

Review

Bioprospection of Enzymes and Microorganisms in Insects to Improve Second-Generation Ethanol Production

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Abstract

The search for increased efficiency and lower costs for second-generation (2G) bioethanol production is generated by concerns regarding food safety and crop expansion, because the first generation of this fuel competes with production of food for consumption. There is also interest in decreasing associated greenhouse gas emissions and dependence on fossil fuels. However, processing 2G ethanol is expensive, inhibiting scale up of production. To produce this second-generation fuel, lignocellulosic materials need to be pretreated and hydrolyzed such that the fermentable sugars can be released from their polysaccharides. This biomass requires efficient enzymes to cleave cellulose and hemicellulose fractions (and sometimes pectin as well) into oligosaccharides (e.g. cellobiose) and monosaccharides (pentoses and hexoses) that depend on yeasts to convert them into ethanol via alcoholic fermentation. Fortunately, herbivorous insects are a potential source of enzymes and microorganisms to perform these functions, because they consume the carbohydrates from the lignocellulosic biomass. This review addresses various strategies for the bioprospection of enzymes (either endogenous or from insect microbiomes) and/or insect isolated yeasts; the efficiency of xylose and cellobiose metabolism of those microorganisms; successful experiments with such biocatalysts; and in particular, the economic impact of bioprospected enzymes and microorganisms exploited for 2G ethanol production.

Keywords: insect guts, glycoside hydrolase, 2G ethanol, metagenome, fermentation

Introduction

Faced with concerns regarding depletion of fossil fuels and the worsening of environmental problems attributable to excessive use of petroleum and its derivatives, worldwide attention has turned to the production of biofuels.¹ Bioethanol has garnered particular attention in the fuel market as a substitute for gasoline. The introduction of flex-fuel-powered vehicles into the market, as well as requirements on the part of some governments with respect to addition of ethanol to levels of up to 25%, has encouraged the increased production of bioethanol worldwide.² World production of bioethanol increased from approximately 22 to 122 billion L between 2000 and 2018.³ The US and Brazil alone contributed 85% of global bioethanol production in 2018.⁴

In Brazil, production is based primarily on the fermentation of sucrose from sugarcane. In the US, it is based on the fermentation of starch, derived mainly from corn.⁵ Nevertheless, in addition to sugar- and starch-based sources, other materials may be used to produce bioethanol, including lignocellulosic biomass, which is rich in polysaccharides that may be hydrolyzed into fermentable sugars and consequently converted to ethanol—so-called second-generation (2G) ethanol.⁶

This raw material is very diverse, and several types of agroindustrial waste, including bagasse, straw, stalks, leaves, grains, husks and even fruit residues, have the potential to be used as raw materials in 2G ethanol production.^{7–8} These residues, produced in large amounts in most developing countries, where agriculture is the main economic activity, have little or no value in productive processes.^{8–9} By contrast, the use of residues in the ethanol production, in addition to solving the problem of food versus energy (which is critical for first-generation production of this fuel), also contributes to the optimization of energy production and to the reduction of costs.^{8,10}

On the other hand, one of the current challenges for 2G ethanol production is making it economically viable. To reach this end, process productivity plays a crucial role. The concept entails achieving high concentrations of final product, with the highest yields and the least possible processing times. This requires glycoside hydrolases (GH) with high activity and low K_m (high affinity), so as to generate product in short periods of time, even with low concentrations of substrate. Simultaneously, yeasts able to rapidly consume the hydrolysis-released monosaccharides are required to allow utilization of all (or almost all) the carbon source through fermentation (instead of metabolizing sugars through the Krebs cycle and the respiratory chain).

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Another challenge is mitigation of the environmental impacts generated by the production processes, particularly those related to freshwater consumption. Despite the fact that 2G ethanol generates smaller water footprints than first-generation fuel (including freshwater for crop irrigation), 2G also demands high volumes of water to fill tanks and must preparation.¹¹ These needs have stimulated the search for alternatives to increase water safety during this process, including the constitution of very high-gravity musts (more concentrated, i.e., with more solutes per liter of water) or musts prepared with seawater instead of freshwater.^{12,13} In order to fulfill this role, however, it is desirable to identify halotolerant enzymes and microorganisms, or at least biocatalysts capable of tolerating high osmotic pressures.

Therefore, the bioprospecting of enzymes and microorganisms in herbivorous insect guts has been proposed. Because these invertebrates absorb sugars as nutrients from lignocellulosic biomass, they rely on biocatalysts in their digestive tracts, either from the resident microflora or from their own cells (endogenous enzymes). The present review will address various strategies and successes in bioprospection, highlighting the main characteristics of the various insect enzymes and microorganisms described in the literature.

Main Challenges for 2G Ethanol Production

Agroindustrial wastes contain compounds with structures that hinder access of microorganisms and enzymes to the polysaccharide fraction available in plant cell walls,⁷ resulting in cellulose, hemicellulose and pectin not being available for hydrolysis, in order to be converted into fermentable sugars.¹⁴ The choice of pretreatment method, or the combination of different methods, depends primarily on the characteristics of the biomass.^{15–18} When the biomass is enriched with lignin, for example, it becomes more resistant to the pretreatment process, and although such characteristics require more aggressive pretreatment processes, it is important to point out that the degradation process of lignin-rich compounds can form furan compounds that may inhibit alcoholic fermentation.¹⁹

Various physical, chemical and physicochemical pretreatment technologies have been employed for the production of second-generation ethanol from lignocellulosic biomass. Physical pretreatments increase the reactivity of cellulose in hydrolysis using microwave, extrusion, heat, ultrasound or a combination of these methods; however, these methods require higher energy consumption than chemical pretreatments.^{20–25} The chemical pretreatments are primarily acid or alkaline. The acid method, in addition to decomposing the lignin structure to increase subsequent cellulase accessibility, may cause hydrolysis of the hemicelluloses. Several acids have been used, including hydrochloric, phosphoric, acetic, formic, nitric, peracetic and sulfuric acid.^{26–30} The acid pretreatment may be carried out with diluted or concentrated acid, the latter being less indicated because of its potential inhibitors and corrosion problems,³¹ observed primarily with the use of sulfuric acid at temperatures above 120°C.³² Alkaline pretreatment breaks down bonds between lignin and hemicellulose, thereby allowing the removal of the former polymer as well as acetyl

groups and uronic acids. Simultaneously, this method generates swelling into cellulose that reduces its crystallinity and degree of polymerization.³³ Such approaches are carried out using NaOH, although reagents as KOH or Ca(OH)₂ may also be effective.³¹ Among the physicochemical pretreatments, steam explosion is one of the most studied methods.³⁴ In this process, lignin and hemicellulose are converted into soluble oligomers using the high-pressure saturated steam auto-hydrolysis reaction,³⁵ affected mainly by temperature, chip size and moisture availability.

Finally, it is also worth noting that pretreatments may liberate compounds such as resins, fatty acids, phenols and tannins that are present in the agro-industrial wastes in small amounts, but are capable of inhibiting enzymes and microorganisms in the subsequent steps of hydrolysis and fermentation in 2G-ethanol production.^{36,37} This is particularly true when treating the biomass at high temperatures and in acidic conditions, favoring the degradation of the sugars in furfural, hydroxymethylfurfural and later in formic acid and levulinic acid, contributing to the hydrolysis of the acetyl group in hemicelluloses to acetic acid.^{38,39}

After pretreatment, the hydrolytic stage or a combination of hydrolysis and fermentation in one single step (the simultaneous saccharification and fermentation process, SSF) takes place. Among the hydrolytic processes described in the literature, enzymatic hydrolysis is a less aggressive and greener method than alkaline or acid hydrolysis, because the enzymes act in milder conditions and generate less impactful waste. Furthermore, through enzymatic hydrolysis, no inhibitory compound is released into the must, thereby restricting the production of those compounds (as by-products of the process) to the pretreatment stage.

The high cost of enzymes makes 2G ethanol production relatively unattractive economically.^{40,41} Therefore, enzymes from alternative sources are compelling strategies for increasing efficiency and reducing operational costs associated with this ethanol production step.⁴²

During enzymatic hydrolysis, various GHs act on cellulose, hemicellulose and pectin, releasing pentoses and hexoses that may be then promptly fermented by yeast cells. Nevertheless, despite the fact that current industrial yeast strains easily ferment glucose, the fermentation of pentoses generate much lower productivity than that of hexose, directly influencing the alcoholic fermentation yield.^{43,44} This is due to the ability to naturally ferment xylose on the part of a few microorganisms, including yeasts of the genera *Spathaspora*⁴⁵ and *Candida*.⁴⁶ Therefore, the search for microorganisms able to efficiently metabolize pentoses has proved to be a challenge for further investigations to increase bioethanol productivity.

Improving 2G Ethanol Production with Endogenous Insect Enzymes

Herbivorous insects play a major role in biogeochemical cycles because of their high capacity for degradation of lignocellulosic biomass.⁴⁷ Throughout evolution, many herbivorous insects have developed the ability to produce endogenous enzymes capable of hydrolyzing lignocellulosic biomass.^{48,49} The evolution of endogenous insect enzymes may be a consequence

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of horizontal gene transfer⁵⁰ or even of co-evolutionary processes between these invertebrates and their symbiotic microorganisms.^{48,51} Ancestral gene duplication processes may have enabled some insects, including those in the family Phasmatoidea, to specialize as folivores and to diverge from their omnivorous ancestors.⁵² Shelomi et al.⁵³ observed high homology among endo- β -1,4-glucanases from distantly-related insect species, not only phasmids but also termites, cockroaches and crickets, suggesting a general distribution of cellulase genes in some endogenous endoglucanase-producing insects.

It should be noted that the degradation of a polysaccharide such as cellulose in the insect gut, as it occurs in the hydrolytic stage of the 2G ethanol production, depends on the synergistic action of various glycoside hydrolases: endoglucanases, exoglucanases and β -glucosidases. Cairo et al.⁵⁴ showed that, in the lower termite *Coptotermes gestroi*, synergy between β -1,4-glucanase (with endo- and exo- activity) and β -1,4-glucosidase (capable of hydrolyzing cellooligosaccharides) increased by 2.5- to 10-fold the complete hydrolytic activity of cellulose compared to the individual application of each of these enzymes. This result suggests that a single insect can provide various enzymes to compose an enzymatic complex that may be used in sugar-alcohol factories.

