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Isolation and characterization of cellulolytic enzymes from the digestive fluid of *Rhynchophorus palmarum* larvae

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INTRODUCTION

Insects and lignocellulosic biomass

Despite the fact that many microorganisms can survive on cellulosic material, it was believed that different animals could not use cellulose. However numerous and increasing evidence has indicated that cellulolytic enzymes are distributed in a wide range of invertebrate animals and in some vertebrates, who use the cellulosic biomass with the help of intestinal symbionts (Martin et al., 1991; Bayané & Guiot, 2011). These animals are primarily ruminants and consume soft herbaceous plants and grasses, but they cannot digest woody plants. In contrast, the wood-eating insects, such as beetles, longhorn beetles and bark beetles, in addition to termites are well adapted to feed on wood (Ferreira et al., 2001; Bashir et al., 2013). Additionally, these insects have evolved through centuries, developing well adapted modifications such as specialized organs of mastication, intestinal digestive structures and symbiotic systems that allow them to thrive on cellulosic materials (Terra, 1990). Additionally, wood-boring insects have efficient microscale bioconversion systems able to degrade lignocellulosic biomass (Watanabe & Tokuda, 2010).

The diet of insects varies depending on the species and habitat and, therefore, insects have evolved through centuries to feed on diverse sources of nutrients, including vegetable tissues, to obtain the nutrients necessary for growth, development and reproduction (Behmer, 2009). This has led to a particular interaction between insects and plants, which means highly specialized ways of feeding in which specific enzymes are involved (Will et al., 2013). Additionally, some microorganisms have adapted to survive inside the gut of some insects and, as Azambuja et al., (2005) report, these microbial communities may exceed even the number of cells present on insect's intestine itself. On the other hand, these symbiotic organisms may grow freely in the lumen or attached to intestine walls of insects (Rosengaus et al., 2011).

Some insects have a specific diet of lignocellulose, varying from plant leaves to wood itself, requiring enzymatic degradation to feed on these polysaccharides and this process is carried out mostly in the hindgut of insects (Odelson & Breznak, 1983). This ubiquitous relation between insects and microbial communities in their guts allows them adapting to a variety of trophic niches (Sabree & Moran, 2014).

Microbiota composition is also wide, from maternally inherited intracellular bacteria to gut-inhabiting bacterial assemblages and even protozoa (Moran et al., 2008), but their contribution allows different substrates to be degraded to digestible molecules. Additionally,

the amount of microbial species may vary depending on the host. Thus, some insects have few bacterial species living in their guts whereas others harbor large groups of diverse bacterial strains (Engel & Moran, 2013). Moreover, the relationship between insects and their microbiota is not limited only to digestive functions, but also influences other aspects such as protection from potential predators and parasites, contributes to intra/inter specific communication and even reproduction (Lemaitre & Hoffmann, 2007; Feldhaar, 2011) and provides also a way to discover novel biocatalysts that eventually would allow the degradation of biomass (Shi et al., 2013).

Insects: a promising source of cellulolytic enzymes

Around the world, insects represent the largest biomass of all terrestrial animals (Gutierrez, 2012). Additionally, insects play key roles in a wide variety of processes from the ecosystem stabilization to waste decomposition and even as source of some industrial or medicinal products (Barry, 2004; Choo, 2008; Capinera, 2008). Furthermore, as Shi et al., (2013) report, insects show a well-known adaptability to different environments. On the other hand, the Ecuadorian Amazon Region has a large tradition on entomophagy. In fact, Paoletti and Dufour (2002) report information on entomophagy of 39 ethnic groups, which represent about 21,4 per cent of the 182 groups known in the Amazon basin. Consequently, the knowledge of relations between native people and ecosystem represents the base for natural and cultural biodiversity preservation.

Among the edible insect species found in the Ecuadorian Amazon region there are *Rhynchophorus palmarum* larvae (DeFoliart, 1992; Paoletti & Dufour, 2002) which are considered a favorite dish by indigenous people, but their consumption has also been extended to city settlers (Gonzales & García, 1993). However, insects are not only a source of nutrients, but they may also be used with biotechnological and industrial purposes (Ramos Elorduy & Pino Moreno, 2009). On the other hand, insects are well-known hosts of a wide variety of microorganisms in their gut (Dillon & Dillon, 2004).

As Mika et al., (2013) report, endosymbiont microbiota plays an essential role in contributing to degrade diverse nutrient sources that insects feed on, by establishing biological and chemical systems where microbial enzymes are a key element.

Among the mentioned enzymes there are cellulases, and due to specific diet some insects have evolved to use cellulosic substrates as source of energy, employing a very complex

pool of enzymes (Martin, 1983; Willis et al., 2010). However, despite some efforts carried out, the relationship between insects and their microbiota, particularly in degradation of cellulose, remains poorly understood even though cellulases from insects may allow the effective use of biomass as a source of energy (Watanabe & Tokuda, 2010).

Research shows that some wood-feeding insects, particularly beetles, depend on a highly specialized pool of enzymes to degrade and efficiently digest cellulose and hemicellulose (Pauchet et al., 2014) and more recently some cellulolytic enzymes have been isolated from the gut of adult and larval stages of beetles, termites and other insects (Tokuda & Watanabe, 2007; Shi et al., 2013). Additionally, some bacteria are crucial for insect nutrition, through provisioning of specific nutrients (Douglas, 2013).

On the other hand, cellulose is a carbohydrate polymer of glucose subunits connected by β-1,4-linkages (O'Sullivan, 1997) and it is the primary structural material of plant cell walls. It is a linear polymer synthesized by higher plants with relative fixing of 6.0-6.3 x 10^{16} g of carbon per year. Natural degradation of the lignocellulosic material is carried out mainly by fungi and bacteria due to hydrolytic and oxidative enzymes which are able to synergistically degrade cellulose, hemicellulose and lignin (Ghio et al., 2012). Thus, insects become potential candidates for the research on new cellulolytic enzymes, because different species of insects are closely adapted to feed on vegetable tissues. Additionally, since the composition of lignocellulose is stretchily related to the plant species and even to the constitution of the different vegetable tissues, insects exhibit a variety of physiological, ethological and synergistic adaptations to feed on lignocellulose and certainly one of these adaptations is expressed in the interaction between insects and their intestinal microbiome in which cellulolytic microorganisms play a key role.

There are many researches focusing on cellulolytic activity in insects (Wharton & Wharton, 1965; Ishaaya & Plaut, 1974; Martin, 1983; Watanabe et al., 1997; Marana et al., 2000 Khademi et al., 2002; Micó et al., 2011), but despite the fact that identification and even cloning of cellulolytic enzymes has been done (Watanabe, et., 1998; Lee et al., 2005; Wei et al., 2006; Kim et al., 2008), only in few cases these enzymes have been characterized (Cazemier et al., 1997) and there is evidence suggesting that both endosymbiont microbiota and insects themselves are responsible for the production of cellulolytic enzymes (Martin et al., 1991; Li et al., 2009).

