termites are unique animals in that they have become specially adapted to survive on sugars and other nutrients obtained from otherwise nutritionally poor lignocellulose diets (Ohkuma, 2003). Lignocellulose is a mixture of the three plant-produced polymers: cellulose, hemicellulose and lignin (structures provided in Scharf & Tartar, 2008). Cellulose consists of extended β-1,4-linked glucose polymers that are held together in bundles by hemicellulose (Ljungdahl & Erickson, 1985; Lange, 2007). Hemicellulose is composed of more compact β-1,4-linked polymers of 5- and 6-carbon sugars and their uronic acids (Saha, 2003). Lignin is a 3-dimensional polymer of phenolic compounds (coumaryl, coniferyl and sinapyl alcohol) that are linked to each other and to hemicellulose by ester bonds. Another attribute of hemicellulose is its esterification with monomers and dimers of phenolic acid esters that are synonymous with the mono-lignols that compose lignin (Saha, 2003; Crepin *et al.*, 2004; Benoit *et al.*, 2008). Hemicellulose is much more water-soluble than cellulose, a property that has been exploited in industrial biorefinery operations (see later).

Termites digest lignocellulose by using endogenous and symbiont-produced digestive enzymes (Breznak & Brune, 1994; Watanabe *et al.*, 1998; Ohkuma, 2003; Scharf & Tartar, 2008; Tartar *et al.*, 2009). Termite gut symbionts consist of diverse micro-organisms such as protozoa, bacteria, spirochetes, fungi and yeast (Cleveland, 1923; Breznak & Brune, 1994; Ohkuma, 2003; Brune, 2006; Warnecke *et al.*, 2007). The order Isoptera is divided into

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the higher and lower termites based mostly on symbiont composition. Lower termites, including *Reticulitermes flavipes*, are hosts to both cellulolytic protozoa and a variety of non-cellulolytic prokaryotes (Lewis & Forschler, 2004; Fisher *et al.*, 2007). Higher termites, alternatively, lack protozoa but serve as hosts to a diverse pool of cellulolytic and non-cellulolytic prokaryotes (Wood & Johnson, 1986; Warnecke *et al.*, 2007).

In recent years, significant research efforts have been directed at understanding termite lignocellulose digestion. The primary reason for this major research thrust is the need for renewable bioenergy. This review addresses one component of renewable bioenergy production: *biomass pre-treatment*. The objectives of this review are to: (i) provide an overview of industrial lignocellulose processing with respect to feedstocks, processing, and economics; (ii) review recent findings from termite research that have revealed candidate biomass pre-treatment enzymes; and (iii) summarize pre-treatment research needs and opportunities for consideration by scientists working in the areas of insect nutrition and lignocellulose digestion.

Industrial biorefining: lignocellulose feedstocks, processing, and economics

Overview

The major processes involved in industrial bioethanol production from lignocellulosic feedstocks are shown in Figure 1. Lignocellulose can serve as a precursor for a number of biorefinery processes such as cellulose/ hemicellulose hydrolysis, pyrolysis, and gasification (Ragauskas *et al.*, 2006; Brown, 2006; Lange, 2007; Yang & Wyman, 2008). However, all three processes depend on biomass pre-treatment strategies that use chemicals and/or heat to delignify feedstocks or to release the sugar polymers cellulose and hemicellulose (Galbe & Zacchi, 2007). Current pre-treatment strategies are both chemical- and energy-intensive, and they are the most costly component of bioethanol production (Wooley *et al.*, 1999a, 1999b; Aden *et al.*, 2002; Brown, 2006; Galbe *et al.*, 2007; Yang & Wyman, 2008). After pre-treatment, simple sugar release is accomplished via enzymatic hydrolysis of β-glycosidic bonds in cellulose and hemicellulose. The release of these fermentable simple sugars is another highly costly phase of bioethanol production (Galbe *et al.*, 2007; Yang & Wyman, 2008). Energy and cost inputs into pre-treatments and hydrolysis can be greatly reduced with effective use of recombinant enzyme technology (Ragauskas *et al.*, 2006; Yang & Wyman, 2008). In particular, enzymes from lignocellulose-digesting insects and their symbionts, especially termites, are useful for pre-treatment and for downstream carbohydrate processing (Rubin, 2008; Scharf & Tartar, 2008).