Tokuda et al.⁵⁵ observed that a species of flagellate-harboring termite (*Reticulitermes speratus*) expressed endogenous cellulase genes specifically in the salivary glands, whereas the termite species that do not have symbiotic organisms (*Nasutitermes takasagoensis*) showed cellulase gene expression only in the midgut. Even with a similar concentration of endo- β -1,4-glucanase activity and luminal volume in the midgut between xylophagous higher termites (*N. takasagoensis*, which digests cellulose without the aid of symbiotic protists) and lower termites (*Coptotermes formosanus*, which requires hindgut protists), the higher termites ingested larger particles of lignocellulosic material.⁵⁶ High cellulolytic activity, similar to endo- β -1,4-glucanase, was observed in the foregut and midgut regions of *Dissosteira carolina*, even when compared to other insect groups.⁴⁹ Endogenous endoglucanases (glycoside hydrolase family 45) were also isolated from the wood-feeding beetles *Oncideres albomarginata chamela*⁵⁷ and *Mesosa myops*⁵⁸ as well as from the salivary gland of *Reticulitermes speratus* and gut of *N. takasagoensis*.⁵⁹ Endogenous endo- β -1,4-glucanase expression levels may differ over the life cycle of the insects, as observed in the termite *Reticulitermes speratus*.⁶⁰

Among all the insect endogenous glycoside hydrolases, β -glucosidases are probably the most prominent. Recently, several studies have characterized these enzymes, especially in termites. Feng et al.⁶¹ found two endogenous β -glucosidases with different substrate specificities in the termite *Coptotermes formosanus*. Both presented maximum activity between 30°C and 40°C and optimum pH at 5.0, corresponding to a well-tolerated temperature and the optimum pH for yeasts—a desirable feature for SSF processes. More recently, Li et al.⁶² characterized a cockroach β -glucosidase with activity against cellobiose, cellotriose and cellohexaose. Although it retains only 50 to 60% of its maximum activity in temperatures ranging from 30°C to 40°C, this enzyme has an optimum pH of 6.0, retaining more than 90% of its maximum activity at pH 5.0.

Among insect endogenous β -glucosidases, two enzymes described in two studies by Uchima et al.^{63,64} deserve to be highlighted. In the second article, the authors presented a β -glucosidase from the higher termite *N. takasagoensis* that displayed maximum activity at pH 5.5, and although it had showed a relatively high optimal temperature (65°C)—not a desired feature for SSF—retaining almost 100% of its activity against 100 mM glucose. Even more impressive were the findings of those authors one year prior: a β -glucosidase from the lower termite *Neotermes koshunensis* with an optimum pH of 5.0 was stimulated 1.3-fold when glucose was present at 200 mM.⁶⁴ Considering that β -glucosidases are expected to be inhibited by glucose,⁶⁵ both β -glucosidases are excellent alternatives for the hydrolytic step in 2G ethanol production. Examples of prominent insect β -glucosidases are summarized in Table 1.

Proteomic studies have also evaluated the presence of endogenous β -glucosidases genes in insects,^{66,67} highlighting their importance for biotechnological purposes through heterologous expression.⁶⁸ Currently in GenBank,⁶⁹ a total of 181 β -glucosidase gene sequences from insects are deposited, of which 113 are endogenous. Figure 1 shows the number of known sequences of β -glucosidase in the animal kingdom, highlighting the number of insect sequences.

Within the class Insecta, the order Isoptera is very likely to be the most prominent for the decomposition of lignocellulosic biomass. Quoting Brune,⁷⁰ termite guts are “the world’s smallest bioreactors.” Indeed, they are thought to assimilate a significant proportion of the cellulose (74–99%) and hemicellulose (65–87%) components of lignocelluloses they ingest.^{47,71} Taking this into account, Ben Guerrero et al.⁷² prospected and evaluated cellulases and hemicellulases from two Argentinian native termite gut digestomes: *Nasutitermes aquilinus* and *Cortaritermes fulviceps*. In that study, the authors found considerable digestion success was due to the cooperation between the endogenous enzymes and those expressed by their microbiota, because the combination in total gut extracts allowed hydrolysis of sugarcane bagasse and Napier grass thanks to their high endoglucanase and xylanase activities. In fact, this profitable relationship had been seen previously by other authors.^{73–75} As reviewed by Taggar,⁷¹ there is compartmentalization of both endogenous and microbial cellulases, with the former ones found especially in the foregut, midgut and salivary glands and the latter ones localized mainly in the hindgut. Taken together, these features underscore the high potential of herbivorous insects to contribute to the improvement of bioethanol production.

Mining Enzymes on Insect Guts Microbiomes

In addition to endogenous insect enzymes, several species of herbivorous insects depend on the symbiotic relationships with bacteria, fungi and/or protozoa in their gut that are also potential sources of important enzymes for the degradation of plant biomass. In this symbiosis, the principle role of microorganisms is to subject the lignocellulosic structure to a variety of enzymes capable of delignifying the material and hydrolyzing its polysaccharide content,⁷⁶ primarily represented by cellulose and hemicellulose. Subsequently, the insect cells

Table 1. Remarkable Features of Microbial and Endogenous Enzymes Prospected in Insects

INSECT SOURCE ENZYME	REMARKABLE FEATURE	REFERENCE
Beetle		
<i>Microbiota</i>		
Cellulase ^a	Na ⁺ -stimulated bi-functional cellulase (with endo- and exoglucanase activities) with wide pH (5.0 to 10.0) and temperature (20 to 60°C) stability, retaining >70% of its maximum activity under such variations of pH and temperature. It is highly stable (ranging from 57 to 105% of activity showed in the control assay) under different organic solvents (at 10% v/v), such as acetone, ethanol, isopropanol, chloroform and DMSO. It presents its maximum cellulase activity at 60°C and pH 5 (9 μmol min ⁻¹ mg ⁻¹).	Hatefi et al. ⁹⁹
XynB119	Endo-1,4-β-xylanase able to retain more than 90% of its maximum activity after proteolytic treatment. It is stable over a broad pH range, retaining >70% activity after incubation at pH 1.0–11.0 for 1 h at 37°C without substrate. Its <i>K_m</i> , <i>V_{max}</i> and <i>k_{cat}</i> were calculated to be 15.1 mg mL ⁻¹ , 441.4 μmol min ⁻¹ mg ⁻¹ , and 251.0 s ⁻¹ , respectively.	Zhou et al. ¹⁰⁴
<i>Endogenous</i>		
Bh-EGase II	Endo-1,4-β-glucanase able to retain ~70% of its maximum activity at 30°C and ~90% of its maximum activity at pH 5.0. It shows the highest activity at 50°C and pH 6.0 (928 μmol min ⁻¹ mg ⁻¹).	Xia et al. ¹⁰⁵
Cockroach		
<i>Endogenous</i>		
PaBG1b	β-glucosidase able to hydrolyze cellooligosaccharide from cellobiose to cellohexaose. It is able to retain ~90% of its maximum activity at pH 5.0. It has high specific activity and catalytic efficiency towards cellobiose with <i>V_{max}</i> , <i>K_m</i> and <i>k_{cat}</i> values of 873.4 μmol min ⁻¹ mg ⁻¹ , 4.1 mM and 450.2 s ⁻¹ , respectively.	Li et al. ⁶²
Fly		
<i>Microbiota</i>		
CS10	Cellulase with thermostability and pH stability preserved at the ranges of 20–50°C and pH 4.0–10.0. It is able to retain ~85% of its maximum activity against 10% ethanol. Its <i>K_m</i> and <i>V_{max}</i> were calculated to be 8.7 mM and 160.6 μmol min ⁻¹ mg ⁻¹ toward CMC, respectively; and 6.1 mM and 364.3 μmol min ⁻¹ mg ⁻¹ against barley glucan, respectively.	Lee et al. ¹⁰⁶
ManEM17	Endo-1,4-β-mannanase able to retain more than 95% of its maximum activity against 10% ethanol. It shows the highest activity at 55°C and pH 6.5 (5,467 μmol min ⁻¹ mg ⁻¹) toward locust bean gum galactomannan.	Song et al. ¹⁰²
Silkworm		
<i>Microbiota</i>		
XynX	Cytoplasmic xylanase released to the bacterial periplasm during osmotic downshock.	Usui et al. ¹⁰⁷
XysB	High-affinity β-xylosidase stable at temperatures below 40°C and pH ranging from 5 to 8. Its <i>K_m</i> and <i>V_{max}</i> were calculated to be 0.34 mM and 33.0 μmol min ⁻¹ mg ⁻¹ , respectively, at 50°C and pH 6.0.	Suzuki et al. ¹⁰⁸
Termite		
<i>Microbiota</i>		
Glu1392	β-glucosidase able to retain more than 80% of its maximum activity under concentrations of up to 1.0 M glucose (<i>K_i</i> value of 2.25 M) and 50% of its maximum activity at 60°C. Its <i>k_{cat}</i> and <i>K_m</i> values were calculated to be 163.2 s ⁻¹ and 134.0 μM, respectively, for cellobiose at 50°C and pH 8.0.	Gao et al. ⁹⁸
Xyl1	Multimodular endo-1,4-β-xylanase with a catalytic domain and two carbohydrate-binding modules which increase hydrolysis rate. Its <i>k_{cat}</i> and <i>K_m</i> values were calculated to be 55.3 s ⁻¹ and 1.2 mg mL ⁻¹ , respectively, for xylan at 50°C and pH 6.0.	Rashamuse et al. ¹⁰⁹
Xyl2	β-xylosidase able to retain ~80% of its maximum activity at 30°C. Its specific activity was calculated to be 11.51 μmol min ⁻¹ mg ⁻¹ at 40°C and pH 5.0 toward pNPβX ^b .	Liu et al. ¹¹⁰
Xyl6	Bifunctional β-xylosidase/β-glucosidase able to retain ~80% of its maximum activity at 30°C. Its specific activity was calculated to be 32.27 μmol min ⁻¹ mg ⁻¹ at 50°C and pH 6.0 toward pNPβX ^b .	Liu et al. ¹¹⁰
Xyl8	Bifunctional β-xylosidase/α-arabinosidase able to retain ~90% of its maximum activity at 30°C. Its specific activity was calculated to be 11.98 μmol min ⁻¹ mg ⁻¹ at 40°C and pH 6.5 toward pNPβX ^b .	Liu et al. ¹¹⁰

(continued)