Furthermore, current world demands the development of industrial-scale processes able to produce fuel from renewable biological resources as an alternative to fossil fuels (Willis et

al., 2010) and today, second-generation bioethanol made from lignocellulosic biomass is considered one of the most promising biofuel (Himmel et al., 2007; Li et al., 2009). The term second-generation biofuels is related to the wide variety of non-food crops proposed for its production. Concerns about potential competition for food crops from which first-generation biofuels are made from, stimulated the development of second-generation biofuels, particularly from lignocellulosic biomass. However, the enzymatic hydrolysis of cellulose leads to an increasing need for hydrolysis technologies that make the production of lignocellulosic ethanol a cost-efficient process (Sims et al., 2010). However, the natural resistance of plant cell walls to microbial and enzymatic degradation, called "biomass recalcitrance" is an essential reason of the high cost of lignocellulose conversion (Himmel, et al. 2007).

Enzymatic degradation is considered an established method to improve the lignocellulose conversion (Zaldivar et al., 2001; Srivastava et al., 2015) and eventually, the reduction of half the amount of enzymes necessary for the process would succeed in decreasing the cost of the process by around 13% (Lynd et al., 2008). However, though the cellulases play a key role in plant-cell-wall polysaccharides degradation, the reduced stability and low performance under industrial conditions show the need for novel cellulases that perform better at large-scale productions (Deejing & Ketkorn, 2009).

The taxonomic orders with cellulolytic activity are Isoptera (Martin & Martin, 1978; Slaytor, 1992; Nakashima et al., 2002), Thysanura (Treves & Martin, 1994), Blattodea (Scrivener et al., 1989; Zhang et al., 1993; Grandcolas & Deleporte, 1996), Lepidoptera (Anand et al., 2010) and most notably Coleoptera (Bayon, 1980; Chararas et al., 1983; Kudor & Martin, 1986; Kudor et al., 1988; Prins & Kreulen, 1991; Genta et al., 2006; Lundgren et al., 2007; Huang et al., 2012). A low number of bacteria in the intestinal regions showed cellulolytic activity in some species of Orthoptera and Phasmatodea (Cazemier et al., 1997). As detailed in Table 1, studies have been conducted in several insect species in order to determine their alleged cellulolytic enzyme activity, using culture media with carboxy-methyl-cellulose (CMC) and microcrystalline cellulose (MMC) (Oppert et al., 2010).

Table 1. Taxonomy, life stage and diet of insects studied for their cellulolytic activity (Oppert et al., 2010).

Order: Family: Subfamily: Genus species[a] (taxonomic authority)	Life-stage(s) screened	Food resource(s)[b] (plant tissues)[c]	Tested replicates CMC, MCC (individuals)[d]	
			Gut fluid	Head fluid
Dictyoptera (suborder Blattaria)				
Cryptocercidae				
Cryptocercus [prob. punctulatus Scudder]	Ad/Ny	Generalist-B, C (W)	3 (3), 3 (3)	
Isoptera				
Rhinotermitidae				
Reticulitermes hageni Banks	Ny[e]	Generalist-B, C (W)	6 (120), 5 (100)	5 (166), 4 (128)
Orthoptera				
Acrididae				
Cyrtacanthacridinae				
Melanoplus differentialis (Thomas)	Ad	Generalist-B, G (S, L)	9 (9), 8 (8)	7 (8), 6 (7)
Melanoplus femurrubrum (DeGeer)	Ad	Generalist-B, G (S, L)	11 (23), 6 (11)	26 (99), 0 (0)
Schistocerca americana (Drury)	Ad	Generalist-B, G (S, L)	7 (7), 4 (4)	6 (8), 4 (5)
S. damnifica (Saussure)	Ad	Generalist-B, G (S, L)	3 (3), 3 (3)	
Gomphrocerinae				
Dicromorpha viridis (Scudder)	Ad	Generalist-G (S, L)	5 (6), 3 (4)	
Syrbula admirabilis (Uhler)	Ad	Generalist-G (S, L)	16 (30), 11 (28)	11 (28), 8 (23)
Oedepodinae				
Chortophaga viridifasciata DeGeer	Ad	Generalist-G (S, L)	14 (42), 6 (18)	13 (50), 11 (44)
Hippiscus ocelote (Saussure)	Ad	Generalist-G (S, L)	12 (N.a.), 8 (N.a.)	10 (N.a.), 4 (N.a.)
Spharagemon bolli Scudder	Ad	Generalist-G (S, L)	3 (N.a.), 0 (0)	
Gryllidae				
Gryllinae				
Gryllus [prob. pennsylvanicus Burmeister]	Ad	Omnivore (S, L)	4 (5), 4 (5)	
Nemobiinae				
Allonemobius [pr. socius (Scudder)]	Ad/Ny	Omnivore (S, L)	17 (57), 8 (17)	6 (39), 0 (0)
Allonemobius [pr. fasciatus (DeGeer)]	Ad/Ny	Omnivore (S, L)	11 (N.a.), 6 (16)	
Psychidae				
Thyridopteryx ephemeraeformis	Lv	Generalist-C, B (L)	4 (11), 4 (11)	
Tettigoniidae				
Conocephalinae				
Conocephalus strictus (Scudder)	Ad/Ny	Generalist-G (L)	3 (4), 0 (0)	
Orchelimum vulgare (Harris)	Ad/Ny	Generalist-B, G (L)	5 (12), 5 (12)	
Copiphorinae				
Neoconocephalus triops (L.)	Ad	Generalist-B, G (L)	4 (4), 3 (3)	
Phanopterinae				
Microcentrum retinerve (Burmeister)	Ad	Generalist-B (L)	3 (3), 0 (0)	
Scudderia [pr. curvicauda (DeGeer)]	Ad	Generalist-B (L)	7 (7), 6 (6)	
Scudderia furcata Brunner	Ad	Generalist-B (L)	4 (4), 3 (3)	
Coleoptera				
Buprestidae				
Chrysobothris sp.	Lv	Generalist-B (hardwoods) (W)	3 (3), 0 (0)	
Cerambycidae				
Elaphidion mucronatum (Say)	Lv	Generalist-B (ex. Cerciscanadensis L.) (W)	5 (14), 4 (12)	
Neoclytus a. acuminatus (Fabricius)	Lv	Generalist-B (ex. Diospyros sp.) (W)5 (8), 3 (5)		
cerambycid sp.	Lv	Undet. (ex. Acer saccharinum L.) (W)	4 (45), 3 (44)	
Curculionidae				
Graphognathus leucoloma	Ad	Generalist-B (W, R, L)	6 (14), 4 (5)	
Scolytinae				
Scolytus [prob. rugulosus (Müller)]	Lv	Ex. Prunus sp. (W)	5 (107), 5 (N.a.)	
Diprionidae				
Neodiprion lecontei	Lv	Ex. Pinus mugho (N)	13 (N.a.), 12 (N.a.)	
Lyctidae				
Lyctus [prob. planicollis Lec.]	Ad/Ny	Generalist (W)	7 (29), 6 (27)	
Scarabaeidae				
Phyllophaga sp.	Lv	Generalist-B, G (L, R)	9 (24), 7 (18)	4 (24), 3 (18)
Tenebrionidae				
Tenebrio molitor	Lv	Generalist[f] (W, R)	6 (N.a.), 12 (N.a.)	3 (21), 3 (21)
Tribolium castaneum	Ad	Generalist[f] (W, R)	3 (45), 3 (45)	3 (51), 0 (0)
Diptera				
Cecidomyiidae				
Monarthropalpus flavus (Schank)	Lv	Ex. Buxus sp. (L)	6 (342), 6 (342)	
Lepidoptera				
Amphisbatidae				
Psilocorsis cryptochiella (Chambers)	Lv	Fagaceae (L)	4 (13), 4 (13)	
Arctiidae				
Arctiinae				
Halysidota tessellaris (J.E. Smith)	Lv	Generalist-B (L)	3 (3), 3 (3)	
Hyphantria cunea (Drury)	Lv	Generalist-B (L)	4 (21), 3 (17)	
Crambidae				
Pyraustinae				
Saucrobotys futilalis (L.)	Lv	Apocynum sp. (L)	5 (20), 5 (20)	
Galacticidae				
Homadaula anisocentra Meyrick	Lv	Albizzia & Gleditsia sp. (L)	6 (30), 6 (30)	