Lignocellulose feedstocks

At present, bioethanol production relies mostly on food-grade sugars and starches obtained from agricultural crops, for example, sugar cane (sucrose) and maize (starch). However, if bioethanol is to compete in the marketplace with petroleum fuels, second-generation feedstocks will have to be developed. Second generation feedstocks are those not derived from grain or sugar-based agricultural commodities (Wyman, 2001; Simmons *et al.*, 2008). There are many second-generation feedstocks emerging from both forestry and agricultural sectors, all of which are viable candidates for consideration and development (Simmons *et al.*, 2008). Combined second-generation feedstock quantities potentially available for bioethanol processing are estimated at 1.3 billion tons per year in the US (0.3 forestry + 1.0 agricultural; Perlack *et al.*, 2005). Emerging forestry feedstocks include slash/loblolly pine,

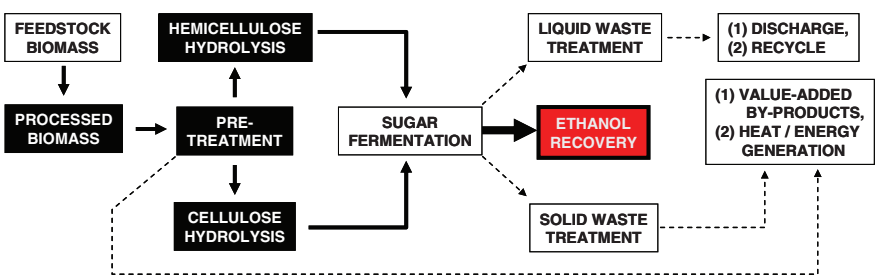

Fig. 1 Flow diagram showing the major processes involved in bioethanol production from second generation (non-food) feedstocks. Black boxes with white text indicate biorefinery processes that are the focus of the current article, and which account for ~40%–45% of total processing costs. Dashed lines indicate pathways for value-added by-products, energy generation, and waste recycling/disposal. Modeled after Wyman (2001), Ragauskas *et al.* (2006), Galbe *et al.* (2007) and Yang & Wyman (2008).

poplar/hybrid poplar, willow, silver maple, black locust, sycamore, sweetgum and eucalyptus (Nowak *et al.*, 2008; Simmons *et al.*, 2008). Agricultural feedstocks of emerging importance include corn stover (Aden, 2008), sugarcane bagasse, switchgrass, reed canary grass, miscanthus, sorghum, and other tropical grasses (Simmons *et al.*, 2008). Each of these feedstocks is economically and strategically viable in specific regions of the US (Nowak *et al.*, 2008; Simmons *et al.*, 2008). Because of the diverse lignocellulose compositions of these different feedstocks, pre-treatment strategies will likely need to be tailored to each of the individual feedstocks (Tartar *et al.*, 2009).

Lignocellulose processing

The major steps involved in industrial bioethanol production from second-generation feedstocks are outlined in Figure 1 (Wyman, 2001; Yang & Wyman, 2008). Biomass feedstock materials are first milled to reduce particle size for subsequent steps (Galbe *et al.*, 2007; Yang & Wyman, 2008). Essentially, this processing step is analogous to the mechanical degradation that would occur via the action of insect/termite mandibles and the gut proventriculus, in which particle sizes of $<50\ \mu$ are achieved (e.g., Itakura *et al.*, 1995). Next, in the process known as pre-treatment, the processed feedstocks are treated to disrupt lignin and to make hemicellulose/cellulose available for biologic degradation. Pre-treatment can take several forms, including biological, chemical, physical and thermal (Yang & Wyman, 2008 and references therein). After pre-treatment, hydrolysates usually contain hemicellulose and solids contain cellulose. Hydrolysates are neutralized and conditioned to remove any lignin metabolites, value-added by-products, and other materials deleterious to the fermentation process.

