Insect science

Insect Science (2010) 13, 303–312, DOI 10.1111/j.1744-7917.2010.01343.x

ORIGINAL ARTICLE

Mining diversity of the natural biorefinery housed within *Tipula abdominalis* larvae for use in an industrial biorefinery for production of lignocellulosic ethanol

Dana M. Cook and Joy Doran-Peterson

Microbiology Department, University of Georgia, Athens, Georgia, USA

Abstract Although they are the largest taxonomic group of animals, relatively few insects have been examined for symbiotic relationships with micro-organisms. However, this is rapidly changing because of the potential for examination of the natural insect–microbe–lignocellulose interactions to provide insights for biofuel technology. Micro-organisms associated with lignocellulose-consuming insects often facilitate the digestion of the recalcitrant plant diet; therefore these microbial communities may be mined for novel lignocellulose-degrading microbes, or for robust and inexpensive biocatalysts necessary for economically feasible biofuel production from lignocellulose. These insect–microbe interactions are influenced by the ecosystem and specific lignocellulose diet, and appreciating the whole ecosystem–insect–microbiota–lignocellulose as a natural biorefinery provides a rich and diverse framework from which to design novel industrial processes. One such natural biorefinery, the *Tipula abdominalis* larvae in riparian ecosystems, is reviewed herein with applications for biochemical processes and overcoming challenges involved in conversion of lignocellulosic biomass to fuel ethanol. From the dense and diverse *T. abdominalis* larval hindgut microbial community, a cellulolytic bacterial isolate, 27C64, demonstrated enzymatic activity toward many model plant polymers and also produced a bacterial antibiotic. 27C64 was co-cultured with yeast in fermentation of pine to ethanol, which allowed for a 20% reduction of commercial enzyme. In this study, a micro-organism from a lignocellulose-consuming insect was successfully applied for improvement of biomass-to-biofuel technology.

Key words ethanol, hydrolytic enzymes, insect-associated microorganisms, lignocellulose

Introduction

Insects are the largest taxonomic group of animals on earth. Although a few thorough studies have shown insects host an environment with high microbial diversity (Buchner, 1965; Tanada & Kaya, 1993; Breznak & Brune, 1994; Kane & Pierce, 1994; Moran, 2001; Dillon & Dillon, 2004; Brune, 2005), less than 1% of described insect species have been examined for micro-organisms (Kane & Mueller, 2002). Insects that degrade lignocellulose and host a gut microbial consortia have become of special interest recently for the application of microbial conversion of lignocellulose to biofuels, including ethanol, butanol and hydrogen. One of the major challenges in lignocellulose conversion is the need for novel, robust and inexpensive enzymes to deconstruct lignocellulose into fermentable sugars; the gut microbial community

Correspondence: Joy Doran-Peterson, Microbiology Department and Biofuels, Biopower, and Biomaterials Initiative, University of Georgia, 527 Biological Sciences Building, 1000 Cedar Street, Athens, GA 30602. Tel: 706 542 4115; fax: 706 542 2674; email: jpeterso@uga.edu

of lignocellulose-degrading insects may be mined for these enzymes, as well as novel micro-organisms themselves for the application of improved biofuel production technology.

As plants have evolved recalcitrant structures to resist predation, so have their consumers evolved mechanisms to overcome that resistance. For micro-organisms, these mechanisms include lignocellulolytic enzymes that deconstruct plant polymers to sugar moieties. Some herbivorous insects host a gut microbial community that facilitates digestion of a recalcitrant lignocellulosic diet. Often in nature lignocellulose degradation is a cooperative activity, which has been reported to be most effective with a mixed culture of cellulolytic and non-cellulolytic bacteria (Odom & Wall, 1983; Haruta *et al.*, 2002; Kato *et al.*, 2004). These studies indicate that non-cellulolytic aerobes enhance cellulose degradation, presumably by establishing and maintaining anaerobic conditions, neutralizing pH and consuming metabolites that might interfere with cellulose degradation.

Individual members of microbial communities are often most metabolically active only when in association with other members of the community. Also, the microbial activity, water chemistry and other biogeochemical processes in the ecosystem, external to the insect itself, can greatly influence functions and productivity of gut microbial communities (Röling, 2007). Therefore, the study of ecosystem–insect–microbiota–lignocellulose interactions should be viewed as a whole process, a natural biorefinery, for greater insight into the individual constituents of microbial species and enzymes.

Macro-invertebrate shredders in riparian streams

Shredders are a functional group of macro-invertebrates that consume lignocellulolosic detritus in small riparian stream ecosystems. In these low-order ecosystems, leaf litter comprises the majority of carbon and energy input (Vannote *et al.*, 1980). Thus, shredders are an important segment of the small stream ecosystem and usually comprise $\approx$ 20% of the total biomass (or 10% numerical abundance) of stream macro-invertebrates (Petersen *et al.*, 1989). Although leaf litter is the primary source of both carbon and energy input into small stream systems, many organisms are unable to degrade this lignocellulosic material, which has low nutritional value due to a high C : N ratio. Furthermore, proteins complexed with tannins, lignins and highly structured plant polysaccharide polymers (cellulose, hemicellulose and lignin) make digestion of leaf litter difficult (Martin *et al.*, 1980). By converting lignocellulose into a form that other organisms can use, shredders influence the bioavailability of carbon and energy within the ecosystem.