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Table 1. <i>continued</i>		
INSECT SOURCE ENZYME	REMARKABLE FEATURE	REFERENCE
<i>Endogenous</i>		
CfGlu1B	β -glucosidase with optimum pH at 5.0. It is able to retain at least 90% of its maximum activity on temperature ranging from 25 to 50°C. Its specific activity, k_{cat} and K_m values were calculated to be 49.5 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, 60.7 s^{-1} and 2.2 mM, respectively, toward $p\text{NP}\beta\text{G}^c$ at 40°C and pH 5.0.	Feng et al. ⁵¹
CfGlu1C	β -glucosidase with optimum pH at 5.0. It is able to retain at least 90% of its maximum activity on temperature ranging from 25 to 40°C. Its specific activity, k_{cat} and K_m values were calculated to be 3.5 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, 3.7 s^{-1} and 1.7 mM, respectively, toward $p\text{NP}\beta\text{G}^c$ at 40°C and pH 5.0.	Feng et al. ⁵¹
CgBG1	High-activity β -glucosidase with optimum pH at 5.5, able to hydrolyze different glucose oligomers. It is also able to retain 88% of its maximum activity at 60°C and 50% of it in the presence of 300 mM of glucose. Its V_{max} , k_{cat} and K_m values were calculated to be 60.55 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, 57.19 s^{-1} and 1.59 mM, respectively, toward $p\text{NP}\beta\text{G}^c$ at 55°C and pH 5.5.	Cairo et al. ⁵⁴
CgEG1	Bi-functional cellulase (with endo- and exo-1,4- β -glucanase activities) with optimum pH 6.0. Its V_{max} , k_{cat} and K_m values were calculated to be 303.10 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, 251.08 s^{-1} and 2.76 mM, respectively, toward β -glucan at 50°C and pH 6.0.	Cairo et al. ⁵⁴
G1mgNtBG1	β -glucosidase with optimum pH at 5.5, able to retain $\sim 100\%$ of its activity against 100 mM glucose ($K_i = 600$ mM). Its V_{max} and K_m values were calculated to be 8.0 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and 0.67 mM, respectively, toward $p\text{NP}\beta\text{G}^c$ at 65°C and pH 5.5.	Uchima et al. ⁶³
G1NkBG	β -glucosidase with optimum pH at 5.0, stimulated by 1.3-fold when glucose is present at a concentration of 200 mM. Its V_{max} and K_m values were calculated to be 16.0 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and 0.77 mM, respectively, toward $p\text{NP}\beta\text{G}^c$ at 50°C and pH 5.0.	Uchima et al. ⁶⁴

^anot named by the authors; ^b*p*-nitrophenyl- β -D-xylopyranoside; ^c*p*-nitrophenyl- β -D-glucopyranoside.

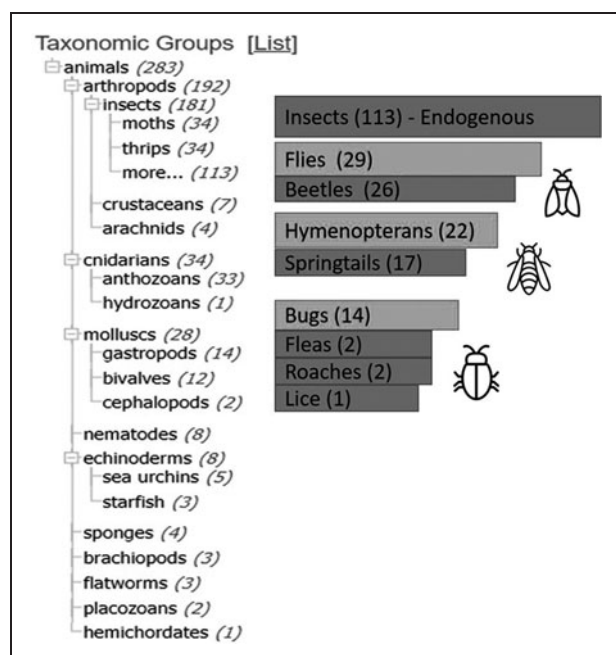


Fig. 1. Total sequences of β -glucosidase gene deposited on GenBank retrieved from the animal kingdom and endogenous β -glucosidase genes from insects (data obtained from <https://ncbi.nlm.nih.gov/gene>).⁶⁹

absorb the monosaccharides generated during the hydrolytic process carried out inside their gut.

Therefore, insect microbiota may be seen as a major source of biotechnological enzymes, especially for 2G ethanol production that uses residual biomass as raw material. As previously mentioned, in the context of 2G ethanol, it is necessary for the lignin to be separated from the polysaccharides for the latter to be hydrolyzed by glycoside hydrolases (GH), so that their monosaccharides can be fermented into ethanol.⁷ Indeed, considering the similarity between the first two stages (pretreatment and hydrolysis) of the 2G ethanol production process and the digestion process of lignocellulosic biomass in the gut of herbivorous insects, several researchers have successfully sought microbial GH in these class of arthropods.

The biodiversity of the gut microbiota is directly linked to the specialization of the diet of each species of insects and to the various stages of its life cycle,^{77–81} directly reflected in the diversity of enzymes. This ends up stimulating bioprospecting of enzymes in the most varied of situations. From a termite gut protist cDNA library, Todaka et al.⁸² cloned 11 cellulases with similar or up to 2.6-fold greater performance than *Trichoderma reesei* endoglucanase I (Genbank accession in M15665), a reference enzyme in the cellulose hydrolysis. Such considerable success was also achieved by Bashir et al.,⁸³ however with enzymes obtained from termite endosymbiont cellulolytic bacteria. Moreover, in this case, the biocatalysts not only presented high activity against carboxymethyl cellulose, but also against

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the mannan and xylan polysaccharides (the main component of the hemicellulose fraction in grasses). In the beetle *Anoplophora glabripennis*, Geib et al.⁸⁴ suggested that the presence of a fungus related to the *Fusarium solani/Nectria haematococca* species complex may be associated with the lignin degradation process that occurs in the digestive tract of this coleoptera. In a study carried out with the cultured microflora of the larvae of the beetle *Euoniticellus intermedius*, Mabhegedhe⁸⁵ generated a cosmid library from which seven clones with cellulase activity were obtained. Shi et al.⁸⁶ collected gut juice from 2,000 third to fifth instar grasshoppers, 100 fourth to fifth instar silkworm larvae and 30 woodborer larvae and analyzed their cellulase and xylanase activities. In general, the enzymatic activities obtained from the grasshopper and woodborer samples were significantly higher than those of the silkworm under various conditions. These authors also verified that both pH and temperature had significant impacts on the enzyme activities in the gut contents. In addition, it is worth noting the xylanase activity of approximately $5,500 \mu\text{M mg}^{-1} \text{min}^{-1}$ in woodborer larvae gut juice at 30°C and pH 7.0 as well as the cellulase activity of approximately $480 \mu\text{M mg}^{-1} \text{min}^{-1}$ in grasshopper gut juice at 50°C and pH 7.0.

Despite the fact that these examples demonstrate the substantial potential of these enzymes in industrial ethanol production, the bioprospection of enzymes of biotechnological interest from the insect microbiomes has been enhanced in the last decade by the application of metagenomic tools. Because it allows access to genetic material even from non-cultured microorganisms, metagenomics allows the screening of up to a 100-fold higher number of microbial species.^{87–92}

To carry out metagenomic analysis in an insect gut, the insect surface needs to be sterilized before beginning the dissection process. Subsequently, the gut is longitudinally cut and the lumen content is gently squeezed into a buffer.⁹³ From this content, it is possible to extract the DNA directly from the resident microorganisms without the need of prior culture to construct metagenomic libraries.⁹⁴ With the library assembled, enzyme screening can be performed using two general strategies: sequence- or functional-based approaches,⁹² as seen in Fig. 2.

Using amplicon and shotgun metagenome sequencing, Scully et al.⁹⁶ analyzed the midgut community derived from third instar larvae of the invasive

beetle *Anoplophora glabripennis* (Asian longhorned beetle) collected while they were actively feeding in the heartwood of sugar maple trees (*Acer saccharum*). The authors detected 4,394 reads of enzymes belonging to 36 families of glycoside hydrolases, with GH1 (~13%), GH3 (~16%) and GH32 (~9%) being most dominant families. It is interesting to note that these three families of GH contain representatives capable of promoting the hydrolysis of cellulose and xylan as well as of their derivative oligosaccharides. In fact, the high number of carbohydrate active enzyme in the microbiome of herbivorous insects has already been observed by other authors. Shi et al.⁷⁸ dissected 2,000 third to fifth instar grasshoppers and 50 fourth to fifth

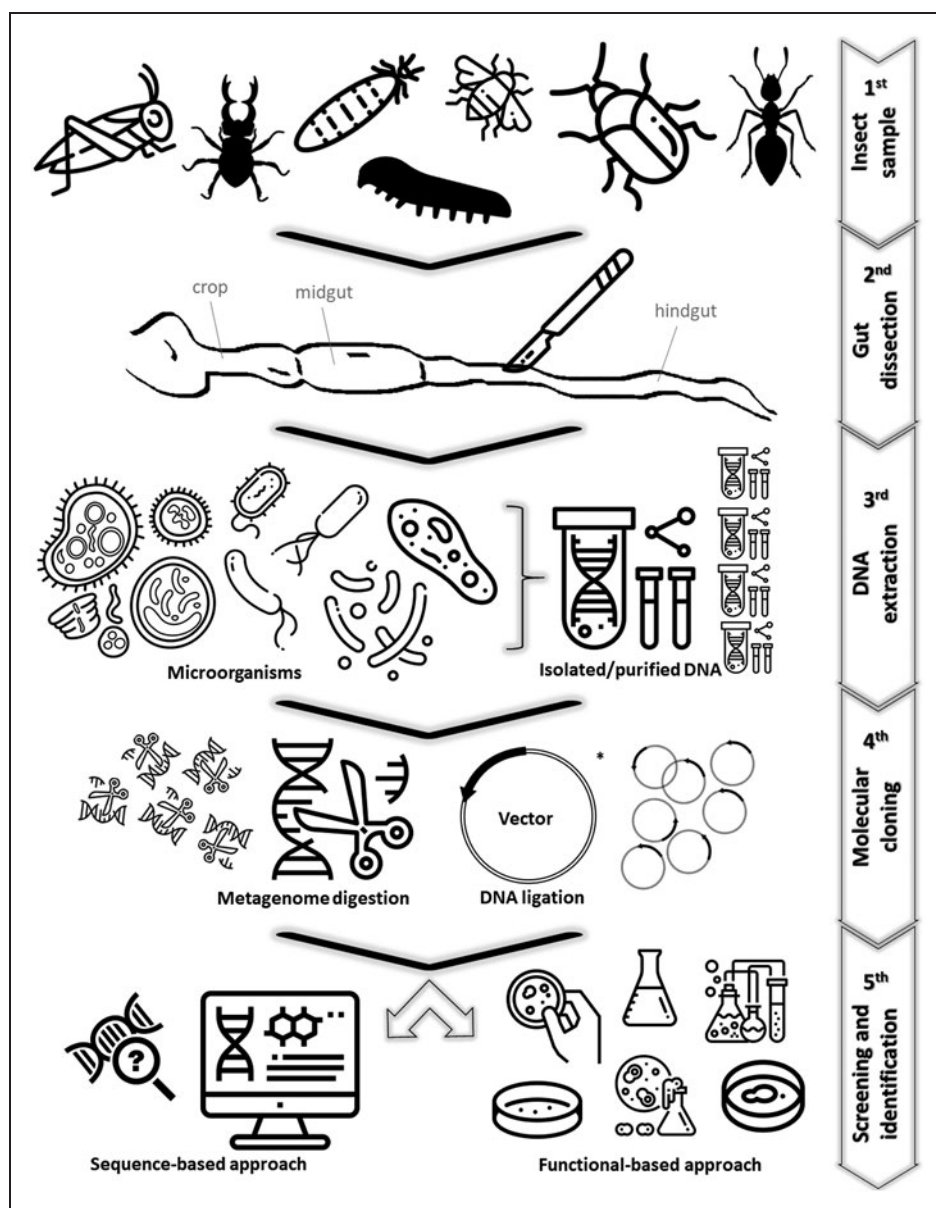


Fig. 2. Bioprospecting enzymes using insect gut microflora metagenomes. Icons were retrieved from FlatIcon, except plasmids* (by Cornelia Scheitz) that were retrieved from the Noun Project, and insect gut that was drawn based on Reid et al.⁹⁵