Next, hemicellulose and/or cellulose are depolymerized into their 5- and 6-carbon monomer sugars, respectively, by enzymes secreted from engineered fungi. In the case of the fungus *Trichoderma*, small amounts of cellulose solids or hemicellulose hydrolysates are pre-incubated with fungal isolates to induce production of cellulases (endoglucanases and β-glucosidases) and hemicellulases, which are then added back to bulk pre-treated materials to depolymerize cellulose into glucose. Engineered bacteria are used primarily for hemicellulose depolymerization. It is also noteworthy that recombinant/engineered bacteria are also being developed for hemicellulose and cellulose depolymerization purposes (Jarboe *et al.*, 2007). The simple sugars liberated from cellulose and hemicellulose are fermented by many types of organisms (e.g., yeast) to produce ethanol, which is then recovered by distillation. The remaining solid materials after distillation (lignin, enzymes, organisms, etc.) are used to feed boilers, produce electricity, and/or create value-added by-products. The resulting waste water is processed and either re-used or discharged. Remaining sludge after waste water treatment feeds the boiler, and then boiler ash is landfilled.

Economics of lignocellulose processing

The primary challenge for bioethanol competitiveness is to reduce the cost of biomass processing for converting raw feedstock materials into product. An estimate from 2001 is that bioethanol could compete with gasoline derived from raw petroleum costing $25/barrel (Wyman, 2001). More recent estimates of this price point are not available; however, recent cost estimates for bioethanol production range from 0.13 to 0.81 US$/L, depending on feedstock (Galbe *et al.*, 2007). For any feedstock, approximately 40% of these total processing costs are linked to the three phases of pre-treatment, enzyme production and enzymatic hydrolysis (Wooley *et al.*, 1999a, 1999b; Aden *et al.*, 2002; Yang & Wyman, 2008). These three phases account for approximately 20%, 10%, and 10%, respectively, of total costs.

Unfortunately, although cost calculation tools and models have been developed for some feedstocks (Wooley *et al*., 1999a; McAloon *et al.*, 2000; Aden, 2008), bioethanol costs are dependent on many regional or local factors, and thus are site-specific and geographically variable (Wyman, 2001; Galbe *et al.*, 2007). For these reasons, it is difficult to predict how specific advances resulting from novel insect or symbiont enzymes could specifically impact processing economics. In particular, of all the options proposed for enzymatic pre-treatments for lignin depolymerization (biological, chemical, physical, thermal), it has long been recognized that biological delignification would be the least energy-intensive and most economical (Kirk & Harkin, 1973; Fan *et al.*, 1982; Detroy *et al.*, 1980; Datta, 1981; Galbe & Zacchi, 2007; Yang & Wyman, 2008). Thus, without question, the development of novel enzymes clearly has the potential to reduce costs of pre-treatment, hydrolysis, fermentation and by-product enhancement/recovery.

Candidate pre-treatment enzymes from *R. flavipes*

Depending on the plant species from which it is derived, wood lignocellulose is composed of approximately 40% cellulose, 25% hemicellulose, 20% lignin, and

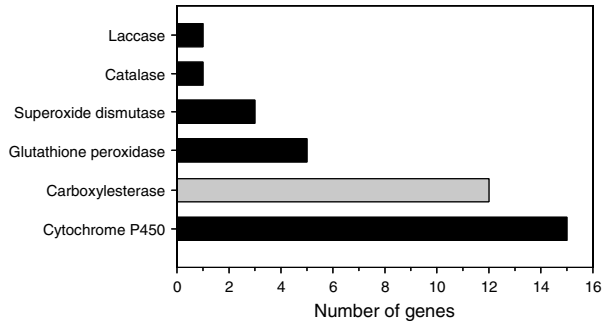

Fig. 2 Summary of genes encoding candidate biomass pre-treatment enzymes identified from a *Reticulitermes flavipes* gut tissue library (Scharf & Tartar, 2008; Tartar *et al.*, 2009). No homologous or functionally similar genes were identified from a hindgut symbiont library. Black and gray bars, respectively, represent candidate lignases and candidate phenolic acid esterases.