Shredders, and detritovores in general (in contrast to some other insects systems such as the termite, Martin & Martin, 1979) do not seem to produce themselves the necessary lignocellulolytic activity to digest the abundant plant polymers of their diets (Barlocher & Kendrick, 1974; Barlocher & Porter, 1986; Walters & Smock, 1991). Some shredders host gut microbial communities that are hypothesized to facilitate digestion of lignocellulose (Cook *et al.*, 2007; Klug & Kotarski, 1980; Sinsabaugh *et al.*, 1985). The necessity of microbial-mediated hydrolysis and fermentation for the digestion and assimilation of plant polymers has been demonstrated with ^{14}C-labelled cellulose in three different genera of shredders (*Pteronarcys proteus*, *Tipula abdominalis*, and *Pycnopsyche luculenta*) (Sinsabaugh *et al.*, 1985). Acetate from microbial fermentation was produced in the guts of shredders *T. abdominalis* and *Pycnopsyche guttifer* and was transported across the gut wall into the hemolymph (Lawson & Klug, 1989).

Tipula abdominalis *larva: A macro-invertebrate shredder hosting a gut microbiota*

Tipula abdominalis is an aquatic crane fly in riparian streams; the larvae are primary shredders of leaf litter detritus. The larvae progress through four larval instar stages. First instar larvae hatch from eggs late in summer and then progress relatively quickly (weeks) through second and third instar stages. They molt into the fourth instar stage in late autumn and persist longest (months) in this final instar (Byers, 1996). Fourth instar larvae consume conditioned leaf litter throughout autumn, winter and spring. The gut morphology of *T. abdominalis* larvae consists of two main compartments: midgut and hindgut. In contrast to the linear gut morphology of other insects (e.g., *Pteronarcys* spp., *Pycnopsyche* spp.), the anterior portion of the hindgut of *T. abdominalis* protrudes from the hindgut where material may be detained for extended digestion; this structure has been termed a "fermentation paunch" or "fermentation chamber" (Klug & Kotarski, 1980) (Fig. 1). The midgut is highly alkaline at pH 11, while the hindgut is neutral at pH 7 (Martin, 1987). Studies suggest that proteolysis occurs in the alkaline conditions in the midgut, dissociating protein complexes from plant polymers, which are then more accessible for saccharification and microbial fermentation in the pH-neutral hindgut (Sinsabaugh *et al.*, 1985; Lawson & Klug, 1989; Garca & Barlocher, 1998; Clark, 1999; Canhoto & Garca, 2006).

Scanning electron microscopy studies revealed that the *T. abdominalis* larval gut hosts a dense and diverse

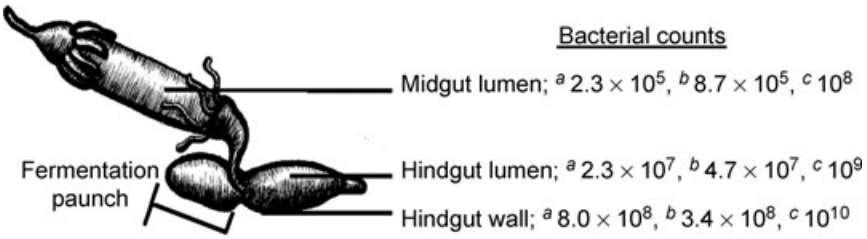

Fig. 1 Drawing of *T. abdominalis* gut tract with viable and direct bacterial cell counts. (*a*) aerobic and (*b*) anaerobic CFU per mg dry weight, (*c*) direct microscope counts per mg dry weight (Klug & Kotarski, 1980). Drawing modified from Rogers (2005).

microbial community (Klug & Kotarski, 1980; Clyde, 1996). The lumen contents of the midgut comprised a microbial diversity similar in morphology to that of ingested leaf detritus. No micro-organisms were associated with the wall (larval epithial tissues) of the midgut. In contrast, the lumen and wall of the hindgut hosted a microbial community of greater density and morphological diversity, which differed from that of the ingested leaf detritus. Aerobic and anaerobic cultivation of bacteria revealed that colony-forming units also increased from midgut lumen to hindgut lumen to hindgut wall. The density and diversity of the microbial community increased with each larval instar stage. Although a portion of the lumen contents was maintained throughout molting, no micro-organisms were associated with the hindgut wall immediately after molting.

Analysis of *T. abdominalis* hindgut bacteria using 16S rRNA gene libraries revealed a phylogenically diverse community (Cook *et al.*, 2007). From a total of 322 clones, 163 phylotypes (operational taxonomic units sharing ≥ 99% sequence similarity), were identified and evaluated for similarity to other ribosomal RNA (rRNA) genes from clones and isolated bacteria using database comparisons. Clones represented nine classes: *Actinobacteria*, *Bacteroidetes*, *Clostridia*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Cyanobacteria*, *Deferribacteres* and *Planctomycetacia*. A closer look at the clone library sequences revealed that the majority of clones had highest sequence similarity to *Clostridia* and *Bacteroidetes*, representing 65% and 19% of the total clones, respectively. *Clostridia* and *Bacteroidetes* clones were the only classes found in all four libraries. Using methods similar to those described (Cook *et al.*, 2007) *Clostridia* and *Bacteroidetes* clones were compared to one another, as well as previously described uncultured and cultured bacteria, at varying percent sequence similarity. Clones were more similar to one another than to previously described sequences, and more similar to uncultured than cultured bacteria (Fig. 2). At ≥ 97% sequence similarity (bacterial species level), 76% of *Clostridia* clones were similar to another clone, while only 4% were similar to previously described uncultured bacteria. No *Clostridia* clones were similar to cultured bacteria at ≥ 97% sequence similarity (Fig. 2A). At ≥ 97% sequence similarity, 92% of *Bacteroidetes* clones were similar to another clone, while no *Bacteroidetes* clones were similar to any previously described sequence (Fig. 2B). The few previously described sequences that were similar to clones in