Order: Family: Subfamily: Genus species[a] (taxonomic authority)	Life-stage(s) screened	Food resource(s)[b] (plant tissues)[c]	Tested replicates CMC, MCC (individuals)[d]	
			Gut fluid	Head fluid
Gelechiidae				
Fascista cercerisella (Chambers)	Lv	Cercis sp. (L)	8 (78), 8 (78)	
Hesperiidae				
Pyrginae				
Epargyreus clarus (Cramer)	Lv	Fabaceae (L)	3 (3), 0 (0)	
Lasiocampidae				
Lasiocampinae				
Malacosoma americana (Fabr.)	Lv	Ex. Prunus sp. (L)	5 (32), 5 (32)	4 (32), 4 (32)
Megalopygidae				
Norape ovina (Sepp)	Lv	Generalist-B (L)	9 (14), 8 (12)	
Noctuidae				
Heliothinae				
Heliothis virescens (Fabricius)	Lv	Generalist-B, G (S, L)[f]	4 (7), 4 (7)	10 (77), 0 (0)
Saturniidae				
Ceratocampinae				
Anisota senatoria (J.E. Smith)	Lv	Quercus sp. (L)	4 (8), 0 (0)	
Anisota virginiensis Drury	Lv	Quercus sp. (L)	4 (4), 4 (4)	
Notodontidae				
Phalerinae				
Datana contracta Walker	Lv	Quercus sp. (L)	3 (10), 3 (10)	
D. integerrima (Grote and Robinson)	Lv	Juglandaceae (L)	6 (19), 5 (16)	3 (15), 3 (15)
Nymphalidae				
Danaina				
Danaus plexippus L.	Lv	Apocynaceae (L)	7 (12), 7 (12)	
Heliconiinae				
Agraulis vanillae L.	Lv	Passiflora sp. (L)	11 (28), 10 (25)	
Nymphalinae				
Junonia coenia Hübner	Lv	Generalist-B (S, L)	3 (3), 3 (3)	
Papilionidae				
Battus philenor L.	Lv	Aristolochia sp. (L)	5 (7), 5 (7)	
Pyralidae				
Galleriinae				
Omphalocera munroei Martin	Lv	Asimina sp. (L)	6 (11), 5 (10)	
Sesiidae				
Melittia satyriniformis	Lv	Cucurbita sp. (R, S)		3 (N.a.), 3 (N.a.)
Synanthedon exitiosa (Say)	Lv	Prunus sp. (W)	4 (11), 4 (11)	
S. scitula (Harris)	Lv	Generalist-B (W)	10 (78), 8 (70)	5 (61), 5 (61)
Sphingidae				
Macroglossinae				
Hemaris diffinis (Boisduval)	Lv	Lonicera sp. (L)	4 (8), 3 (7)	3 (8), 3 (8)
Tortricidae				
[poss. Archips sp]	Lv	Ex. Urtica sp. (L)	3 (4), 0 (0)	
Yponomeutidae				
Attevinae				
Atteva punctella (Cramer)	Lv	Ailanthus sp. (L)	3 (7), 3 (7)	
Hymenoptera				
Tenthredinidae				
Allantinae				
Allantus cinctus (L.)	Lv	Rosa sp. (L)	7 (33), 5 (25)	3 (38), 3 (38)
Macremphytus tarsatus (Say)	Lv	Cornus sp. (L)	7 (48), 6 (43)	
Nematinae				
Cladius difformis (Panzer)	Lv	Rosa sp. (L)	3 (15), 3 (15)	

Life-stage sampled (Ad. = adult, Lv./Ny. = larvae/nymphs).
(B = broadleaf forbs, G = grasses, C = conifers, undet. = undetermined).

[a] Several specimens were collected either as larvae in an uncharacteristic developmental stadium or for which insufficient descriptive keys could be found, thus not all individuals could be conclusively identified. When adult insects could not be reared from the host plant, genera were confirmed and tentative identifications were made [denoted by brackets]. Bracketed names represent the most probable species identities based either on preferred host plant range, insect's reported geographic distribution or local occurrence evidenced by pinned specimens preserved in the University of Tennessee's Institute of Agriculture insect collection.

[b] Plant tissues commonly consumed by sampled life-stage (L = leaves and leaf petioles, N = needles, R = roots, S = stems, W = wood).

[c] ex. = Host plant species from which sampled larvae were recovered.

[d] Number of individuals for all biological replicates. N.a. = data not available.

[e] Samples included larvae from both alate and worker castes and excluded soldiers.

[f] Collected from laboratory cultures reared on artificial diet.

The importance of microbial cellulolytic enzymes

Cellulose is widely used as a raw matter for a variety of industrial processes, and recently it has gained much more importance as a substrate for the production of biofuels, single cell proteins and various other chemicals through enzymatic degradation by microbial cellulases (Goyal & Soni, 2011).

Throughout the world, several microorganisms have been identified as producers of these enzymes, including fungi and bacteria (Nakashima et al., 2002; Narasimha et al., 2006). The conversion of cellulose into low-weight fermentable sugars requires an enzymatic complex

that combines β-1,4-endoglucanase (EC 3.4.1.4), β-1,4-exoglucanase (EC 3.2.1.91) and β-1,4-glucosidase (EC 3.2.1.21) (O'Sullivan, 1997; Alvarez et al., 2013).

The growing need of cellulose-derived substances means thus the increment in scientific research to develop novel biocatalysts able to degrade the cellulose-rich biomass, not only as source of fermentable sugars for biofuels, but also as feedstock for chemical conversion at industrial levels (Lynd et al., 2002; Bhalla,et al., 2013). In addition, the permanent research on cellulases, pectinases and hemicellulases tends to increase our knowledge about their interaction with substrates and so makes sense of their biotechnological importance (Bhat, 2000; Haki & Rakshit, 2003; Kuhad et al., 2011).

Research on cellulases began around the early 1950's considering the prospect of cellulosic biomass as energy source (Coughlan, 1992). However, over the next decades studies showed results regarding the low effectiveness and uneconomical aspects of cellulose fermentation (Walker & Wilson, 1991). However, despite the lack of initial success, fundamental researches along with applications of cellulases at industrial level grew through decades, particularly in in the textile, paper and food industries (Cavaco-Paulo, 1998; Kirk, et al, 2002; Weber, et al, 2010).

Furthermore, along with already existing research upon cellulases worldwide, scientific concern about the ecological consideration of burning fossil fuels and its effects on increasing amounts of CO_2 in the atmosphere brought a renewed international interest on lignocellulose as raw matter for biofuels (Bhalla et al., 2013; Hsu et al., 2011; Zhang et al., 2012). The variety of processes in which cellulases are needed led to a major demand of these enzymes from industries such as brewery, laundry, pulp and even for agricultural purposes. Indeed, as Bhat (2000) reports, the demand of cellulases is growing more rapidly than ever before.