15% other miscellaneous components (Ragauskas *et al.*, 2006; Lange, 2007 and references therein). A recent survey of host and symbiont transcriptomes from the *R. flavipes* gut revealed over 175 genes encoding cellulases, hemicellulases, candidate lignases, and other potentially relevant digestive enzymes (Scharf & Tartar, 2008; Tartar *et al.*, 2009). The following section reviews research findings on two candidate groups of *R. flavipes* gut enzymes with potential relevance in industrial lignocellulose pre-treatment: (i) candidate lignases; and (ii) candidate phenolic acid esterases (Fig. 2).

Candidate lignases: laccases, catalases, peroxidases, cytochrome P450s and superoxide dismutases

The rationale for investigation of lignase activities in termites comes from several decades of research documenting lignin modification and/or degradation capabilities in termite guts (Esenther & Kirk, 1974; Butler & Buckerfield, 1979; Cookson, 1987, 1988; Breznak & Brune, 1994; Brune *et al.*, 1995a; Itakura *et al.*, 1995; Kuhnigk & König, 1997; Taprab *et al.*, 2005; Katsumata *et al.*, 2007; Geib *et al.*, 2008). Lignin breakdown also requires oxygen (Breznak & Brune, 1994), which is supported by evidence that termite guts are not completely anaerobic environments, especially the foregut and hindgut periphery (Brune *et al.*, 1995b). Although no termite genes or gene products have yet been proven to participate in lignin degradation, several candidate genes were recently identified from the *R. flavipes* host gut transcriptome (Fig. 2).

Of the candidate lignases, the most relevant are laccases, catalases and peroxidases, each of which is known to degrade lignin and related phenolic compounds in fungi (Erikkson *et al.*, 1990; Ohkuma, 2003; Baldrian, 2006; Anderson & Akin, 2008; Sutay-Kocabas *et al.*, 2008). Of these, the laccase enzyme isoform Rflac6 identified from *R. flavipes* is particularly interesting. Rflac6 possesses a secretory signal peptide, is highly expressed in salivary gland tissue, and readily oxidizes numerous lignin-like phenolic substrates, including the lignin monomer sinapinic acid (Tartar *et al.*, 2009, unpublished results). Unlike other insect laccases that act predominantly in cuticle melanization, Rflac6 is virtually inactive toward the melanin precursors L-DOPA and L-tyrosine (unpublished results).

Another potentially important set of genes identified from the *R. flavipes* gut transcriptome encode a number of cytochrome P450 mono-oxygenases (Tartar *et al.*, 2009). The P450s are a well-represented family of oxidative enzymes in all life-forms that are known for their roles in detoxification and xenobiotic metabolism (Feyereisen, 2005). With respect to lignin degradation, bacterial P450s have been shown to directly oxidize lignin-like phenolic compounds (e.g., Sutherland, 1986). Additionally, insect P450s are well known for catalyzing *O*-demethylations such as the lignin side-chain oxidations noted previously by Geib *et al.* (2008) in the guts of termites and xylophagous beetle larvae. Finally, because lignin digestion generates potentially cytotoxic free radicals (Breznak & Brune, 1994), antioxidant enzymes that reduce free radicals also may have uses in industrial lignocellulose pre-treatment. In this regard, previous sequencing work revealed one catalase and three superoxide dismutase (SOD)-coding genes from the *R. flavipes* host gut transcriptome (Tartar *et al.*, 2009). Under native conditions in the termite gut, antioxidant enzymes such as catalases and SODs may protect termite gut symbionts from toxic lignin degradation products. Thus, antioxidant enzymes may protect cellulolytic and fermentative microbes from oxidative damage caused by lignin degradation products.