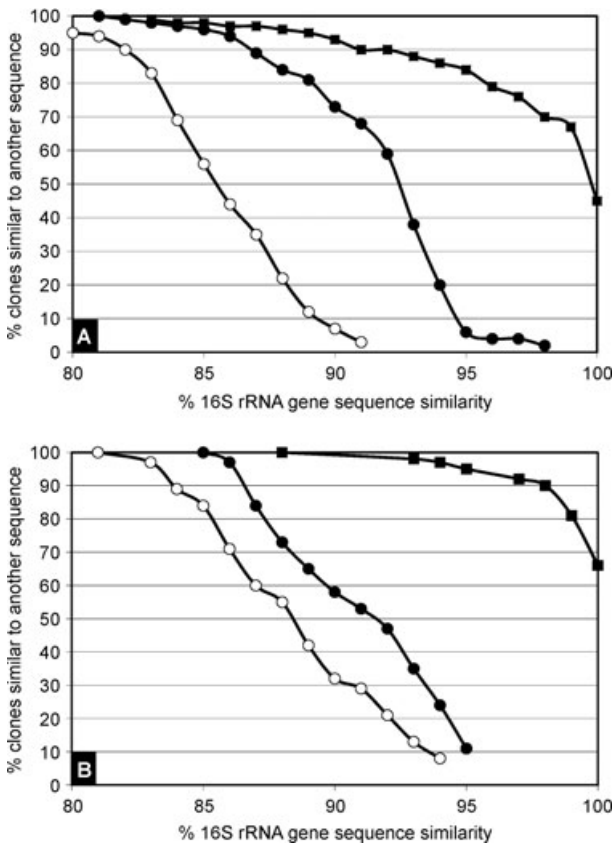

Fig. 2 Percentage of (A) *Clostridia* clones, and (B) *Bacteroidetes* clones similar to another clone from this study (squares), previously described uncultured (closed circles), or cultured (open circles) bacteria at *x*% sequence similarity. Data from Cook *et al.* (2007).

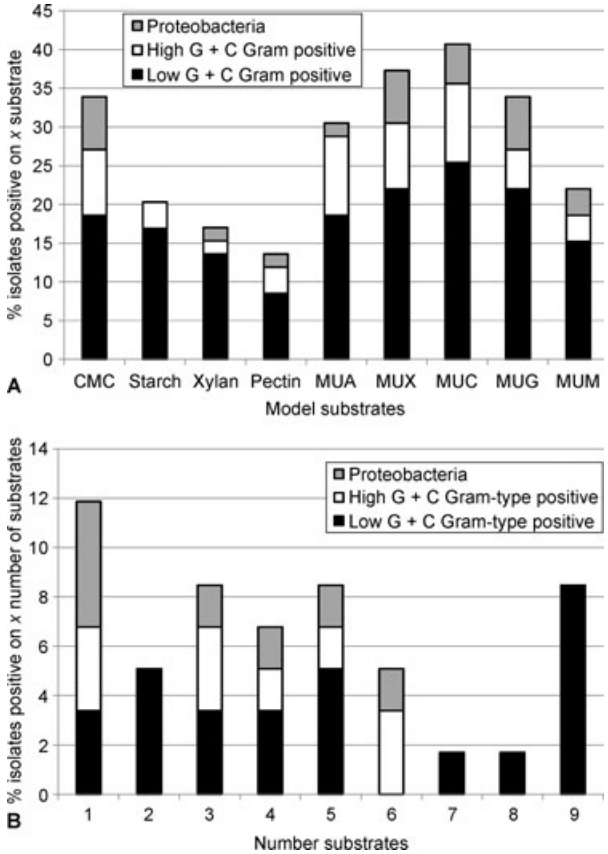

Fig. 3 (A) Summary of isolates' enzymatic activities on nine model plant polymer substrates: carboxymethylcellulose (CMC), starch, xylan, polygalacturonate (PGA), and methylumbelliferyl conjugated to cellobiopyranoside (MUC), arabinofuranoside (MUA), glucoside (MUG), mannopyranoside (MUM), and xyloside (MUX). (B) Summary of isolates positive on 1 to 9 model substrates. Data from Cook *et al.* (2007).

that study were sequences from other uncultured bacteria, most often cloned from other insect guts (data not shown). These analyses of the 16S rRNA genes from the *T. abdominalis* larval hindgut bacteria indicated that this microbial community was unique from previously described micro-organisms.

Further investigations of the *T. abdominalis* larval hindgut microbiota included isolation and characterization of bacteria (Cook *et al.*, 2007). Using simple aerobic cultivation techniques, 59 isolates representing four classes of bacteria were obtained. Those isolates were screened for enzymatic activity on model plant polymer substrates. Many of the isolates were able to hydrolyze several of the substrates (Fig. 3A). A group of five isolates with identical 16S rRNA gene sequences was able to degrade all model substrates (Fig. 3B). A representative was chosen, isolate 27C64, and putatively identified by 16S rRNA gene sequence as *Paenibacillus amolyticus.* This isolate was able to degrade all substrates tested.