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instar cutworms to extract the genomic DNA of their gut symbionts. The metagenomic DNA was then analyzed and compared to the termite gut metagenome (JGI IMG Database GOLD ID: GM00013 and Sample ID: GS0000048).⁹⁷ In this analysis, the authors found a total of 31, 40, and 52 different GH families in the grasshoppers, cutworms and termite metagenomes, respectively. The termite gut featured the most abundant putative cellulases and hemicellulases, with 125 GH5 cellulases and 101 GH10 xylanases. Liu et al.⁹⁴ combined large-scale functional screening of fosmid libraries, shotgun sequencing, and biochemical assays to interrogate the gut microbiota of about 100 whole guts of wood-feeding termite *Globitermes brachycerastes*, and detected polysaccharide-degrading activities in 464 clones from the assembled metagenomic cosmid library. Of this total, they identified dozens of clones with endoglucanase (267), exoglucanase (24), β -glucosidase (72) and endoxylanase (101) activities. Therefore, the metagenome of only one insect provided enzymes able to hydrolyze the three major polysaccharides in the residual plant biomass: cellulose, hemicellulose (xylan) and pectin.

During hydrolysis of the lignocellulosic biomass, a set of enzymes must act in synergy to enable the release of monosaccharides resulting from polysaccharide breakdown. In the case of cellulose, glucose (the final product of cellulose hydrolysis) inhibits β -glucosidases,⁶⁵ reducing the efficiency of the hydrolytic process and, consequently, decreasing the yield of ethanol per ton of biomass. In this scenario it is worth noting the high glucose-tolerant glucosidase Glu1392 found in a hindgut microbiome of the termite *Coptotermes formosanus* that presented an inhibition constant (K_i) value of 2.25 M for glucose. This glucosidase retained more than 80% of its maximum activity at concentrations of up to 1.0 M glucose.⁹⁸ For this, those authors dissected ~500 worker termites to isolate the DNA and construct a plasmid metagenomic library in the *Escherichia coli* DH5 α as the cloning host.

Another enzyme that deserves to be highlighted is the bifunctional cellulase purified by Hatefi et al.⁹⁹ from cultured bacteria (*Bacillus* sp.) isolated from the digestive tract of third instar larvae of the beetle *Osphranteria coerulescens*, collected from infected apricot and almond gardens. The enzyme characterized by these authors was stable in a wide range of temperatures (from 20°C to 60°C) and presented its maximum activity at pH 5.0, making it an excellent choice for SSF processes, given the need to meet temperature and pH ranges amenable to yeasts. In addition, the cellulase characterized by Hatefi et al.⁹⁹ was stimulated by Na⁺, thereby demonstrating its potential for application in alternative processes of 2G ethanol production that aim to reduce the water footprint by using sea water as a total or partial substituent for the freshwater employed in bioreactors.^{12,13}

In the context of the search for cleaner, renewable energies, third-generation (3G) ethanol is beginning to gain attention. 3G ethanol is derived from algal carbohydrate biomass as the substrate for alcoholic fermentation.¹⁰⁰ This raw material, however, may present polysaccharides different from those of terrestrial plants, requiring different hydrolases for the hydrolytic step. In red algae, mannan is one example.¹⁰¹ This polymer must be cleaved by mannanases to release mannose residues to be fermented by yeasts. Through the analysis of gut microflora me-

tagenome from ~1,000 larvae of black soldier flies (*Hermetia illucens*), Song et al.¹⁰² discovered an endo-1,4- β -mannanase belonging to the GH26 family, with high activity against galactomannan. Interestingly, when tested against various organic solvents, this enzyme maintained more than 90% of its maximum activity against 10% (v/v) ethanol. Considering that SSF processes currently have a certain predilection to produce cellulosic ethanol,¹⁰³ the tolerance of hydrolytic enzymes to ethanol becomes an extremely desirable feature. In this way, insects may contribute to the production of third-generation ethanol.

Many of the putative enzymes found in the metagenomic libraries of insect gut microbiota have been tested and characterized, demonstrating their high potential for biotechnological application. Examples of enzymes (prospected from insect microbiomes or from the own insect cells—endogenous enzymes, as previously discussed) with potential application in the production of bioethanol are summarized in *Table 1*. It is worth noting that some of them have extremely desirable features for process that aim to achieve low water footprints, considering their high-salt tolerance (halotolerant enzymes). Moreover, most of the enzymes presented in *Table 1* also present characteristics that made them suitable for SSF, because they are highly stable and active in pH and temperature ranges that meet yeast requirements for efficient alcoholic fermentation.

It is important to highlight that some of the enzymes in *Table 1* showed better performance than some commercial enzymes. Uchima et al.,⁶³ for example, compared the thermostability and glucose tolerance of G1mgNtBG1 with those of the most common commercial β -glucosidase preparation from *Aspergillus niger*, Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark). After 1 h of incubation at 60°C, G1mgNtBG1 retained 93% of its total activity while Novozym 188 was quickly inactivated and retained only 24% of its total activity. Regarding inhibition by glucose, while G1mgNtBG1 retained ~100% and 50% of its activity at 0.1 M and 0.5 M glucose respectively, only 26% and 5% of Novozym 188 activity was retained at the same concentrations of this hexose.⁶³ In *Table 1*, there are other examples of β -glucosidases (Glu1392 and CgBG1) whose thermostability and glucose tolerance are higher than those of Novozyme 188, and one of these was even stimulated by such monosaccharide (G1nkBG). Therefore, at least four β -glucosidases listed in *Table 1* appears to be a better choice than the very common Novozyme 188.

Normally, commercially-available β -glucosidases are used as supplements in enzyme cocktails for hydrolysis of plant biomass,¹¹¹ with Novozyme 188 commonly employed with Celluclast® (Novozymes A/S) that consists of cellobiohydrolases and endo-1,4- β -glucanases.^{112–114} Considering the current predilection for processes whose hydrolysis step occurs simultaneously with fermentation,¹⁰³ some features presented in *Table 1* are better fits for SSF than Celluclast. According to Herlet et al.,¹¹³ Celluclast retains 50–65% of its maximum activity at 35°C and pH 5.0 depending on the cellulosic analyzed substrate. On the other hand, as shown in *Table 1*, the cellulase described by Hatefi et al.⁹⁹ shows maximum activity at pH 5.0 and almost 90% of it at 30°C, and the endo-1,4- β -glucanase Bh-EGase II¹⁰⁵ retained ~70% and ~90% of its maximum activity at 30°C and pH 5.0, both pH and temperature levels being optimum for yeast cells.

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Therefore, various mixes of insect β -glucosidases and cellulases may show better performance in terms of hydrolysis of vegetal biomass than the commonly used commercial mix Novozyme 188 + Celluclast, especially in SSF processes.

Cellobiose- and Xylose-Fermenting Yeasts in Plant-Associated Insects

Research in pursuit of new microbial strains is relevant primarily because of their predisposition to produce enzymes of high commercial value, thereby constituting alternatives that reduce biotechnological costs. The screening of new strains usually occurs via isolation of microorganisms of interest in the most varied habitats and hosts.^{115,116} In fact, the microbiological biodiversity existing in the natural ecosystem is huge; however, identification of new strains with industrial value generates significant impact on the development of globally-relevant bioprocesses, including 2G ethanol production.^{117,118}

In the case of biodiversity, insects can be considered intermediates in a large microbial community forming consortia of symbiotes among these constituents, including bacteria, yeasts, filamentous fungi and protists. Indeed, the environment of the insect intestinal microflora is extremely abundant in organisms that produce enzymes capable of metabolizing lignocellulosic material that are ingested as food.^{116,119,120}

Therefore, the insect gut may also be considered a source of various yeast species.^{121–123} Yeasts are linked to environments that have sugar sources, and that makes herbivorous insects a perfect niche, because these invertebrates feed on rich-carbohydrate biomasses.^{124–126} Studies with wasps, beetles and termites have shown that most yeast species consumed by these insects survive and remain in the gastrointestinal system and in the cuticles, thereby making the insects vectors of new yeasts among the most varied ecosystems. Therefore, isolation of yeast from the intestine of insects is a promising alternative for discovering new strains with potential for various applications.^{126–129}

Insect-isolated yeasts present the possibility of producing a series of enzymes essential for a range of industrial processes, including those that allow metabolism of xylose (the main product of hemicellulose hydroly-

sis and the second-most abundant sugar in biomass hydrolysates) as well as hydrolysis of cellobiose (a disaccharide found on cellulose hydrolysates).^{130,131} For the fermentation of the pentose, yeast cells must harbor a xylose reductase (XR) and a xylitol dehydrogenase (XDH) that share the same coenzyme—i.e., that allows NADH/NAD⁺ recycling—as well as an overexpressed xylulokinase. These three enzymes work in sequence, allowing the conversion of xylose into xylulose-5P that then accesses pentose-phosphate pathway before generating glycolysis intermediates to enable the conversion of pyruvate into ethanol.⁴³ Regarding cellobiose, normally the third-most abundant sugar in biomass hydrolysates after glucose and xylose,^{132,133} yeasts need to express a β -glucosidase that can be secreted by the cells or located either in the periplasm or in the cytosol. β -glucosidases can also be part of a cellulase complex; however, in such cases, it has been shown that it may be insufficient for the amount of cellobiose formed during the hydrolysis of cellulose. Therefore, this disaccharide accumulates and may inhibit the action of endo- and exoglucanases by negative feedback, reducing the saccharification rate.^{134–136} In this scenario, a direct metabolization of cellobiose by yeasts during the production of 2G ethanol is crucial for the optimization of the process.¹³⁷