As Saratale & Oh (2012) report, the increment of human population would eventually reach 8 billion by 2030 and hence the worldwide demand of energy would then increase by 42%. This perspective of our dependence on fossil fuels comprises shortcomings to develop novel energy sources to reduce either necessity of these fuels or their adverse impact over greenhouse effect. This situation makes lignocellulose very attractive for energy production and demands further research on cellulases to allow the development of a cost-effective conversion of lignocellulosic biomass into biofuels and other derivatives (Hsu et al., 2011; De Souza, 2013)

Additionally, some microorganisms are described to produce enzymes able to degrade lignocellulose. These microorganisms may be either aerobic or anaerobic as well as mesophiles or thermophiles (Haki & Rakshit, 2003). Some particular fungal and bacterial genera are well known cellulase-producers, such as *Aspergillus, Penicillium, Cellulomonas, Clostridium, Acetobacter,* and *Terendinibacter* (Watanabe et al., 2000; Wang et al., 2005; Maki et al., 2009). The diversity of cellulolytic microorganisms means that the specific enzymes they produce are widely diverse as well, so we can find bacterial genera such as *Acetovibrio, Bacillus* and *Cellulomonas* producing cellulases effectively, though some anaerobic species of *Clostridium* and *Bacteroides* produce highly-specific-activity cellulases but at a lower growing rate (Saratale et al., 2008).

On the other hand, although the production lignocellulosic biofuels such as ethanol is the principal aim of most of the research on microbial cellulolytic enzymes, it also comprises a new field of scientific interest concerning alternative energy sources, particularly the production of biohydrogen through dark-fermentation procedures (Saratale et al., 2008; Chong et al., 2009). In addition, most of the cellulolytic enzymes available at biotechnological level come from mesophilic bacteria and fungi. However, recent studies show that some extremophilic microorganisms, are another good source of cellulases, capable to cope with current chemical pretreatment procedures applied to lignocellulose (Haki & Rakshit, 2003; Wang et al., 2009; Saratale & Oh, 2011).

The need of novel cellulases is also due to the intricate degradation of lignocellulose, due to its particular characteristics that render it impossible to be degraded by a single enzyme as it is carried out in corn-derived ethanol. This requires, instead, a complex of multiple synergistic enzymes able to degrade lignocellulose biomass to fermentable sugars (Lee, 1997; Goho, 2008). Additionally, it is well known that, at biological level, lignocellulose is naturally degraded by those organisms with a specific diet containing cellulose or other high-density polysaccharides. Henceforth, since the 1960's some cellulolytic bacteria have been isolated from cattle rumen (Dehority, 1965) Whereas, some invertebrates such as termites and beetles have even more specific lignocellulose-based diets which could only be degraded by highly specialized microbial enzymes (Bashir et al., 2013).

Evolution also led to changes in the way microorganisms degrade lignocellulosic biomass. The enzymatic activity and the effectiveness in degrading lignocellulose will vary depending on the amount of lignin found on the biomass (De Souza, 2013). Nonetheless, the complex molecular nature of cellulose associated with hemicellulose and lignin makes it

hard to biodegrade (Anwar, Gulfraz, & Irshad, 2014). However, some microorganisms, particularly basidiomycetes and actinomycetes are quite effective in degrading lignin to achieve a more efficient lignocellulosic degradation (El-Gammal et al., 1998).

Composition of lignocellulosic biomass

Lignocellulosic biomass is the most abundant organic matter on Earth. It refers to plant dry matter composed of long chain carbohydrate polymers containing five and/or six carbon sugar monomers (cellulose, hemicellulose) which are tightly bound to an aromatic polymer (lignin) (Malherbe & Cloete, 2002). Cellulose and hemicellulose, typically comprise two-thirds of the dry mass of lignocellulose, and both can be hydrolyzed to sugars and eventually fermented to ethanol, whilst lignin cannot be used for ethanol production (Lee, 1997; Hamelinck et al., 2005).

Cellulose

In plant cell walls cellulose is formed of thousands β-1,4-linked glucose molecules. The β-glycosidic chains are hydrogen-bonded. Cellulose in higher plants is organized into elongated subunits called micelles, which are further arranged into larger structures called microfibrils, each measuring about 3 to 6 nm in diameter and containing up to 36 cellulose chains (Figure 1) (Lynd et al., 2002). On the other hand, despite being a polymer, cellulose possesses a particular crystalline structure. However, as the single fibers are not crystalline themselves, it creates amorphous regions, allowing the penetration of relatively large molecules including enzymes. Additionally, cellulose microfibrils are bonded among them with hydrogen linkages and tightly packaged by hemicellulosic polymers such as mannan and xylan which are merged with cellulose either by hydrogen or covalent bonds extremely resistant to chemical and biological degradation (Coughlan, 1992). Furthermore, those amorphous regions within the cellulose crystalline structure are very heterogeneous with a diversity of bonds and shall become essential during the biodegradation process (Malherbe & Cloete, 2002).
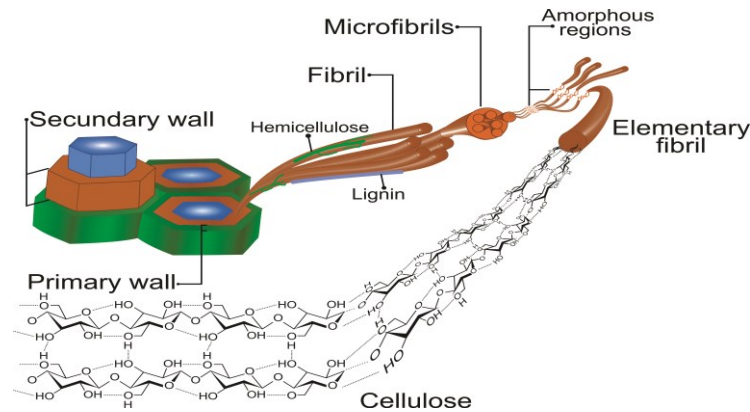
Figure 1. Arrangement of fibrils, microfibrils and cellulose in cell walls (Rojas et al., 2015)

Hemicellulose

Hemicellulose is a hetero-polymer consisting of various arabino-xylan saccharides. It has a random, low-strength, amorphous structure, rich of branches, which are very easy to be removed from the main stem and hydrolyzed either by dilute acid or base as well as by hemicellulases (Yang et al., 2007).

Hemicelluloses from woody plants are constructed from a few select sugar residues, commonly D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-D-glucuronic acid, D-galacturonic acid, and D-glucuronic acid (Figure 2). In contrast, the hemicellulose sugar residues, found in grasses and cereals, are mostly D-xylose, L-arabinose, D-glucose and D-galactose (Puls, 1997). Furthermore, the hemicelluloses possess a great variety of linkages and branching types, depending on the species and the tissue within a single species (Bauer et al., 1973).