Bacterial isolate 27C64

Bacterium 27C64 was isolated from the *T. abdominalis* larva hindgut and characterized as described (Cook *et al.*, 2007). Briefly, hindgut homogenates were serially diluted and plated onto tryptic soy agar and incubated for up to 3 weeks at 22°C. Colonies were subcultured until pure cultures were obtained. Sequencing of the 16S rRNA genes from the cultured isolates was performed at MIDI Labs (Newark, Delaware, USA). Isolate 27C64 had enzymatic activity (hydrolysis of substrate) on model substrates carboxymethylcellulose (CMC) (Wood & Kellogg, 1988); starch (Difco 272100); xylan (Mondou *et al.*, 1986); polygalacturonate (PGA) (Starr *et al.*, 1977); and methylumbelliferyl conjugated to cellobiopyranoside (MUC), arabinofuranoside (MUA), glucoside (MUG), mannopyranoside (MUM), and xyloside (MUX) (Sharrock, 1988). Further study revealed that 27C64 also produced the polymyxin antibiotics E1 and E2 (Henriksen *et al.*, 2007). For further characterization of 27C64, carbohydrate utilization was assayed and enzymatic activity was quantified. Because of its carbohydrate preferences, its possession of many plant polymer-degrading enzymes, and its production of bacterial antibiotics, 27C64 was investigated in biomass fermentations to fuel ethanol.

Biomass fermentations to fuel ethanol

Biochemical conversion processes such as using enzymes to deconstruct plant cell walls followed by fermentation of the carbohydrates to generate a useful product such as ethanol, offer great potential for expanding our renewable chemicals and fuels capacity. Current commercial ethanol production from starch involves two enzymes, α-amylase and glucoamylase that easily depolymerize starch into glucose, which is then fermented to ethanol by *Saccharomyces* yeasts. Plant cell wall deconstruction from lignocellulosic biomass is more challenging due to the recalcitrance and complexity of the biomass itself. Depending upon the biomass some type of physical and/or chemical pretreatment is necessary to open the fiber structures to allow enzyme access to the plant carbohydrate polymers (Wright, 1989; Gray *et al.*, 2006; Farrell *et al.*, 2006). To illustrate the use of insect-associated micro-organisms in biofuel production, we will discuss fermentation of pretreated pine biomass using a

Saccharomyces yeast strain together in co-culture with a bacterium (27C64) isolated from the *Tipula abdominalis* hindgut microbial community (Patents-Pending).

The use of enzymes to saccharify lignocellulosic biomass, such as pine, is typically performed after other physical and/or chemical methods of pretreatment and can be accomplished prior to or in conjunction with fermentation. Pretreatment breaks down biomass to allow access to the enzymes, which can then hydrolyze the remaining cellulose, hemicellulose and pectin polymers (Galbe & Zacchi, 2007; Himmel *et al.*, 2007). Most enzymatic saccharifications are performed with commercially available cell-free extracts of fungal cultures, or in some cases, bacterial cultures, designed to provide hydrolysis of the lignocellulose. If the fermentation biocatalyst and the enzyme mixtures function optimally at the same pH and temperature range, then simultaneous saccharification and fermentation (SSF) is the desired method. SSF is advantageous because the fermenting organism, in this example the yeast, and the enzymes, most often commercial mixtures from fungi, are added at the same time and as the enzymes liberate sugars, the yeast converts the sugars to ethanol, alleviating any end-product inhibition of the enzymes due to sugar accumulation (Gauss *et al.*, 1976; Takagi *et al.*, 1977; Doran-Peterson *et al.*, 2009 and references therein).

Degradation of cellulose is achieved through the action of three types of enzymes: endo-glucanases, cellobiohydrolases (or exo-glucanases) and β-glucosidases (Chang, 2007; Turner *et al.*, 2007). Endo-glucanases and cellobiohydrolases cleave within or at the end of the glucan chain, respectively (the latter releasing units of cellobiose), and are classified based on both their structural fold and catalytic mechanism (Henrissat & Davies, 1997). β-glucosidases cleave cellobiose to monomeric glucose and are essential for overall cellulose degradation to glucose because accumulated cellobiose and/or glucose inhibit the activity of glucanases (Bayer *et al.*, 1998). Additional enzyme activities useful for pretreated pine biomass include, but are not limited to, mannanases and xylanases (Turner *et al.*, 2007).

To improve ethanol yields from pine biomass, 27C64 was co-cultured with yeast. It was hypothesized that enzymes produced by 27C64 would help to saccharify the substrate and allow for a reduction in the fungal enzyme loading required to make sugars available for the yeast conversion to ethanol. Also, antibiotic production from 27C64 would reduce bacterial contamination of the fermentation without impacting the performance of the yeast.

Materials and methods

27C64 carbohydrate utilization and enzyme activity

27C64 was grown on basal minimal media with 1% (w/v) carbon sources as listed in the Table 1. The defined basal medium was based on modified Davis minimal media containing the following ingredients per liter: 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate and 0.1 g $MgSO_4$-$7H_2O$. The original recipe includes glucose. Glucose was not added, and the sugars listed in Table 1 were individually added to result in 11 separate media. After 48 h, supernatant was collected via centrifugation and assayed for xylanase, polygalacturonase and CMCase activity by measuring degradation of oat spelt xylan, polygalacturonic acid or carboxymethylcellulose, a modified cellulose with methylated hydroxyl groups

Table 1 Enzyme activity units (IU) per mL of culture supernatant using various substrates.

Substrate	Xylanase	Polygalacturonase	Carboxymethylcellulase
Glucose	ND[†]	ND	0.12
Mannose	0.19	0.25	0.46
Xylose	0.13	0.23	0.09
Arabinose	0.13	0.23	0.15
Cellulose	0.33	0.18	ND
Pectin	0.14	ND	0.17
Starch	ND	0.19	ND
Xylan	0.38	0.06	0.12
Carboxymethylcellulose	0.17	0.21	0.08
Pine acid hydrolysate[‡]	0.13	ND	0.19

[†]ND = none detected.