Depending on the yeast species, its use in the 2G ethanol production can be as only an enzyme supplier—because their enzymes can be heterologously expressed in industrial yeast strains already employed in first-generation ethanol plants—or directly as the fermenting microorganism itself. In fact, yeasts of the genera *Blastobotrys*, *Candida*, *Pachysolen*, *Hamamotoa*, *Meyerozyma*, *Nematodospora*, *Pichia*, *Scheffersomyces*, *Schizosaccharomyces*,

Table 2. Xylose- and Cellobiose-Fermentative Performances of Yeasts Isolated from Different Insect Guts

SUGAR YEAST SOURCE	YEAST	SUGAR CONSUMPTION (%) ^a	ETHANOL YIELD (G G ⁻¹) ^b	CONVERSION EFFICIENCY (%) ^c	REFERENCE
<i>Xylose</i>					
Beetle	<i>Scheffersomyces illinoensis</i>	90.3	0.26	56.5	Cadete et al. ¹¹⁸
	<i>Scheffersomyces stipites</i>	100.0	0.44	95.6	Jeffries et al. ¹⁴⁷
	<i>Spathaspora passalidarum</i>	100.0	0.41	90.6	Long et al. ¹³⁸
	<i>Spathaspora passalidarum</i>	90.0	0.45	97.8	Su et al. ¹⁴⁸
Termite	<i>Candida pseudorhagii</i>	95.2	0.31	67.4	Ali et al. ¹¹⁶
	<i>Hamamotoa lignophila</i>	90.6	0.22	47.8	Ali et al. ¹¹⁶
<i>Cellobiose</i>					
Wood-boring insect	<i>Candida queiroziae</i>	100.0	0.32	59.4	Santos et al. ¹⁴⁵
<i>Xylose + Cellobiose^d</i>					
Beetle	<i>Spathaspora passalidarum</i>	100.0	0.43	-	Long et al. ¹³⁸

^aPercentage of the total sugar available to the yeast cells; ^bRatio between ethanol concentration (g L⁻¹) and xylose and/or cellobiose consumed (g L⁻¹); ^cPercentage of the maximum theoretical yield of ethanol, considering 0.46 g_{ethanol}/g_{xylose} and 0.538 g_{ethanol}/g_{cellobiose}.^{149–150}; ^dCofeimentation of xylose and cellobiose.

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Spathaspora and *Sugiyamaella* have been found in insect guts (especially beetles and termites) and were shown to be able to ferment xylose.^{116,118,127,138–144} Some of them showed high efficiency in converting this sugar into ethanol (Table 2). Furthermore, most of the aforementioned genera (especially *Candida* and *Spathaspora*) have representatives that can hydrolyze and ferment cellobiose.^{138,145–146} Table 2 summarizes the most notable yeast fermentative performances in xylose- and cellobiose-containing media.

In the Brazilian ethanol industry, approximately 150 to 200 g L⁻¹ of fermentable sugar are required. These concentrations can cause osmotic stress in many yeasts, thereby decreasing fermentation yields,^{151–156} because the carbon source is eventually diverted to glycerol synthesis.¹⁵⁷ In fact, it has been demonstrated that current Brazilian industrial yeasts have adaptations that make them more apt to these conditions, including greater copy numbers of *SNO1*/*SNZ* genes involved in the sequential synthesis of pyridoxine and thiamin.¹⁵² Therefore, a high resistance to osmotic stress caused by high sugar concentrations becomes a desirable feature of the fermenting microorganism. The yeasts presented in Table 2 were subjected to various concentrations of xylose, from 50 g/L^{116,118} to 150 g/L.¹⁴⁸ Especially for *S. passalidarum*, an increase in the concentration of this pentose from 90 g/L¹³⁸ to 150 g/L¹⁴⁸ did not cause any detrimental effect on the fermentative yield of this yeast; to the contrary, ethanol yields in the context of xylose concentrations were approximately 10% higher (Table 2).

Economic Potential of Insect Enzymes and Yeasts

Currently, endogenous and symbiotic insect enzymes are not yet commercialized. However, insects have high economic potential, because of the growing demand for enzymes capable of degrading lignocellulosic materials. In addition to their uses in the food and beverage, household care, and pharmaceutical industries, the growing demand for renewable fuels, especially 2G ethanol, has driven the market for enzymes aiming for higher efficiency of the production process combined with reduction in costs.¹⁵⁸ The global enzymes market was valued at \$7.08 billion in 2017, and is projected to reach \$10.5 billion by 2024, with an annual growth rate of 5.7% over this period.¹⁵⁹ The biofuel enzymes market was worth over \$900 million in 2018, and is forecast to grow annually at around 9.0% to reach \$1.5 billion by 2024.¹⁶⁰

However, enzymes should never be considered in isolation, but rather as a part of a biocatalyst technology. Microorganisms also play an important role in the ethanol production. Currently, Fermentec (Brazil)¹⁶¹ has the largest bank of *Saccharomyces cerevisiae* in the world, with more than 2,800 yeasts isolated from industrial fermentation in several different regions. Fermentec and LNF Latin America¹⁶² have commercially available three-selected yeasts responsible for 70% of all ethanol produced in Brazil: CAT-1, PE-2 and FT858. Between 1997 and 2007, Fermentec showed a net profit of around \$3.5 billion, only from the increase of the ethanol efficiency production in 33 plants.¹⁶³ According to the authors, in 2007, the company introduced customized yeasts that allowed greater stability of the fermentation process because of the greater adaptation (more

persistence and dominance) of the yeasts isolated and re-introduced at the same plant. Over the subsequent seven years, the production of ethanol by these yeasts increased to over 3 billion L, representing a fermentation yield improvement from 75% in 1977 to 92% in 2014.¹⁶³ Customized yeast by Fermentec increased from 2 to 29 strains over the last ten years.¹⁶⁴ Furthermore, in addition to *S. cerevisiae*, very recently other yeasts^{165–169} and filamentous fungi^{150,170–172} have been shown to be efficient in the production of ethanol, either by improving hydrolysis of lignocellulosic biomass or by fermenting sugars such as glucose, cellobiose and xylose.

Enzyme and microorganism bioprospection in insect guts for 2G ethanol production may be, thus, considered a profitable biotechnology market, since one single insect organism may perfectly work as provider of enzyme complexes and yeast strains that can increase productivity and reduce costs in this biofuel production, transforming the dream of second-generation ethanol into reality.

Final Considerations

The biofuels industry has glimpsed the possibility of locating in insects a way to increase the economic viability of 2G ethanol production, because herbivorous insects emerge as a great supplier of biocatalysts capable of fully guaranteeing the production of this second-generation biofuel. The target enzymes for sugar-alcohol factories are produced inside insect guts, either endogenously or by their intestinal microbiota. Several examples of success, presented in this review, confirm the potential of their hydrolases to ensure that the hydrolytic stage of the 2G ethanol production occurs with high efficiency, considering costs, water footprint and reaction time. Moreover, the potential of insects to optimize bioethanol production is not restricted to the bioprospection of enzymes. Several yeast species have already been isolated from their guts and have demonstrated the ability to ferment the sugars found in lignocellulosic hydrolysates. Some of these yeasts have a fermentation efficiency higher than 90%. Therefore, enzymes and yeasts found in insects complement each other in such a way that a single insect species might provide all the biocatalysts necessary for optimized production of second-generation ethanol.

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REFERENCES

1. Canabarro NI, Foletto EL, Kuhn RC, et al. Ethanol production by solid-state saccharification and fermentation in a packed-bed bioreactor. *Renew Energy* 2017;102:9–14.
2. Abud AKS and Silva CEF. Bioethanol in Brazil: Challenges and perspectives to improve the production. In: Ray R and Ramachandran S (eds). *Bioethanol Production from Food Crops: Sustainable Sources, Interventions, and Challenges*. Elsevier Inc, 2019;417–443.