Candidate phenolic acid esterases: carboxylesterases

A number of microbial and fungal esterases recently have been identified that enable hemicellulose solubilization (and subsequent depolymerization) by functioning as phenolic acid esterases (Crepin *et al.*, 2004; Benoit *et al.*, 2008). These enzymes, also known as "feruloyl" or "ferulic acid" esterases, hydrolyze carboxylester bonds between hemicellulose sugars and lignin monomers (de Vries *et al.*, 1997, 2002) and possibly ester linkages in the lignin polymer itself (Scharf & Tartar, 2008). In the case

of hemicellulose, this hydrolysis reaction constitutes a one-step hemicellulose solubilization reaction, which has tremendous relevance to industrial biomass pre-treatment. Previous gut transcriptome sequencing work revealed 12 gut carboxylesterase genes from *R. flavipes* (Fig. 2; Tartar *et al.*, 2009).

The rationale for investigating termite gut carboxylesterases as potential phenolic acid esterases is that: (i) the termite diet contains significant amounts of hemicellulose; (ii) there are at least 45 hemicellulase genes encoded in the *R. flavipes* gut meta-transcriptome; and (iii) there is strong esterase activity and substantial isoform diversity in the *R. flavipes* gut (Tartar *et al.*, 2009; Wheeler *et al.*, 2009). Four of the twelve identified esterase genes (named *RfEst1, 2, 3* and *4*) have been studied in detail (Wheeler *et al.*, 2009). The four genes encode two larger juvenile hormone-like esterases (*RfEst1* and *2*) and two shorter esterases with homology to insect carboxylesterases and the fungal phenolic acid esterases (*RfEst3* and *4*). Gene expression studies for the four esterases revealed significant expression of each gene in symbiont-free midgut, foregut, and salivary gland tissues, with reduced hindgut expression. These findings, in addition to the fact that the esterases were only identified from a symbiont-free gut library (not a separate symbiont library), and that considerable esterase activity exists in symbiont-free gut regions, strongly suggest that the *R. flavipes* gut esterases are not symbiont-derived. Thus, searches for culturable symbionts that possess phenolic acid esterase activity, or larger-scale symbiont metatranscriptome sequencing are not likely to be fruitful for phenolic acid esterase identification.

Research needs and opportunities

As highlighted throughout this article, the most critical research needs in the biomass pre-treatment area center on the development of low-cost enzymatic strategies for delignification and cellulose/hemicellulose solubilization. Because pre-treatment represents the most expensive component of bioethanol production (~20% of total costs), this is the area in which significant opportunities for innovation exist. Specifically, biologically-based strategies are needed to replace harsh chemical and energy-intensive heat treatments for delignification and hemicellulose solubilization.

One area of opportunity is the prospecting of upstream termite gut regions where biological pre-treatment naturally occurs (salivary gland, foregut and midgut; Fig. 3). It is well established that termite lignocellulose digestion generally results from collaboration between host- and symbiont-derived digestive enzymes (reviewed by Scharf & Tartar, 2008). For instance, the candidate host-derived pre-treatment factors studied to date (laccases and esterases) are produced in symbiont-free tissues, well upstream of the symbiont-rich hindgut paunch (Fig. 3) (Wheeler *et al.*, 2009; Tartar *et al*., 2009). Thus, symbiont-free tissues such as the salivary glands, foregut and midgut, likely hold the greatest potential for identification of candidate pre-treatment enzymes, model processes and strategies.