[‡]hydrolysate = the liquid fraction of the two step-sulfur dioxide, steam explosion pretreatment.

for increased solubility of the substrate compared to native cellulose. Degradation of carboxymethylcellulose is suggestive of endoglucanase activity, but it is not specific for endoglucanase activity only, therefore the term "CMCase" activity is often used (Sharrock, 1988). These model biomass substrates were added at 1% w/v concentrations in 50 mmol/L sodium acetate buffer, pH 4.8 and assayed for xylanase, polygalacturonase and CMCase activity, respectively, using published methods (Berlin *et al.*, 2005; Ximenes *et al.*, 2007). Filter paper activity (FPAase) was assayed as described by Mandels *et al.* (1976). Release of reducing sugars was determined according to Miller (1959). Briefly, reactions were incubated at 50°C for 15 min and stopped by adding 1.0 mL DNS reagent (g/L: 10 dinitrosalicylic acid, 16 sodium hydroxide pellets, 300 potassium sodium tartarate), then incubated in a boiling water bath for 5 min. Absorbance was measured at 540 nm. Standard curves were constructed with known glucose (CMCase and amylase), galacturonic acid (pectinase) and xylose (xylanase) concentrations. Reducing sugars were extrapolated from standard curves to determine the amount of reducing sugars liberated from the different biomass model substrates. One unit of cellulase (for CMC as substrate), xylanase and polygalacturonase activity was defined as the release of one μmol of glucose, xylose or galacturonic acid, repectively, per min.

Pine fermentation with co-culture yeast and 27C64

Pretreated G3S2 pine was produced as follows. Loblolly pine from Georgia, USA, was chipped to a particle size of 10 mm or less. Chips were then pretreated with gaseous sulfur dioxide in two steps. A batch of a known amount of chips was treated with 2.5% SO_2 w/w of moisture content in chips, at a temperature of 190°C for 5 min. Following this pretreatment step, the material was pressed using a hydraulic press to collect liquid. This liquid was not used in the experiments described herein. The pretreated solids (material remaining after the liquid was pressed out and removed), was then washed with water and pressed to a dry matter content of 40%. A second impregnation with 2.5% SO_2 w/w of moisture content in the solids followed, and the materials were allowed to react at a temperature of 210°C for 5 min. The sample obtained using these two steps of pretreatment were used in co-culture fermentations. Moisture content of the pretreated pine was 71.53%.

Fermentation with yeast with and without addition of 27C64 co-culture: four bioreactors each containing 20 g dry weight (DW; 10% solids) of pretreated pine were autoclaved at 121°C. Enzymes were added on a unit per gram dry weight of pretreated pine basis. Novozyme cellulase cocktail (12 FPU/g or 15 FPU/g as indicated) and Cellobiase (60 U/g) (Novozymes, Inc. Franklinton, NC, US) were used. Active dried yeast (ADY, North American Bioproducts Corporation, Duluth, GA, US) was inoculated at a concentration of 2 g/L in each vessel. Either sterile water or resuspended bacterial pellets of 27C64 were added to the bioreactor to determine the effect of co-culture on ethanol yield. Five hundred milliliters of overnight-grown culture of 27C64 was centrifuged, the pellet resuspended in a small volume of 2 × tryptic soy broth and 5×10^7 cells (roughly 0.2 g DW of bacteria) were added to the bioreactor. The total volume of each fermentation was 200 mL. Fermentation reaction was incubated at 37°C for 48 h with constant stirring.

To quantify ethanol production, gas chromatography (GC) was performed (Shimadzu GC-8A; Shimadzu Corp., Kyoto, Japan) with column DB-624 (Agilent Technologies, Inc., Santa Clara, CA, US) as described previously (Doran-Peterson *et al.*, 2009). The grams of ethanol produced per FPU of cellulase calculated were calculated as follows: g ethanol/total FPU; g ethanol = ethanol (g/L) × 0.2 L (reaction volume); total FPU = g solids (pine substrate) × FPU/g (12 or 15).

Results and discussion

27C64 carbohydrate utilization and enzyme activity

Cellulase enzyme activity measured as filter paper units of activity (FPU) using xylose, mannose, or both sugars together as growth substrates in minimal media, resulted in 0.26, 0.20 and 0.24 FPU/mL of culture supernatant, respectively. Strain 27C64 produces xylanase, pectinase and cellulase when grown in the minimal basal media in the presence of various carbon sources (Table 1).

Pine fermentation with co-culture yeast and 27C64

Ethanol production from pretreated pine at 10% w/v solids with and without co-inoculation with the bacterium 27C64 is presented in Table 2. The theoretical maximum for ethanol production from pretreated pine under these conditions was 31.8 g ethanol per liter of fermentation broth. All bioreactor-run data are the average of duplicates due to limited pretreated pine availability. Yeast cells were evaluated for their ability to produce ethanol without the addition of any enzymes (neither fungal nor from 27C64) as a baseline. Yeast cells plus an inoculum of 27C64 cells only (without the additional fungal enzymes and without the 27C64 culture supernatant) were cultivated together to determine whether 27C64 could grow in the pine substrate without decreasing the ethanol produced by the fermenting yeasts. Where fungal enzymes were added,

Table 2 Ethanol production in g/L after 24 and 48 h of fermentation using commercially available fungal enzymes and either yeast cells alone or in combination with 27C64 cells.