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3. Organisation for Economic Co-operation and Development and Food and Agriculture Organization. *Agriculture statistics (database)* In: OECD-FAO Agricultural Outlook. 2018. Available at: <http://dx.doi.org/10.1787/agr-outl-data-en>. (Last accessed March 2019).
4. Renewable Fuels Association. *World Fuel Ethanol Production*. 2019. Available at: <https://ethanolrfa.org/resources/industry/statistics/> (Last accessed March 2019).
5. Sarkar N, Ghosh SK, Bannerjee S, et al. Bioethanol production from agricultural wastes: an overview. *Renew Energ* 2012;37:19–27.
6. Neves PV, Pitarello AP, Ramos LR. Production of cellulosic ethanol from sugarcane bagasse by steam explosion: effect of extractives content, acid catalysis and different fermentation technologies. *Bioresour Technol* 2016; 208:184–194.
7. Antunes FAF, Chandel AK, Brumano LP, et al. A novel process intensification strategy for second-generation ethanol production from sugarcane bagasse in fluidized bed reactor. *Renew Energ* 2018;124:189–196.
8. Bonatto C, Venturin B, Mayer DA, et al. Introduction on Bioethanol. In: Treichel H, Alves Jr SL, Fongaro G, Müller C (eds). *Ethanol as a Green Alternative Fuel: Insight and Perspectives*. Nova Publishers 2019 pp. 1–20.
9. Guerrero AB and Muñoz E. Life cycle assessment of second generation ethanol derived from banana agricultural waste: Environmental impacts and energy balance. *J Clean Prod* 2018;174:710–717.
10. Bechara R, Gomez A, Saint-Antonin, et al. Review of design works for the conversion of sugarcane to first and second-generation ethanol and electricity. *Renew Sust Energ Rev* 2018;91:152–164.
11. Camargo AF, Scapini T, Stefanski FS, et al. In: Treichel H, Alves Jr SL, Fongaro G, Müller C (eds). *Ethanol as a Green Alternative Fuel: Insight and Perspectives*. Nova Publishers 2019 pp. 203–224.
12. Zaky AS, Greetham D, Louis EJ, et al. A New isolation and evaluation method for marine-derived yeast spp. with potential applications in industrial biotechnology. *J Microbiol Biotechnol* 2016;26(11):1891–1907.
13. Zaky AS, Greetham D, Tucker GA, et al. The establishment of a marine focused biorefinery for bioethanol production using seawater and a novel marine yeast strain. *Sci Rep* 2018;8(1):12127.
14. Silveira, MHL, Morais RC, Costa M, et al. Current pretreatment technologies for the development of cellulosic ethanol and biorefineries. *ChemSusChem* 2015;8:3366–3390.
15. Naik SN, Goud VV, Rout PK, et al. Production of first and second generation biofuels: A comprehensive review. *Renew Sust Energ Rev* 2010; 14:578–597.
16. Taherzadeh MJ and Karimi K. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review. *BioResources* 2007;3:472–499.
17. Arenas-Cárdenas P, López-López A, Moeller-Chávez GE, et al. Current pretreatments of lignocellulosic residues in the production of bioethanol. *Waste Biomass Valorization* 2016;8:161–181.
18. Shirkavand E, Baroutian S, Gapes DJ, et al. Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment—A review. *Renew Sust Energ Rev* 2016;54:217–234.
19. Akhtar N, Kanika, Jain AK, et al. Surfactant-assisted microwave-acid pretreatment of leaf litter biomass for enhanced enzymatic release of sugars. *Cellulose Chem Technol* 2016;50:127–137.
20. Ghosh P, Ghose TK. Microorganism in sustainable agriculture and biotechnology. *Adv Biochem Eng Biotechnol* 2003;85:1–27.
21. Xu J, Chen H, Kádár Z, et al. Optimization of microwave pretreatment on wheat straw for ethanol production. *Biomass Bioenerg* 2011;35:3859–3864.
22. Yoo J, Alavi S, Vadlani P, et al. Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. *Bioresour Technol* 2011; 102:7583–7590.
23. Moro MK, Teixeira RSS, Silva AS, et al. Continuous pretreatment of sugarcane biomass using a twin-screw extruder. *Ind Crops Prod* 2016;97:509–517.
24. Bang JH and Suslick KS. Applications of ultrasound to the synthesis of nanostructured materials. *Adv Mater* 2010;22:1039–1059.
25. Karimi M, Jenkins B, Stroeve P. Ultrasound irradiation in the production of ethanol from biomass. *Renew Sust Energ Rev* 2014;40:400–421.
26. Kabel MA, Bos G, Zeevalking J, et al. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresour Technol* 2007;98:2034–2042.
27. Ballesteros I, Ballesteros M, Manzanares P, et al. Dilute sulfuric acid pretreatment of cardoon for ethanol production. *Biochem Eng J* 2008;42: 84–91.
28. Kootstra MJ, Beefink HH, Scott EL, et al. Comparison of dilute mineral and organic acid pretreatment for enzymatic hydrolysis of wheat straw. *Biochem Eng J* 2009;46:126–131.
29. Castro RCA, Roberto IC. Effect of nutrient supplementation on ethanol production in different strategies of saccharification and fermentation from acid pretreated rice straw. *Biomass Bioenergy* 2015;78:156–163.
30. Skiba EA, Budaeva VV, Baibakova OV, et al. Dilute nitric-acid pretreatment of oat hulls for ethanol production. *Biochem Eng J* 2016;126:118–125.
31. Kennes D, Abubakar HN, Diaz M, et al. Bioethanol production from biomass: Carbohydrate vs syngas fermentation. *J Chem Technol Biotechnol* 2016;91: 304–317.
32. Agrawal R, Sattlewal A, Gaur R, et al. Pilot scale pretreatment of wheat straw and comparative evaluation of commercial enzyme preparations for biomass saccharification and fermentation. *Biochem Eng J* 2015;102: 54–61.
33. Kim JK, Lee YY, Kim TH. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour Technol* 2016;199:42–48.
34. Srivastava N, Rawat R, Oberoi HS, et al. A Review on Fuel Ethanol Production from Lignocellulosic Biomass. *Int J Green Energy* 2015;12:949–960.
35. Kumar P, Barrett DM, Delwiche MJ, et al. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res* 2009;48:3713–3729.
36. Li J, Zhang S, Li H, et al. Cellulase pretreatment for enhancing cold caustic extraction-based separation of hemicelluloses and cellulose from cellulosic fibers. *Bioresour Technol* 2018;251:1–6.
37. Venturin B, Camargo AF, Scapini T, et al. Effect of pretreatments on corn stalk chemical properties for biogas production purposes. *Bioresour Technol* 2018; 266:116–124.
38. Dias MOS, Cavalett O, Maciel RF, et al. Integrated first and second generation ethanol production from sugarcane. *Chem Eng* 2014;37:445–450.
39. Islam SMM, Li Q, Loman, et al. CO₂-H₂O based pretreatment and enzyme hydrolysis of soybean hulls. *Enzyme Microb Technol* 2017;106:18–27.
40. Aditiya HB, Mahlia, TMI, Chong WT, et al. Second generation bioethanol production: A critical review. *Renew Sust Energ Rev* 2016; 66:631–653.
41. Mupondwa E, Li X, Tabil L, et al. Status of Canada's lignocellulosic ethanol: Part II- Hydrolysis and fermentation technologies. *Renew Sust Energ Rev* 2017;79:1535–1555.
42. Hendriks ATWM and Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour Technol* 2009;100:10–18.
43. Stambuk BU, Eleutherio ECA, Florez-Pardo LM, et al. Brazilian potential for biomass ethanol: Challenge of using hexose and pentose co-fermenting yeast strains. *J Sci Ind Res* 2008;67:918–926.
44. Neto AC, Guimarães MJOC, Freire E. Business models for commercial scale second-generation bioethanol production. *J Clean Prod* 2018;184:168–178.
45. Souza RDFR, Dutra ED, Leite FCB, et al. Production of ethanol fuel from enzyme-treated sugarcane bagasse hydrolysate using d-xylose-fermenting wild yeast isolated from Brazilian biomes. *3 Biotech* 2018;8(7):312.

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46. Martins GM, Bocchini-Martins DA, Bezzerra-Bussoli C, et al. The isolation of pentose-assimilating yeasts and their xylose fermentation potential. *Braz J Microbiol* 2018;49:162–168.
47. Ohkuma M. Termite symbiotic systems: efficient biorecycling of lignocelluloses. *Appl Microbiol Biotechnol* 2003;61:1–9.
48. Watanabe H, Tokuda G. Cellulolytic systems in insects. *Annu Rev Entomol* 2010;55:609–632.
49. Willis JD, Oppert C, Jurat-Fuentes JL. Methods for discovery and characterization of cellulolytic enzymes from insects. *Insect Sci* 2010;17: 184–198.
50. Pauchet Y and Heckel DG. The genome of the mustard leaf beetle encodes two active xylanases originally acquired from bacteria through horizontal gene transfer. *Proc R Soc B* 2013;280:20131021.
51. Martin MM. The evolution of insect–fungus associations: from contact to stable symbiosis. *Am Zool* 1992;32:593–605.
52. Shelomi M, Heckel DG, Pauchet Y. Ancestral gene duplication enabled the evolution of multifunctional cellulases in stick insects (Phasmatodea). *Insect Biochem Mol Biol* 2016;71:1–11.
53. Shelomi M, Watanabe H, Arakawa G. Endogenous cellulase enzymes in the stick insect (Phasmatodea) gut. *J Insect Physiol* 2014;60:25–30.
54. Cairo JPLF, Oliveira LC, Uchima CA, et al. Deciphering the synergism of endogenous glycoside hydrolase families 1 and 9 from *Coptotermes gestroi*. *Insect Biochem Mol Biol* 2013;43(10):970–981.
55. Tokuda G, Lo N, Watanabe H, et al. Major alteration of the expression site of endogenous cellulases in members of an apical termite lineage. *Mol Ecol* 2004;13(10):3219–3228.
56. Tokuda G, Watanabe H, Hojo M, et al. Cellulolytic environment in the midgut of the wood-feeding higher termite *Nasutitermes takasagoensis*. *J Insect Physiol* 2012;58(1):147–154.
57. Calderón-Cortés N, Watanabe H, Cano-Camacho H, et al. cDNA cloning, homology modelling and evolutionary insights into novel endogenous cellulases of the borer beetle *Oncideres albomarginata* chamele (Cerambycidae). *Insect Mol Biol* 2010;19(3):323–336.
58. Liu J, Song K, Teng H, et al. Endogenous cellulolytic enzyme systems in the longhorn beetle *Mesosa myops* (Insecta: Coleoptera) studied by transcriptomic analysis. *Acta Biochim Biophys Sin.* 2015;47(9):741–748.
59. Hirayama K, Watanabe H, Tokuda G, et al. Purification and characterization of termite endogenous β -1,4-endoglucanases produced in *Aspergillus oryzae*. *Biosci Biotechnol Biochem* 2010;74(8):1680–1686.
60. Shimada K and Maekaw K. Changes in endogenous cellulase gene expression levels and reproductive characteristics of primary and secondary reproductives with colony development of the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). *J Insect Physiol* 2010;56(9):1118–1124.
61. Feng T, Liu H, Xu Q, et al. Identification and characterization of two endogenous β -Glucosidases from the termite *Coptotermes formosanus*. *Appl Biochem Biotechnol* 2015;176:2039–2052.
62. Li Y, Arakawa G, Tokuda G, et al. Heterologous expression in *Pichia pastoris* and characterization of a β -glucosidase from the xylophagous cockroach *Panesthia angustipennis spadica* displaying high specific activity for cellobiose. *Enzyme Microb Technol* 2017;97:104–113.
63. Uchima CA, Tokkuda G, Watanabe H, et al. Heterologous Expression in *Pichia pastoris* and characterization of an endogenous thermostable and high-glucose-tolerant β -glucosidase from the termite *Nasutitermes takasagoensis*. *Appl Environ Microbiol* 2012;78(12):4288–4293.
64. Uchima CA, Tokkuda G, Watanabe H, et al. Heterologous expression and characterization of a glucose-stimulated β -glucosidase from the termite *Neotermes koshunensis* in *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 2011;89:1761–1771.
65. Skory S, Freer SN, Bothast RJ. Expression and secretion of the *Candida wickerhamii* extracellular β -glucosidase gene, bglB, in *Saccharomyces cerevisiae*. *Curr Genet* 1996;30(5):417–422.
66. Kannan M, Suryaaathanmanathan V, Saravanakumar M, et al. Proteomic analysis of the silkworm midgut during larval-pupal transition. *Invertebrate Surviv J* 2016;13:191–204.
67. Kannan M, Ramya T, Anbalagan S, et al. Proteomic analysis of pupal gut serine protease of silkworm, *Bombyx mori*: Partial purification and biochemical characterization. *Biocatal Agric Biotechnol* 2017;12:159–165.
68. Ni J, Tokuda G, Takehara M, et al. Heterologous expression and enzymatic characterization of β -glucosidase from the drywood-eating termite, *Neotermes koshunensis*. *Appl Entomol Zool* 2007;42(3):457–463.
69. NCBI - National Center for Biotechnology Information. GeneBank. Sequences of β -glucosidase. 2019. Available at: <https://ncbi.nlm.nih.gov/gene>. (Last accessed March 2019).
70. Brune A. Termite guts: The world's smallest bioreactors. *Trends Biotechnol* 1998;16:16–21.
71. Taggar MS. Insect cellulolytic enzymes: Novel sources for degradation of lignocellulosic biomass. *JANS* 2015;7(2):625–630.
72. Ben Guerrero E, Arneodo J, Campanha RB, et al. Prospection and evaluation of (hemi) cellulolytic enzymes using untreated and pretreated biomasses in two Argentinean native termites. *PLoS One* 2015;10(8):e0136573.
73. Nakashima K, Watanabe H, Satoh H, et al. Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochem Mol Biol* 2002;32(7):777–784.
74. Tokuda G and Watanabe H. Hidden cellulases in termites: Revision of an old hypothesis. *Biol Lett* 2007;3(3):336–339.
75. Zhou X, Smith JA, Oi FM, et al. Correlation of cellulose gene expression and cellulolytic activity throughout the gut of the termite *Reticulitermes flavipes*. *Gene* 2007;395:29–39.
76. Ceja-Navarro JA, Karaouz U, Bill M, et al. Gut anatomical properties and microbial functional assembly promote lignocellulose deconstruction and colony subsistence of a wood-feeding beetle. *Nat Microbiol* 2019;doi: 10.1038/s41564-019-0384-y.
77. Arias-Cordero E, Ping L, Reichwald K, et al. Comparative evaluation of the gut microbiota associated with the below- and above-ground life stages (larvae and beetles) of the forest cockchafer, *Melolontha hippocastani*. *PLoS One* 2012;7(12):e51557.
78. Shi W, Xie S, Chen X, et al. Comparative genomic analysis of the endosymbionts of herbivorous insects reveals eco-environmental adaptations: Biotechnology applications. *PLoS One* 2013;9(1):e1003131.
79. Chen B, The B-S, Sun C, et al. Biodiversity and activity of the gut microbiota across the life history of the insect herbivore *Spodoptera littoralis*. *Sci Rep* 2016;6:29505.
80. Shukla SP, Sanders JG, Byrne MJ, et al. Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Mol Ecol* 2016;25: 6092–6106.
81. Mohammed WS, Ziganshina EE, Shagimardanova EI, et al. Comparison of intestinal bacterial and fungal communities across various xylophagous beetle larvae (Coleoptera: Cerambycidae). *Sci Rep* 2018;8:10073.
82. Todaka N, Nakamura R, Moriya S, et al. Screening of optimal cellulases from symbiotic protists of termites through expression in the secretory pathway of *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem* 2011;75(11):2260–2263.
83. Bashir Z, Kondapalli VK, Adlakha N, et al. Diversity and functional significance of cellulolytic microbes living in termite, pill-bug and stem-borer guts. *Sci Rep* 2013;3:2558.
84. Geib SM, Filley TR, Hatcher PG. Lignin degradation in wood-feeding insects. *PNAS* 2008;105(5):12932–12937.
85. Mabhegedhe M. Cellulolytic activities of the dung beetle, *Euoniticellus intermedius*, larva gut micro-flora. *Open Biotechnol J* 2017;11:105–113.
86. Shi W, Ding S-Y, Yuan JS. Comparison of insect gut cellulase and xylanase activity across different insect species with distinct food sources. *Bioenerg Res* 2011;4:1–10.