Hardwood hemicelluloses contain mostly xylans, whereas softwood contains mostly glucomannans (Shibuya & Misaki, 2014). Xylans of many vegetable tissues are heteropolysaccharides with homopolymeric backbone chains of 1,4-β-D-xylanopyranose units (Aspinall, 1959; Timell, 1965). It is known that xylan may play a significant role in structural integrity of cell walls, due to both covalent and non-covalent bonds (Bastawde, 1992; Thomson, 1993; Saha, 2003)
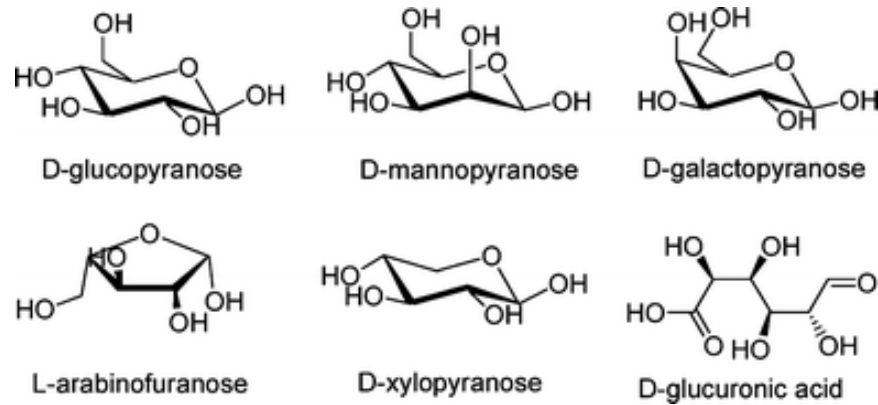
Figure 2. Monomers in hemicellulose (Gandini, 2011).

Lignin

Lignin is a generic term used to refer to a diverse group of aromatic polymers resulting from the oxidative combination of 4-hydroxyphenylpropanoids. These aromatic alcohols are also known as monolignols (Vanholme et al., 2010). Lignification of plant cell walls begins with formation of the secondary wall. Starting from the lamella, it impregnates both the primary and secondary walls filling the spaces between cellulose, hemicellulose, and pectin and linked to them through covalent bonds (Figure 3), resulting in a strong arrangement that confers stiffness to vegetable tissues and protects cell wall against depolymerization (Pearl, 1967).

However, the synthesis of lignin is a very complex process and in contrast with the other components of plant cell walls, its polymerization is still subject to study. The biosynthesis of lignin is guided by the oxidative coupling of three types of repeating units: coumaryl (H), guaiacil (G), and syringyl (S). They are derived from known monolignols, *p*-coumaric, coniferyl and sinapyl alcohols (Vanholme et al., 2008). However lignin may also derive from other monomers and conjugates different to those mentioned (Chabannes et al., 2001).
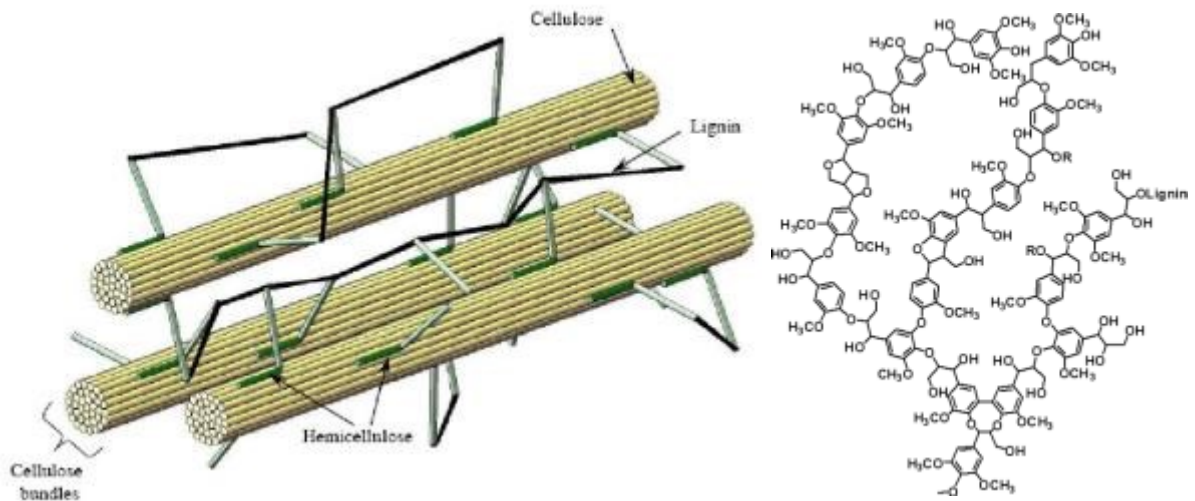
Figure 3. Lignin structure and its interaction with cellulose and hemicellulose. Adapted from (Tabil et al., 2011)

Enzymatic degradation of lignocellulosic biomass

The abundance of lignocellulosic biomass makes it attractive for energy purposes, as a potential source of fermentable sugars that can be further transformed into biofuels (Nathan Mosier et al., 2005). However, its complex structure and the recalcitrance of lignocellulosic biomass represent a barrier to overcome prior to its feasible fermentation.

Nevertheless, not only biofuels are available to be obtained from lignocellulosic biomass, but another chemical feedstock as well, making it a low-cost and uniquely sustainable resource (Wyman, 2003). On the other hand, as fossil fuel sources are rapidly going scarce, it becomes necessary to develop procedures that render lignocellulosic transformation economically sustainable to compete with fossil fuels (Lynd et al., 2008).

Hence, the degradation of lignocellulosic biomass requires pretreatment to transform the complex polysaccharides into monomers able to be fermented. In this sense, pretreatment of lignocellulosic biomass is a key step. The current fundamental pretreatments are the steam explosion, the enzymatic hydrolysis and the use of diluted or concentrated acids (Mosier et al., 2005; van Maris et al., 2006). Additionally some other chemicals such as organic solvent, ammonia, sulfur dioxide may also be used to improve enzymatic degradation of lignocellulosic biomass (Deejing & Ketkorn, 2009).

On the other hand, the different components of lignocellulosic biomass do not degrade in the same way through pretreatment process. For instance, hemicellulose can be readily

hydrolyzed by dilute acids under moderate conditions, but cellulose hydrolysis requires even more extreme pressure and temperature conditions and the yields of cellulose obtained would be conditioned by these factors (Kumar et al., 2009).

The purpose of pretreatment is to disrupt the highly polymerized structure of lignocellulosic biomass and render it more hydrolysable or bioavailable (Hendriks & Zeeman, 2009). However, as mentioned before, the difference on the now-available hydrolysis techniques employed is funded in retention times, the procedure conditions and the yield of fermentable sugars (Girard & Fallot, 2006). Therefore, these and some other structural and compositional factors hinder the pretreatment process.

The use of enzymes for pretreatment of lignocellulosic biomass is well known. In fact, it is a promising pretreatment method (Lynd et al., 2002). In this sense, cellulolytic enzymes produced by a variety of microorganisms are widely studied in order to improve enzymatic degradation (Figure 4). Furthermore, because of its insolubility in water, the use of enzymes as pretreatment entails direct contact between the substrate and the enzyme (Hendriks & Zeeman, 2009).
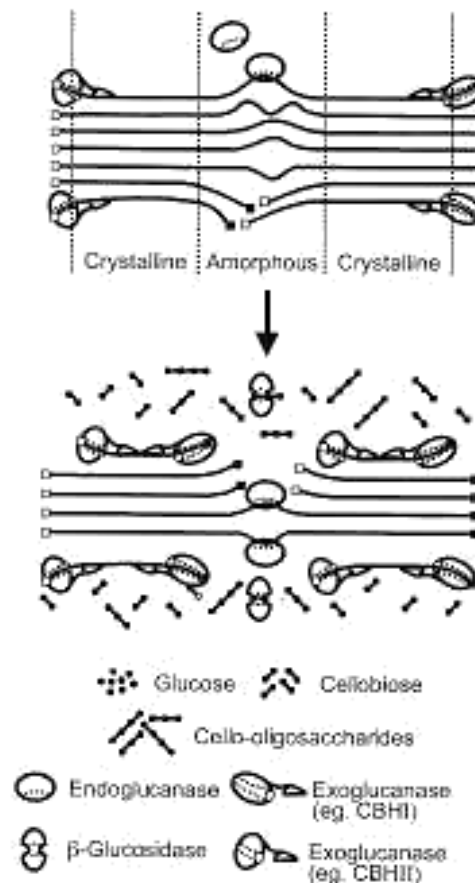

Figure 4. Scheme of an enzymatic cellulose hydrolysis (Lynd et al., 2002).