	Fungal cellulase FPU/g†	Yeast cells only			Yeast and 27C64	
		0	12	15	0	12
g/L ethanol	Time (h) 0	0.6	0.55	0.7	0.7	0.7
	24	0.9	17.8	22.9	3.0	23.5
	48	1.3	25.4	26.6	6.2	29.8
g ethanol/FPU fungal cellulase		NA	0.021	0.018	NA	0.025

Each value represents the average of duplicates for G3S2 pretreated pine fermentations. †FPU = filter paper units of activity per gram dry weight of pretreated pine.

fermentations contained 60 U fungal cellobiase per gram dry weight (gdw) of pretreated pine solids. Four bioreactors contained 12 FPU cellulase/gdw pretreated pine in addition to the cellobiase and two of the bioreactors contained 15 FPU cellulase/gdw of pretreated pine in addition to the cellobiase. Two of the 12 FPU/gdw pretreated pine bioreactors were inoculated with 27C64 cells at the same time as yeast and commercial enzyme addition. Two additional bioreactors were inoculated with yeast and 27C64 cells at the same time without any additional fungal commercial enzymes.

A small but significant amount of ethanol was produced during the 48 h of fermentation with the 27C64 inoculum compared to the yeast inoculum alone. This suggests that 27C64 was able to produce enzymes able, at least to some extent, to degrade the pretreated pine substrate. In addition, approximately 17% more ethanol was produced from the fermentation where 27C64 was added together with the yeast during fermentations using 12 FPU cellulose/gdw pretreated pine. In contrast, increasing the fungal enzyme loading from 12 FPU to 15 FPU/gdw pretreated pine increased the ethanol concentration maximum by only 5% (1.2 g/L). The 17% increase in ethanol production in the fermentations with added 27C64 may not be a major increase; however, it does show that adding 27C64 cells at the start of the fermentation does not negatively impact ethanol production from the fermenting yeast. This is a bit surprising because 27C64 is capable of metabolizing some of the same sugars as the yeast. However, the 27C64 strain can use sugars that the yeast is unable to metabolize, such as pentoses. Ongoing studies with 27C64 (data not shown) suggest that this organism can use acetate and other potentially inhibitory compounds as a carbon source, perhaps helping to detoxify the yeast's environment; however, more studies are needed to confirm this hypothesis.

Although these conditions have not been optimized for the mixture of 27C64 bacteria, fungal enzymes and fermenting yeasts, the authors believe the results support the proof in principle of using insect-associated micro-organisms for enhancing biofuel production.

Tipula abdominalis *larva: a natural biorefinery*

The model of the *T. abdominalis* larva as a natural biorefinery can be applied toward developments in technology for industrial biomass refinery processes (Fig. 4). In this model, the substrate (conditioned leaf litter) is ingested by larvae. Maceration of the substrate during ingestion decreases particle size and increases surface area-to-volume ratios. Upon entering the alkaline midgut, proteolysis degrades complexed proteins making polysaccharide polymers more accessible for further processing. In the neutral pH hindgut, bacterial enzymes saccharify cellulose and hemicellulose. These sugars are then consumed by bacteria and converted to acetate and other fermentation products, which can be transported across the gut to the hemolymph to support larval energy and growth requirements (Lawson & Klug, 1989). In the fermentation paunch, material may be retained for extended processing (Klug & Kotarski, 1980). Lastly, waste and by-products are excreted and are valuable to other organisms in the ecosystem.

The simplicity of this model should not overshadow the true complexity of this system. In this natural biorefinery, numerous bacterial species are interacting in complex relationships to degrade and ferment a heterogeneous substrate in a simultaneous saccharification and fermentation (SSF) approach. In another possible approach employed by industrial biomass refineries, a separate hydrolysis followed by fermentation (SHF) approach, in which biomass is converted in discrete and separate steps: enzymatic saccharification followed by fermentation. In a third process strategy, partial saccharification and co-fermentation (PSCF), enzyme saccharification is begun

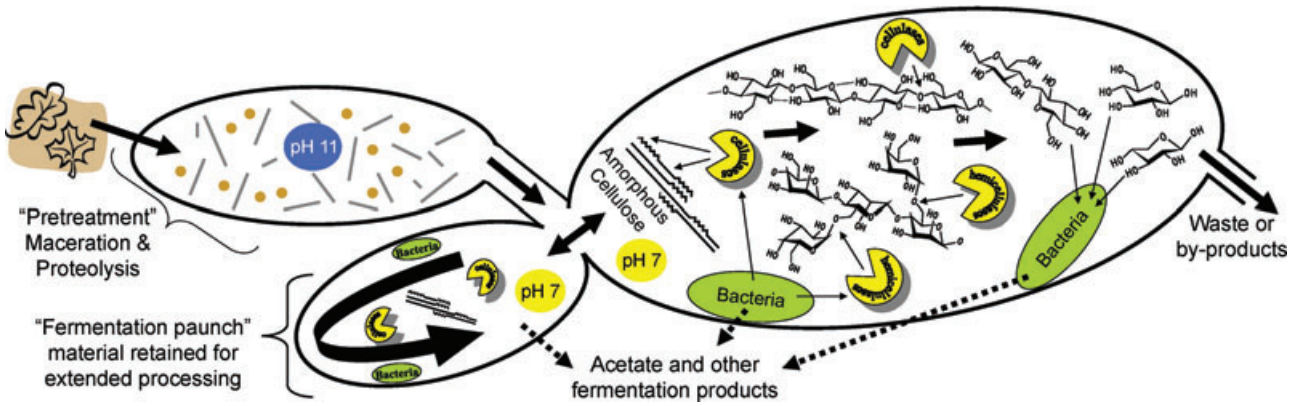

Fig. 4 Model of the *T. abdominalis* larva as a natural biorefinery.

prior to fermentation, then allowed to proceed (though at decreased efficiency) during the fermentation process. In contrast to the natural biorefinery of the *T. abdominalis* hindgut, industrial biomass refineries typically require fungal enzymes for saccharification and a single microbial species in monoculture is employed for fermentation of a relatively homogeneous substrate. This can present problems for SSF processes, as fungal enzymes and fermenting micro-organisms often have different optimal conditions. Study of the natural biorefinery can provide insights to microbe interactions during lignocellulose conversion, including cooperative cellulose degradation and how to deal with inhibitors produced during lignocellulose deconstruction. Like the natural biorefinery, industrial biomass refineries should seek value in processing by-products that may otherwise be considered waste. This natural biorefinery is also a reservoir for potentially novel enzymes that can enhance biomass degradation processes. Another application is to genetically engineer the genes for these enzymes into designer fermenting organisms capable of selected pre-treatment of the biomass to be converted. Novel enzymes, and possibly even novel metabolic pathways, can be genetically engineered into fermenting micro-organisms to increase their value as industrial biocatalysts.