ALVES ET AL.

87. Langer M, Gabor EM, Liebeton K, et al. Metagenomics: An inexhaustible access to nature's diversity. *Biotechnol J* 2006;1(7–8):815–821.
88. Sleator RD, Shortall C, Hill C. Metagenomics. *Lett Appl Microbiol* 2008;47:361–366.
89. Jeon JH, Kim JT, Kim YJ, et al. Cloning and characterization of a new cold-active lipase from a deep-sea sediment metagenome. *Appl Microbiol Biotechnol* 2009;81(5):865–874.
90. Uchiyama T, Miyazaki K. Functional metagenomics for enzyme discovery: Challenges to efficient screening. *Curr Opin Biotechnol* 2009;20(6):616–622.
91. Guazzaroni M-E, Silva-Rocha R, Ward RJ. Synthetic biology approaches to improve biocatalyst identification in metagenomic library screening. *Microb Biotechnol* 2014;8(1):52–64.
92. Alma'abadi AD, Gojobori T, Mineta K. Marine metagenome as a resource for novel enzymes. *Genom Proteom Bioinf* 2015;13(5):290–295.
93. Li H, Dietrich C, Zhu N, et al. Age polyethism drives community structure of the bacterial gut microbiota in the fungus-cultivating termite *Odontotermes formosanus*. *Environ Microbiol* 2016;18(5):1440–1451.
94. Liu N, Li H, Chevrette MG, et al. Functional metagenomics reveals abundant polysaccharide-degrading gene clusters and cellobiose utilization pathways within gut microbiota of a wood-feeding higher termite. *ISME J* 2019;13:104–117.
95. Reid NM, Addison SL, Macdonald LJ, et al. Biodiversity of active and inactive bacteria in the gut flora of wood-feeding huhu beetle larvae (*Prionoplus reticularis*). *Appl Environ Microbiol* 2011;77(19):700–7006.
96. Scully ED, Hoover K, Carlson JE, et al. Midgut transcriptome profiling of *Anoplophora glabripennis*, a lignocellulose degrading cerambycid beetle. *BMC Genomics* 2013;14:850.
97. Warnecke F, Luginbühl P, Leadbetter JR, et al. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* 2007;560–565.
98. Gao G, Wang A, Gong B-L, et al. A novel metagenome-derived gene cluster from termite hindgut: encoding phosphotransferase system components and high glucose tolerant glucosidase. *Enzyme Microb Technol* 2016;84:24–31.
99. Hatefi A, Makhdoumi A, Asoodeh A, et al. Characterization of a bi-functional cellulase produced by a gut bacterial resident of Rosaceae branch borer beetle, *Osphranteria coerulea* (Coleoptera: Cerambycidae). *Int J Biol Macromol* 2017;103:158–164.
100. Li-Beisson Y, Peltier G. Third-generation biofuels: Current and future research on microalgal lipid biotechnology. *OCL* 2013;20(6):D606.
101. Balabanova L, Slepchenko L, Son O, et al. Biotechnology potential of marine fungi degrading plant and algae polymeric substrates. *Front Microbiol* 2018;9:1527.
102. Song J, Kim S-Y, Kim D-H, et al. Characterization of an inhibitor-resistant endo-1,4- β -mannanase from the gut microflora metagenome of *Hermetia illucens*. *Biotechnol Lett* 2018;40:1377–1387.
103. Loaces I, Schein S, Nova F. Ethanol production by *Escherichia coli* from *Arundo donax* biomass under SSF, SHF or CBP process configurations and in situ production of a multifunctional glucanase and xylanase. *Bioresour Technol* 2017;224:307–313.
104. Zhou J, Shi P, Zhang R, et al. Symbiotic *Streptomyces* sp. TN119 GH 11 xylanase: a new pH-stable, protease- and SDS-resistant xylanase. *J Ind Microbiol Biotechnol* 2011;38:523–530.
105. Xia D, Wei Y, Zhang G, et al. cDNA cloning, expression, and enzymatic activity of a novel endogenous cellulose from the beetle *Batocera horsfieldi*. *Gene* 2013;514(1):62–68.
106. Lee C-M, Lee Y-S, Seo S-H, et al. Screening and characterization of a novel cellulase gene from the gut microflora of *Hermetia illucens* using metagenomic library. *J Microbiol Biotechnol* 2014;24(9):1196–1206.
107. Usui K, Suzuki T, Akisaka T, et al. A cytoplasmic xylanase (XynX) of *Aeromonas caviae* ME-1 is released from the cytoplasm to the periplasm by osmotic downshock. *J Biosci Bioeng* 2003;95(5):488–495.
108. Suzuki T, Kitagawa E, Sakakibara F, et al. Cloning, expression, and characterization of a family 52 β -xylosidase gene (xysB) of a multiple-xylanase-producing bacterium, *Aeromonas caviae* ME-1. *Biosci Biotechnol Biochem* 2001;65(3):487–494.
109. Rashamuse K, Tendai WS, Mathiba K, et al. Metagenomic mining of glycoside hydrolases from the hindgut bacterial symbionts of a termite (*Trinervitermes trinervoides*) and the characterization of a multimodular β -1,4-xylanase (GH11). *Biotechnol Appl Biochem* 2017;64(2):174–186.
110. Liu C, Zou G, Yan X, et al. Screening of multimeric β -xylosidases from the gut microbiome of a higher termite, *Globitermes brachycaerastes*. *Int J Biol Sci* 2018;14(6):608–615.
111. Del Pozo MV, Fernández-Arrojo L, Gil-Martínez J, et al. Microbial β -glucosidases from cow rumen metagenome enhance the saccharification of lignocellulose in combination with commercial cellulase cocktail. *Biotechnol Biofuels* 2012;5:73.
112. Arantes V, Saddler JN. Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. *Biotechnol Biofuels* 2011;4:3.
113. Herlet J, Kornberger P, Roessler B, et al. A new method to evaluate temperature vs. pH activity profiles for biotechnological relevant enzymes. *Biotechnol Biofuels* 2017;10:234.
114. Basit A, Liu J, Miao T, et al. Characterization of two Endo- β -1, 4-Xylanases from *Myceliophthora thermophila* and their saccharification efficiencies, synergistic with commercial cellulase. *Front Microbiol* 2018;9:233.
115. Boekhout T. Gut feeling for yeasts. *Nature* 2005;434:449–450.
116. Ali SS, Wu J, Xie R, et al. Screening and characterization of xylanolytic and xylose-fermenting yeasts isolated from the wood-feeding termite, *Reticulitermes chinensis*. *PLoS One* 2017;12:1–21.
117. Harner NK, Wen X, Bajwa PK, et al. Genetic improvement of native xylose-fermenting yeasts for ethanol production. *J Ind Microbiol Biotechnol* 2015;42(1):1–20.
118. Cadete RM, Melo-Cheab MA, Dussán KJ, et al. Production of bioethanol in sugarcane bagasse hemicellulosic hydrolysate by *Scheffersomyces parashehatae*, *Scheffersomyces illinoensis* and *Spathospora arborariae* isolated from Brazilian ecosystems. *J Appl Microbiol* 2017;123:1203–1213.
119. Katsumata K, Jin Z, Hori K, et al. Structural changes in lignin of tropical woods during digestion by termite *Cryptotermes brevis*. *J Wood Sci* 2007;53:419–426.
120. Scharf ME and Tartar A. Termite digestomes as sources for novel lignocellulases. *Biofuels Bioprod Bioref* 2008;2:540–552.
121. Chandler JA, Eisen JA, Kopp A. Yeast communities of diverse *Drosophila* species: comparison of two symbiont groups in the same hosts. *Appl Environ Microbiol* 2012;78: 7327–7336.
122. Six DL. The bark beetle holobiont: Why microbes matter. *J Chem Ecol* 2013;39:989–1002.
123. Günther CS, Goddard MR. Do yeasts and *Drosophila* interact just by chance? *Fungal Ecol* 2019;38:37–43.
124. Dierks A, Fischer K. Feeding responses and food preferences in the tropical, fruit-feeding butterfly, *Bicyclus anynana*. *J Insect Physiol* 2008;54(9):1363–1370.
125. Lievens B, Hallsworth JE, Pozo MI, et al. Microbiology of sugar-rich environments: diversity, ecology and system constraints: Microbiology of sugar-rich environments. *Environ Microbiol* 2015;17:278–298.
126. Madden AA, Epps MJ, Fukami T, et al. The ecology of insect-yeast relationships and its relevance to human industry. *Proc R Soc B* 2018;285:1–8.
127. Nguyen NH, Suh SO, Marshall CJ, et al. Morphological and ecological similarities: woodboring beetles associated with novel xylose fermenting