However, as Chang, et al., (2001) report, enzymatic degradation of lignocellulosic biomass may also be hampered by the crystallinity of cellulose, the degree of polymerization, moisture content, lignin content and even the surface area.

On the other hand, the biological pretreatment offers advantages in relation with other pretreatment methods, as the majority of them require expensive facilities, high amounts of energy and specialized materials or equipment (Kumar et al., 2009). Biological pretreatment is been considered a low-impact method as it does not demand high energy and performs delignification process particularly well (Okano, et al., 2005).

The biological pretreatment is usually performed by fungi, but even bacteria are suitable to be used for this purpose. Furthermore, commercial preparations of bacterial and fungal cellulolytic enzymes can be readily used in this process (Mtui, 2009).

Bacterial pretreatment also involves both aerobic and anaerobic conditions. Strictly anaerobic pretreatment is carried out mostly by mesophilic rumen-derived bacteria, whilst employing aerobic-anaerobic combined pretreatment may render more efficient lignin degradation (Z.-H. Hu et al., 2008). On the other hand, strictly aerobic systems use actinomycetes such as *Streptomyces griseus* or genetically-modified strains of *Escherichia coli* and *Klebsiella oxytoca*, with high yields of extracellular cellulolytic enzymes (Arora, et al., 2005; Peterson & Ingram, 2008).

Meanwhile, the nature of vegetable tissues itself entails another obstacle: the proportion of the basic components of lignocellulosic biomass (cellulose, hemicellulose and lignin) is not specific and the degree of mixture of the mentioned components supposes the use of particular enzymes to achieve deserved levels of depolymerization (Ammary, 2004).

Therefore, a complex combination of enzymes is required during pretreatment process. Endoglucanases (endo-1,4-glucanohydrolase) acts over the low crystalinity regions of cellulose fibbers exposing the free chain ends. Exoglucanase or cellobiohydrolase (1,4-β-glucan cellobiohydrolase) then removes cellobiose units from the free ends. Finally, β-glucosidase hydrolases cellobiose to glucose subunits (Mtui, 2009).

However, another group of enzymes also participates during biodegradation of lignocellulose. Among this group are xylanases, laccases, acethyl-esterases, lignin-peroxidases, glucuronidase, feruloylesterase, β-xylosidase, galactomannanase and glucomannanase (Nikolov et al., 2000; Draude, et al., 2001; Sánchez-Ramírez et al., 2014).

As seen, the degradation of lignocellulosic biomass involves a wide diversity of enzymes which degrade the intricate structure of the mentioned substrates into low weight molecules

that shall be fermented or subject to further chemical or biological treatment. In addition, the assortment of enzymes is related with a variety of microorganisms producing particular enzymes with different yields that degrade lignocellulosic biomass in specific manners.

Cellulolytic enzymes

Biodegradation of lignocellulosic biomass has been occurring in nature for thousands of years as part of the natural cycle of carbon. Furthermore, there are numerous organisms, particularly insects that evolved through centuries to achieve a diet relying specifically on lignocellulosic substances (Bayané & Guiot, 2011). As aforementioned there is not a single enzyme that renders vegetable biomass into monomers, but this process compels an interaction of enzymes that attack the major components of vegetable tissues (Hoshino, et al., 1993). Hence, it is necessary to make a review of these enzymes in order to achieve better comprehension of their mechanism of degradation.

Regarding the microbiological level, bacteria depend on complex anabolic and metabolic processes where enzymes are always present. Hence, there are two major groups of enzymes, considering their medium of action: endoenzymes and exoenzymes. Endoenzymes are restricted to the interior of bacteria. On the other hand, exoenzymes though internally synthetized, act exclusively in the extracellular medium in Gram (+) bacteria or in the periplasmic space in Gram (-) bacteria and its role is to degrade macromolecules which cannot trespass the cell wall (Suga, et al., 1975; Claeyssens & Henrissat, 1992).

Essentially, lignocellulosic biomass is degraded by two enzymatic systems produced by cellulolytic microorganisms. The aggregative systems are formed by a group of cellulolytic enzymes distributed around the surface of their cell walls in complex arrangements surrounded by a membrane called cellulosomes (Figure 5), consisting of at least 14 different peptides including xylanases, cellobiohydrolases and at least one β-glucosidase attached to a cellulose binding protein A (CbpA) with no enzymatic activity, though it acts as sensor recognizing amorphous structures present in cellulose fibers (Bayer, et al., 1983; Gilbert & Hazlewood, 1993).
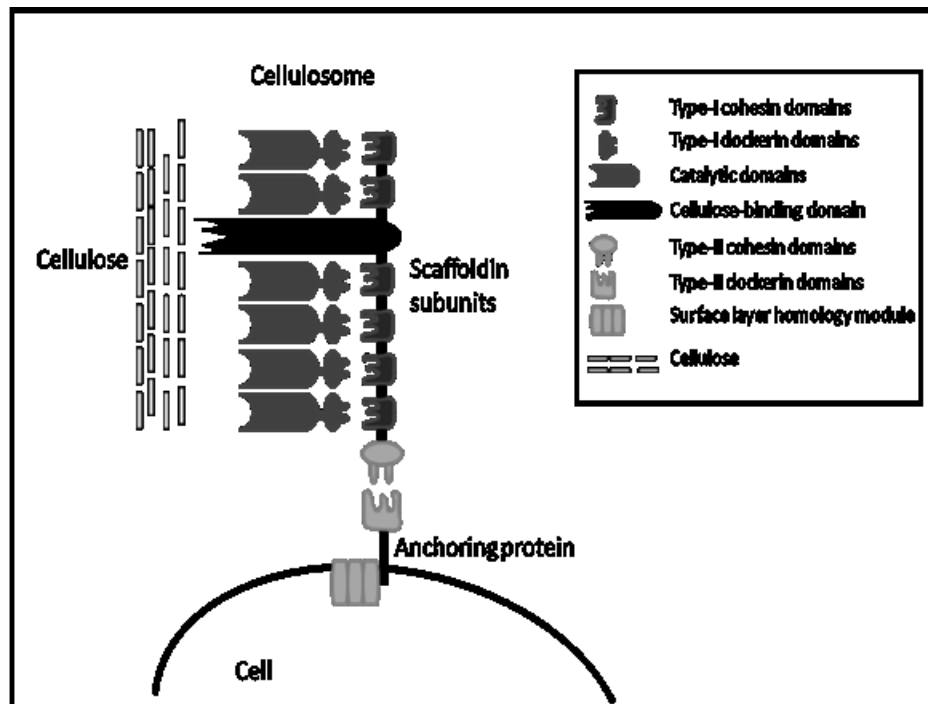
Figure 5. Scheme of a cellulosome (Willson et al., 2016).