Acknowledgments

This work was funded in part by the University of Georgia Microbiology Department, C2 Biofuels (Atlanta, GA), and an award from the Technology Commercialization Office of the University of Georgia Research Foundation, Inc. The authors wish to thank anonymous reviewers for their contributions toward improving the manuscript.

References

Barlocher, F. and Kendrick, B. (1974) Assimilation efficiency of *Gammarus pseudolimnaeus* (Amphipoda) feeding on fungal mycelium or autumn-shed leaves. *Oikos*, 26, 55–59.

Barlocher, F. and Porter, C.W. (1986) Digestive enzymes and feeding strategies of three stream invertebrates. *Journal of the North American Benthological Society*, 5, 58–66.

Bayer, E.A., Chanzy, H., Lamed, R. and Shoham, Y. (1998) Cellulose, cellulases, and cellulosomes. *Current Opinion in Structural Biology*, 8, 548–557.

Berlin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M., Kilburn, D. and Saddler, J.N. (2005) Weak lignin-binding enzymes: a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. *Applied Biochemistry and Biotechnology*, 121, 163–170.

Breznak, J.A. and Brune, A. (1994) Role of microorganisms in the digestion of lignocellulose. *Annual Review of Entomology*, 39, 453–487.

Brune, A. (2005) Symbiotic associations between termites and prokaryotes. *The Prokaryotes: an Online Electronic Resource for the Microbiological Community* (eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandts), 3rd edn. Springer-SBM, New York.

Buchner, P. (1965) *Endosymbiosis of Animals with Plant Microorganisms*. Interscience, New York. 909 pp.

Byers, G.W. (1996) Tipulidae. *An Introduction to the Aquatic Insects of North America*, 3rd edn (eds. R.W. Merritt & K.W. Cumminss), pp. 549–570. Kendall/Hunt Publishing Company, Dubuque, Iowa.

Canhoto, C. and Garca, M.A.S. (2006) Digestive tract and leaf processing capacity of the stream invertebrate *Tipula lateralis*. *Canadian Journal of Zoology*, 84, 1087–1095.

Chang, M.C. (2007) Harnessing energy from plant biomass. *Current Opinion in Structural Biology*, 11, 677–684.

Clark, T.M. (1999) Evolution and adaptive significance of larval midgut alkalinization in the insect superorder Mecopterida. *Journal of Chemical Ecology*, 25, 1945–1960.

Clyde, E.P. (1996) Analysis of hindgut microbial communities in *Tipula abdominalis* (Diptera; Tipulidae) larvae collected from streams in maple and coniferous forests. Master Thesis, 123 pp. Central Michigan University, MI.

Cook, D.M., Henriksen, E.D., Upchurch, R. and Peterson, J.B.D. (2007) Isolation of polymer-degrading bacteria and characterization of the hindgut bacterial community from the detritus-feeding larvae of *Tipula abdominalis* (Diptera: Tipulidae). *Applied Environmental Microbiology*, 73, 5683–5686.

Dillon, R.J. and Dillon, V.M. (2004) The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology*, 49, 71–92.

Doran-Peterson, J., Jangid, A., Brandon, S.K., Henriksen, E.D., Dien, B. and Ingram, L.O. (2009) Simultaneous saccharification and fermentation and partial saccharification and cofermentation of lignocellulosic biomass for ethanol production. *Biofuels: Methods and Protocols, Methods in Molecular Biology* (ed. J.R. Mielenz), vol. 581, pp. 263–280. Humana Press, New York, NY.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M. and Kammen, D.M. (2006) Ethanol can contribute to energy and environmental goals. *Science*, 311, 506–508.

Galbe, M. and Zacchi, G. (2007) Pretreatment of lignocellulosic materials for efficient bioethanol production. *Advances in Biochemical Engineering/Biotechnology*, 108, 41–65.

Garca, M.A.S. and Barlocher, F. (1998) Proteolytic gut enzymes in *Tipula caloptera* – interaction with phenolics. *Aquatic Insects*, 21, 11–18.

Gauss, W.F., Suzuki, S. and Takagi, M. (1976) Manufacture of alcohol from cellulosic materials using plural ferments. United States Patent, 3990944.

Gray, K.A., Zhao, L. and Emptage, M. (2006) Bioethanol. *Current Opinion in Structural Biology*, 10, 141–146.

Haruta, S., Cui, Z., Huang, Z., Li, M., Ishii, M. and Igarashi, Y. (2002) Construction of a stable microbial community with high cellulose-degradation ability. *Applied Microbiology and Biotechnology*, 59, 529–534.

Henriksen, E.D., Phillips, D.R. and Doran-Peterson, J.B. (2007) Polymyxin E production by *P. amylolyticus*. *Letters in Applied Microbiology*, 45, 491–496.