BIOCATALYSTS IN INSECTS FOR 2G ETHANOL PRODUCTION

- yeasts, *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov. *Mycol Res* 2006;110:1232–1241.
128. Houseknecht JL, Hart EL, Suh SO, et al. Yeasts in the *Sugiyamaella* clade associated with wood-ingesting beetles and the proposal of *Candida bullrunensis* sp. nov. *Int J Syst Evol Microbiol* 2011;61:1751–1756.
 129. Guzmán B, Lachance MA, Herrera CM. Phylogenetic analysis of the angiosperm-floricolous insect–yeast association: Have yeast and angiosperm lineages co-diversified? *Mol Phylogenet Evol* 2013;68(2):161–175.
 130. Urbina H and Blackwell M. Multilocus phylogenetic study of the *Scheffersomyces* yeast clade and characterization of the n-terminal region of xylose reductase gene. *PLoS One* 2012;7(6):e39128.
 131. Molnárová J, Vadkertiová R and Stratilová E Extracellular enzymatic activities and physiological profiles of yeasts colonizing fruit trees. *J Basic Microbiol* 2014;54:574–584.
 132. Buzala KP, Kalinowska H, Przybysz P, et al. Conversion of various types of lignocellulosic biomass to fermentable sugars using kraft pulping and enzymatic hydrolysis. *Wood Sci Technol* 2017;51(4):873–885.
 133. Yu X, Bao X, Zhou C, et al. Ultrasound-ionic liquid enhanced enzymatic and acid hydrolysis of biomass cellulose. *Ultrason Sonochem* 2018;41:410–418.
 134. Kilikian BV, Afonso LC, Souza TFC, et al. Filamentous fungi and media for cellulase production in solid state cultures. *Braz J Microbiol* 2014;45: 279–286.
 135. Srivastava N, Srivastava M, Mishra PK, et al. Applications of fungal cellulases in biofuel production: Advances and limitations. *Renew Sust Energ Rev* 2018; 82:2379–2386.
 136. Chi Z, Chi Z, Liu G, et al. *Saccharomycopsis fibuligera* and its applications in biotechnology. *Biotechnol Adv* 2009;27:423–431.
 137. Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JHD. Cellodextrin transport in yeast for improved biofuel production. *Science* 2010;330: 84–86.
 138. Long TM, Su Y-K, Headman J, et al. Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. *Appl Environ Microbiol* 2012;78(16):5492–5500.
 139. Mussato SI, Machado EMS, Carneiro LM, et al. Sugar metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. *Appl Energy* 2012;92:763–768.
 140. Ren YC, Liu XJ, Yi ZH, et al. *Nematodospora anomalae* sp. nov., a novel and D-xylose-fermenting yeast species in the *Lodderomyces* clade. *Int J Syst Evol Microbiol* 2016;66:4046–4050.
 141. Wang Y, Ren YC, Zhang ZT, et al. *Spathaspora allomyrinae* sp. nov., a D-xylose fermenting yeast species isolated from a scarabeid beetle *Allomyrina dichotoma*. *Int J Syst Evol Microbiol* 2016;66:2008–2012.
 142. Mohd Azhar SH, Abdulla R, Jambo SA, et al. Yeasts in sustainable bioethanol production: A review. *Biochem Biophys Rep* 2017;10:52–61.
 143. Souza GFL, Valentim LTCN, Nogueira SRP, et al. Efficient production of second generation ethanol and xylitol by yeasts from Amazonian beetles (Coleoptera) and their galleries. *Afr J Microbiol Res* 2017;11(20): 814–824.
 144. Barretto DA, Avchar R, Carvalho C, et al. *Blastobotrys bombycis* sp. nov., a D-xylose-fermenting yeast isolated from the gut of the silkworm larva *Bombyx mori*. *Int J Syst Evol Microbiol* 2018;68:2638–2643.
 145. Santos RO, Cadete RM, Badotti F, et al. *Candida queiroziae* sp. nov., a cellobiose-fermenting yeast species isolated from rotting wood in Atlantic Rain Forest. *Antonie van Leeuwenhoek* 2011;99:635–642.
 146. Urbina H, Schuster J, Blackwell M. The gut of Guatemalan passalid beetles: A habitat colonized by cellobiose- and xylose-fermenting yeasts. *Fungal Ecol* 2013;6:339–355.
 147. Jeffries TW, Grogoriev IV, Grimwood J, et al. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat Biotechnol* 2007;25(3):319–326.
 148. Su YK, Willis LB, Jeffries TW. Effects of aeration on growth, ethanol and polyol accumulation by *Spathaspora passalidarum* NRRL Y-27907 and *Scheffersomyces stipitis* NRRL Y-7124. *Biotechnol Bioeng* 2015;112(3):457–469.
 149. Lee TH, Kim MY, Ryu YM, et al. Estimation of theoretical yield for ethanol production from d-xylose by recombinant *Saccharomyces cerevisiae* using metabolic pathway synthesis algorithm. *J Microbiol Biotechnol* 2001;11(3):384–388.
 150. Im KH, Nguyen TK, Choi J, et al. Ethanol production from various sugars and cellulosic biomass by white rot fungus *Lenzites betulinus*. *Mycobiology* 2016; 44(1):48–53.
 151. Basso LC, de Amorim HV, de Oliveira AJ, et al. Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Res* 2008;8(7):1155–1163.
 152. Stambuk BU, Dunn B, Alves Jr SL, et al. Industrial fuel ethanol yeasts contain adaptive copy number changes in genes involved in vitamin B1 and B6 biosynthesis. *Genome Res* 2009;19(12):2271–2278.
 153. Brown NA, Castro PA, Figueiredo BCP, et al. Transcriptional profiling of Brazilian *Saccharomyces cerevisiae* strains selected for semi-continuous fermentation of sugarcane must. *FEMS Yeast Res* 2013;13(3): 277–290.
 154. Della-Bianca BE, Basso TO, Stambuk BU, et al. What do we know about the yeast strains from the Brazilian fuel ethanol industry? *Appl Microbiol Biotechnol* 2013;97(3):979–991.
 155. Lopes ML, Paulillo SCL, Godoy A, et al. Ethanol production in Brazil: A bridge between science and industry. *Braz J Microbiol* 2016;47:64–76.
 156. Raghavendran V, Basso TP, da Silva JB, et al. A simple scaled down system to mimic the industrial production of first generation fuel ethanol in Brazil. *Antonie Van Leeuwenhoek* 2017;110(7):971–983.
 157. Saito H, Posas F. Response to hyperosmotic stress. *Genetics* 2012;192(2):289–318.
 158. Patel AK, Singhania RR, Pandey A. Production, purification, and application of microbial enzymes. In: Brahmachari G (ed). *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications*. Elsevier Inc. 2017;13–41.
 159. Srivastava A, Srivastava M. *Enzymes Market—Global Opportunity Analysis and Industry Forecast, 2017–2024* (2018). Available at: <https://alliedmarketresearch.com/enzymes-market> (Last accessed March 2019)
 160. Research and Markets. *Biofuel Enzymes Market—Forecasts from 2019 to 2024*. Available at: https://researchandmarkets.com/research/lkctfl/global_biofuel?w=4 (Last accessed March 2019).
 161. Fermentec. *Leveduras personalizadas*. Available at: www.fermentec.com.br/capa.asp?p=259. (Last accessed March 2019). 2019.
 162. LNF Latino Americana. *Soluções para açúcar e etanol*. Available at: <https://inf.com.br/acucar-e-etanol.html>. (Last accessed March 2019).
 163. Borges EP, Lopes ML, Bernardino CD et al. The benefits of applied research: 37 years of discoveries, adaptations and solutions. *Sugar Industry* 2015;140(4): 209–216.
 164. Amorim HV, Lopes ML, Paulillo SCL, et al. Yeast strains for ethanol production in Brazil: from laboratories to the industries. In: Treichel H, Alves Jr SL, Fongaro G, Müller C (eds). *Ethanol as a Green Alternative Fuel: Insight and Perspectives*. Nova Publishers 2019 pp. 93–106.
 165. Li YX, Yi P, Wang NN, et al. High level expression of β -mannanase (RmMan5A) in *Pichia pastoris* for partially hydrolyzed guar gum production. *Int J Biol Macromol* 2017;105(Pt 1):1171–1179.
 166. Cadete RM, Rosa CA. The yeasts of the genus *Spathaspora*: Potential candidates for second-generation biofuel production. *Yeast* 2018;35(2):191–199.
 167. Ding J, Liang G, Zhang K, et al. Extra metabolic burden by displaying over secreting: Growth, fermentation and enzymatic activity in cellobiose of recombinant yeast expressing β -glucosidase. *Bioresour Technol* 2018;254:107–114.
 168. Du C, Li Y, Zhao X, et al. The production of ethanol from lignocellulosic biomass by *Kluyveromyces marxianus* CICC 1727-5 and *Spathaspora passalidarum* ATCC MYA-4345. *Appl Microbiol Biotechnol* 2019;doi: 10.1007/s00253-019-09625-1.

ALVES ET AL.

169. Kwak S, Jo JH, Yun EJ, et al. Production of biofuels and chemicals from xylose using native and engineered yeast strains. *Biotechnol Adv* 2019;37(2):271–283.
170. Boyce A, Walsh G. Purification and characterization of a thermostable β -xylosidase from *Aspergillus niger* van tieghem of potential application in lignocellulosic bioethanol production. *Appl Biochem Biotechnol* 2018;186(3):712–730.
171. Jagtap SS and Rao CV. Microbial conversion of xylose into useful bioproducts. *Appl Microbiol Biotechnol* 2018;102(21):9015–9036.
172. Maehara L, Pereira SC, Silva AJ, et al. One-pot strategy for on-site enzyme production, biomass hydrolysis, and ethanol production using the whole solid-state fermentation medium of mixed filamentous fungi. *Biotechnol Prog* 2018;34(3):671–680.

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AU1: change ok?

AU2: change to units ok?