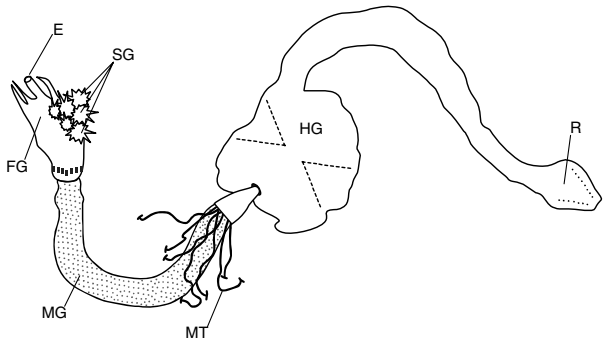

Fig. 3 Line drawing of the *Reticulitermes flavipes* gut. E, esophagus; FG, foregut; SG, salivary gland; MG, midgut; MT, Malpighian tubules; HG, hindgut paunch; R, rectum.

As noted previously, no single dominant bioethanol feedstock has yet emerged nationwide, but rather, many regionally important feedstocks have materialized and each is likely to remain at least regionally important (Perlack *et al.*, 2005; Aden, 2008; Nowak *et al.*, 2008; Simmons *et al.*, 2008). Furthermore, lignocellulose composition varies significantly between agricultural and forestry feedstocks, as well as within each of these two groups (www.wisbiorefine.org; Karp & Shield, 2008; Xu *et al.*, 2009). Thus, another area of substantial opportunity is the development of biological pre-treatment strategies that are specifically tailored to regionally important feedstocks. Selective feeding studies with live termites would provide an excellent model system for identifying feedstock-specific enzyme blends (e.g., Smith *et al.*, 2009). In such studies, termite colonies are fed exclusive diets of different feedstock materials for defined time periods before gene expression and/or relevant enzyme activities are measured. Gene expression can be investigated using semi-quantitative or quantitative real-time polymerase chain reaction (PCR) for smaller groups of genes (e.g., Wheeler *et al.*, 2007), whereas broader-scale gene expression can be investigated with whole genome or gut meta-transcriptome micro-arrays (e.g., Whitfield *et al.*, 2002) or next-generation sequencing (e.g., Warnecke *et al.*,

2007). Also, if desired, changes in symbiont species composition can be investigated using PCR-based 16S sequence surveys (e.g., Fisher *et al.*, 2007; Miyata *et al.*, 2007; Hu *et al.*, 2009). Finally, activities of responsive enzymes can be investigated using model substrates that target key digestive enzymes, for example, phenoloxidases, esterases, hemicellulases and cellulases (Smith *et al.*, 2009; Tartar *et al.*, 2009; Wheeler *et al.*, 2007, 2009).

Finally, for optimal release of fermentable sugars from specific lignocellulose feedstocks, we hypothesize that co-evolved pre-treatment and cellulase/hemicellulase enzymes from the same termite species will be more efficient than mixtures of enzymes from different organisms. For example, suites of enzymes from a single termite digestome (Scharf & Tartar, 2008) are likely to be more efficient than mixtures of termite pre-treatment enzymes and fungal cellulases and hemicellulases (Tartar *et al.*, 2009). Thus, selective feeding studies that consider global digestome expression changes and enzyme activity shifts in response to feedstock diets are likely to identify optimal enzyme cocktails that work with maximal efficiency to depolymerize lignocellulose feedstocks into fermentable simple sugars.

Conclusions

At ∼20% of total costs, feedstock pre-treatment is presently the most costly component of industrial bioethanol production. There are currently several regionally important bioethanol feedstocks, all in need of innovations that reduce pre-treatment costs and improve processing efficiency. This paper has provided an overview of industrial biorefinery operations, examples of candidate termite-derived pre-treatment enzymes, and a scientific, hypothesis-driven approach to develop novel biocatalyst + feedstock combinations for use in bioenergy production.

This is a unique time for bioenergy technology development. The selective feeding approach proposed here can create unprecedented opportunities to develop novel recombinant enzymes that collaborate to enable efficient digestion and fermentation of bioethanol feedstocks. Gaining such knowledge is important because it will enhance the development of sustainable, economically viable and environmentally friendly bioethanol feedstocks. Ultimately, developing optimal feedstock-specific biorefinery processes can lead to significantly reduced production costs, greater competitiveness for bioethanol in the marketplace, and most importantly, reduced dependence on petroleum-based energy.

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