Henrissat, B. and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases. *Current Opinion in Structural Biology*, 7, 637–644.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W. and Foust, T.D. (2007) Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science*, 315, 804–807.

Kane, M.D. and Mueller, U.G. (2002) Insights from insect-microbe symbioses. *Biodiversity of Microbial Life* (eds. J.T. Staley & A.-L. Reysenbachs), pp. 289–313. Wiley-Liss, Inc., New York.

Kane, M.D. and Pierce, N.E. (1994) Diversity within diversity: Molecular approaches to studying microbial interactions with insects. *Molecular Ecology and Evolution: Approaches and Applications* (eds. B. Schiewater, B. Streit, G.P. Wagner & R. DeSalles), pp. 509–524. Birkhauser Verlag, Basel, Switzerland.

Kato, S., Haruta, S., Jun Cui, Z., Ishii, M. and Igarashi, Y. (2004) Effective cellulose degradation by a mixed-culture system composed of a cellulolytic *Clostridium* and aerobic non-cellulolytic bacteria. *FEMS Microbiology Ecology*, 51, 133–142.

Klug, M.J. and Kotarski, S. (1980) Bacteria associated with the gut tract of larval stages of the aquatic cranefly *Tipula abdominalis* (Diptera: Tipulidae). *Applied and Environmental Microbiology*, 40, 408–416.

Lawson, D.L. and Klug, M.J. (1989) Microbial fermentation in the hindgut of two stream detritivores. *Journal of the North American Benthological Society*, 8, 85–91.

Mandels, M., Andreotti, R. and Reese, E.T. (1976) Measurement of saccharifying cellulose. *Biotechnology Bioengineering*, 6, 17–34.

Martin, M.M. and Martin, J.S. (1979) The distribution and origins of cellulolytic enzymes of the higher termite, *Macrotermes natalensis*. *Physiological Zoology*, 52, 11–21.

Martin, M.M. (1987) *Invertebrate-Microbial Interactions: Ingested Fungal Enzymes in Arthropod Biology*. Cornell University Press, Ithaca, NY. 176 pp.

Martin, M.M., Martin, J.S., Kukor, J.J. and Merritt, R.W. (1980) The digestion of protein and carbohydrate by the stream detritivore, *Tipula abdominalis* (Diptera, Tipulidae). *Oecologia*, 46, 360–364.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426–428.

Mondou, F., Shareck, F., Morosoli, R. and Kluepfel, D. (1986) Cloning of the xylanase gene of *Streptomyces lividans*. *Gene*, 49, 323–330.

Moran, N.A. (2001) The coevolution of bacterial endosymbionts and phloem-feeding insects. *Annals of the Missouri Botanical Garden*, 88, 35–44.

Odom, J.M. and Wall, J.D. (1983) Photoreduction of H_2 from cellulose by an anaerobic coculture. *Applied and Environmental Microbiology*, 45, 1300–1395.

Petersen, R.C., Cummins, K.W. and Ward, G.M. (1989) Microbial and animal processing of detritus in a woodland stream. *Ecological Monographs*, 59, 21–39.

Rogers, T.E. (2005) Cellulase and hemicellulase activity within the *Tipula abdominalis* larval gut. *Microbiology*. University of Georgia, Athens, GA, p. 84.

Röling, W.F. (2007) Do microbial numbers count? Quantifying the regulation of biogeochemical fluxes by population size and cellular activity. *FEMS Microbiology Ecology*, 62, 202–210.

Sharrock, K.R. (1988) Cellulase assay methods: a review. *Journal of Biochemical and Biophysical Methods*, 17, 81–106.

Sinsabaugh, R.L., Linkins, A.E. and Benfield, E.F. (1985) Cellulose digestion and assimilation by three leaf-shredding aquatic insects. *Ecology*, 66, 1464–1471.

Starr, M.P., Chatterjee, A.K., Starr, P.B. and Buchanan, G.E. (1977) Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* species in relation to clinical laboratory procedures. *Journal of Clinical Microbiology*, 6, 379–386.

Takagi, M., Abe, S., Suzuki, S., Emert, G.H. and Yata, N. (1977) A method for production of alcohol directly from cellulose using cellulase and yeast. *Proceedings of Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Protein* (ed. T.K. Ghose), pp. 551–571. I.I.T., New Delhi.

Tanada, Y. and Kaya, H.K. (1993) *Insect Pathology*, Academic Press, London. 666 pp.

Turner, P., Mamo, G. and Karlsson, E.N. (2007) Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbial Cell Factories*, 6, 9–32.

Vannote, R., Minshall, G., Cummins, K.W., Sedell, J. and Cushing, C. (1980) The river continuum concept. *Canadian Journal of Fisheries and Aquatic Sciences*, 37, 130–137.

Walters, K.H. and Smock, L.A. (1991) Cellulase activity of leaf litter and stream-dwelling, shredder macroinvertebrates. *Hydrobiologia*, 220, 29–35.

Wood, W.A. and Kellogg, S.T. (1988) *Methods in Enzymology, Vol. 160: Biomass Part A, Cellulose and Hemicellulose*. Academic Press, San Diego, CA. 774 pp.

Wright, J.D. (1989) Evaluation of enzymatic hydrolysis processes. *Energy from Biomass and Wastes XII* (ed. D.L. Klass), pp. 1247–1276. Institute of Gas Technology, Chicago.

Ximenes, E.A., Dien, B.S., Ladish, M.R., Mosier, N., Cotta, M.A. and Li, X.-L. (2007) Enzyme production by industrially relevant fungi cultured on coproduct from corn dry grind ethanol plants. *Applied Biochemistry and Biotechnology*, 137, 171–183.

Accepted April 12, 2010