Cellulosomes can be found either attached to cell walls or acting freely in solution, but its basic purpose is to disrupt insoluble cellulosic substrates into soluble ones that are further absorbed. However, despite cellulosomes have been studied in bacteria such as *Cellulomonas thermocellum, C. cellulolyticum,* and *C. cellulovorans* their intricate structure complicates better comprehension about their structure and function (Schwarz, 2001).

However, another system is called non aggregative and is found particularly in fungi. In this case no enzymatic complex is found, allowing fungi to penetrate in cellulosic fibers by releasing enzymes in the internal sites of these fibers. This system includes β-1,4-glucanases, cellobiohydrolases and β-glucosidases. On the contrary, as mentionded most bacteria lack of this penetration capacity and rely mostly in aggregative systems (Sinsabaugh, 1994; Davies & Henrissat, 1995; Warren, 1996)

As Zhang, et al., (2006) mention, the basic process of biodegradation of cellulose involves three major enzymatic groups: β-1,4-glucanases, cellobiohydrolases and β-glucosidases. Each of these groups have specific roles throughout the degradation process, working in synergy and sequencially.

Endoglucanases

These enzymes act randomly over the β-1,4-glycosidic linkages, hydrolising the available internal linkages and disrupting macromolecules of cellulose into variable-length oligosaccharides (Figure 6). These disrupts reduce the high level of polymerization of cellulose, resulting in reduction of viscosity and the formation of glucose, cellobiose (disaccharide) and cellotriobiose (trisaccharide) (Yennamalli, et al., 2013).

Endoglucanases act over substrates such as carboxymethyl cellulose (CMC), amorphous cellulose or celloolygosaccharides but are unable to significantly disrupt higly-crystalline cellulose like cotton fibers. Furthermore, their hydrolysis rate is conditioned by the ability to act over the amorphous region of crystalline cellulose therefore creating reducing substrates which shall be further hydrolysed by exoglucanases (Horn, et al., 2012).

Despite the fact that endoglucanase activity can be measured either by reduction of viscosity of the substrate and/or by the amount of reducing ends, exoglucanases also produce reducing ends, so becomes necessary to measure endoglucanase activity by both means.

However, another semi-quantitative method requires carboxymethylcellulose-based substrates and the use of colorants like Congo Red, which shows affinity with long-chain polysaccharides and are only absorbed by them, making it ideal to non-specific endoglucanase activity essays (Teather & Wood, 1982a).
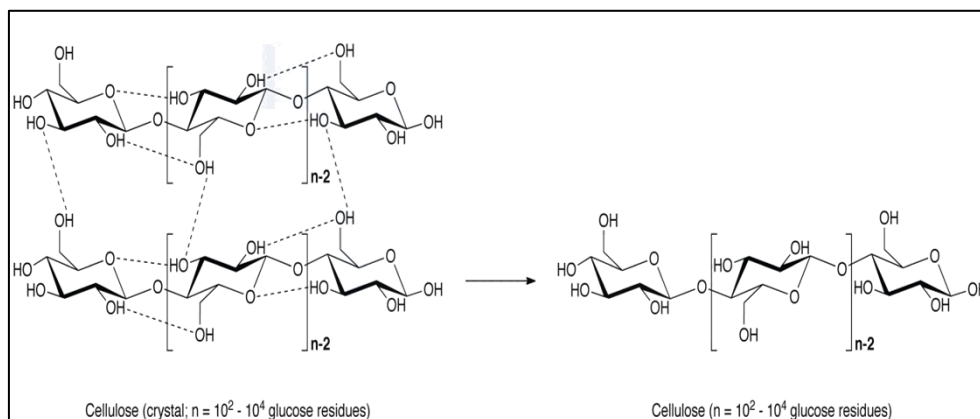
Figure 6. Schematic representation of endoglucanase activity.

Exoglucanases

These enzymes, also known as cellobiohydrolases (CBH), cleave the accessible ends of cellulose molecules, producing exohydrolysis of 1,4-β-D-glucosidic linkages in cellulose substrates, with subsequent formation of oligosaccharides (mostly tetrasaccharides or disaccharides) such as cellobiose (Figure 7). They disrupt two to four units from the ends of

the exposed chains produced by endocellulase and there are basically two main types of exocellulases: CBHI works processively from the reducing end, and CBHII works processively from the nonreducing end of cellulose (Medve, et al., 1998).

Figure 7. Schematic representation of CBHI and CBHII enzymatic activity (Pei et al., 2010).

β-Glucosidases

These enzymes acts over β-1,4 linkages that bond glucose subunits of soluble cellobiose or other cellodextrins. It is an exocellulase with specificity for a var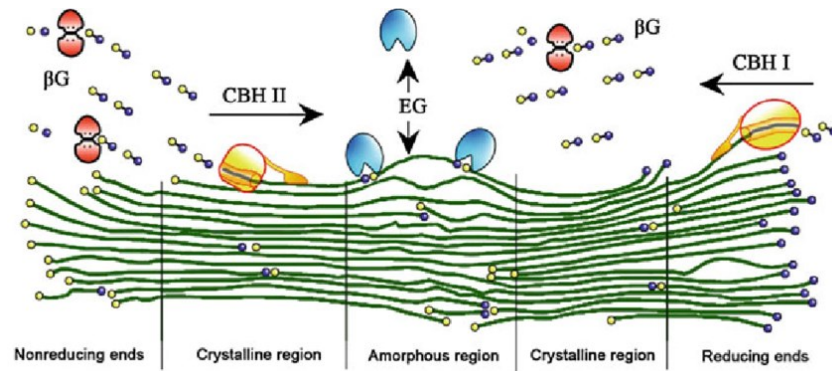iety of β-D-glycoside substrates with a polymerization degree up to 6. It catalyzes the hydrolysis of terminal non-reducing residues in β-D-glucosides subsequently releasing glucose.

The result of this enzymatic synergy is the degradation of cellulosic fibers. As mentioned the process begins with a first hydrolysis carried out by endoglucanases which bond randomly upon the amorphous regions, cleaving β-1,4-glycosidic linkages and creating multiple sites in which exoglucanases shall hydrolyse the non reducing ends in the chains. As result, several subunits of cellobiose are produced (Figure 8). At this point β-glucosidase intervenes disrupting cellobiose and such oligomers to finally hydrolyse them into glucose (Carvalho, et al., 2009)
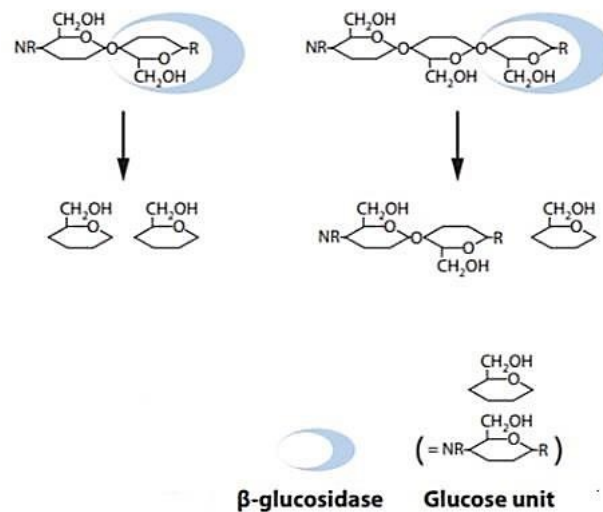
Figure 8. Schematic representation of β-D-glucosidase activity. Adapted from (Watanabe & Tokuda, 2010)

The synergism of cellulolytic enzymes is based on complementary activities among them, which in terms of hydrolysis means that their combined activity is superior in comparison to separate single activity of each enzyme. However, the degree of synergism observed depends on several factors, including the type of substrate and the nature of a particular cellulase component (Woodward, 1991).

Factors influencing enzymatic degradation of lignocellulosic biomass

Regarding lignocellulosic biodegradation, many factors affect the enzymes and the optimisation of the hydrolysis process, such as enzyme/substrate ratio, substrate loadings, enzyme loadings, inhibitors, adsorption and surfactants (Van Dyk & Pletschke, 2012). On the other hand, though in recent years advances in the study of microbial enzymes had lead us to comprehend the structures, kinetics, catalytic action, interactions of enzymes and their substrates and even the ability and limitation of those enzymes to hydrolyze lignocellulosic biomass, no single mechanism of total lignocellulosic saccharification has been established (Mansfield, et al., 1999).

Among the additional factors that have been identified to affect the hydrolysis of cellulose are porosity of lignocellulosic materials, degree of crystallinity in cellulose fibers, and the content of lignin and hemicellulose (Kumar et al., 2009). As the presence of lignin and hemicellulose difficult the access of cellulase enzymes to cellulose, the efficiency of the hydrolysis is reduced (Yoshida et al., 2008). Hence, enhancements in pretreatment of biomass leading to removal of lignin and hemicellulose, and increase of porosity accompanied by optimization of the cellulase enzymes may improve the hydrolysis (Sun & Cheng, 2002).

On the other hand, pretreatment of lignocellulosic biomass is fundamental to render it suitable for enzymatic degradation. The type of lignocellulosic biomass also determines the results of pretreatment, particularly in increasing surface accessible area, reducing cristallynity and polymerization of cellulose as well as the solubilization of lignin and hemicellulose. Physical, chemical and biological pretreatments are described in literature for enhancing saccharification of lignocellulosic biomass. However, no single pretreatment is able to overcome recalcitrance of lignocellulose and disrupting the array of covalent cross-linkages and non covalent forces that bind cellulose to hemicellulose and lignin.

Physical pretreatments include mechanical techniques to reduce particle size, such as milling and grinding. Other physical pretreatments include steam explosion (autohydrolysis), liquid hot water pretreatment (hydrothermolysis) irradiation with ultrasound and microwaves and extrusion (Zheng et al., 2014). Chemical pretreatments involve the use of acids, alkalis and ionic liquids. Physical and chemical pretreatments, however, also produce inhibitors that affect further enzymatic hydrolysis of cellulose and require a large input of energy as well as special infrastructure (Mosier et al., 2005)

Biological pretreatments involve the use of microorganisms and their metabolites. White rot, brown rot and sot rot fungi are the most common microorganisms used for this purpose. In addition, biological pretreatment is an eco-friendly process and has additional advantages such as no generation of fermentation inhibitors, no generation of toxic compounds to the environment and no effluent generation during the process. Major drawbacks, however, are the treatment time and sugar consumption (Sindhu et al., 2016)

On the other hand, a reduced crystallinity of cellulose, increases the yield of monosaccharides. This reflects that cellulases hydrolyse better amorphous regions of cellulose rather than crystalline regions (Yoshida et al., 2008). Therefore, mechanical increasing of porosity may also increase the number of substrate sites in the cellulose microfibrils, leading to a higher saccharification yield, as it has been established that the rate and extent of saccharification is governed by particle size, surface area, and degree of polymerization, since crystallinity effects alone are not decisive in the saccharification process (Puri, 1984).

Regarding temperature it is worth to say that lignocellulosic microorganisms work in diverse conditions of temperature, ranging from those temperatures close to congelation up to temperatures above 50°C. Depending on temperature, microorganisms are adapted to lignocellulose degradation, because as temperature rises, degradation of lingocellulosic substrates increases, having a direct effect upon enzymatic action (Bhalla et al., 2013). Hence those thermophilic microbes growing at temperature of 50−80 C are sources of highly active and thermostable enzymes (Zambare, et al., 2011).

Several factors influence the enzymatic system of cellulases. These factors are related with inhibition of cellulolytic enzyme complex by the final product, synergysm and adsorption of the enzymes (Mansfield et al., 1999). Regarding the synergysm, it has been studied that interactions among endoglucanases and exoglucanases influences hydrolysis rate. Thus, when combining two or more enzymes their combined action is higher than that of a single enzyme itself. In this sense, Wang, et al. (2013) found that, when treating cellulosic fibers with a mixture of three *Trichoderma reesei* cellulases (one endo and two exoglucanases), a high rate of almost 100% hydrolisis was observed.

As described by Sun & Cheng (2002), the substrate concentration is one of the main factors affecting the yield and initial rate of cellulose enzymatic hydrolysis. At low substrate levels, an increase of the substrate concentration normally results in an increase of the yield and reaction rate of hydrolysis. However Huang & Penner (1991) reported that higher substrate

concentration causes substrate inhibition, which substantially lowers the rate of hydrolysis. Inhibition of enzymatic hydrolysis is one of the major concerns for the saccharification of lignocellulosic biomass. As aforementioned, pretreatment processes produce inhibitor compounds such as aliphatic carboxylic acids, acetic acid, phenylic compounds, furans, etc. These substances are produced by side-reactions taking place during pretreatment and their inhibitory effect is given by the accumulation of lignocellulose-by-products, as result of water recirculation and/or the sustained increase of lignocellulose concentration during the process. Moreover, the type of pretreatment also influences the generation of certain inhibitors. For instance, acid-based pretreatments are prone to generate mostly aliphatic carboxylic acids, phenylic compounds and furans, whereas hydrothermal processing (steam explosion) produce mostly acetic acid, but minor amounts of furan aldehydes (Jönsson & Martín, 2016).

Additionally, though enzyme and substrate concentration and the end-product inhibition influence the rate of hydrolysis, this effect tends to be less pronounced during the initial stages, due to the adsortion of most of the cellulases by the unhydrolyzed residue (Gregg & Saddler, 1996). Hence, recycling the cellulases adsorbed onto the residual substrate has been suggested as an alternative to reduce the amount of enzymes required for the saccharification process as well as elevating rates of hydrolysis (Alvira, et al., 2010). However, the number of times that residues can be recycled is limited by the content of lignin associated with softwood-derived cellulose (Gregg & Saddler, 1996).

The particular structure of cellulases, which are formed by at least one catalytic and a non-catalytic cellulose-binding domain (CBD) also regulates their enzymatic activity. The arrangement of the catalytic and CBD's results in a large possibility of combinations and, therefore, variable selectivity and synergism with cellulose (Henrissat, 1994). This situation is also observed in insoluble carbohydrates such as raw starch or chitin, suggesting that the binding domains not only improve the binding with cellulose but also facilitate the activity of the catalytic domain on the insoluble but not on soluble substrates (Linder & Teeri, 1997).

On the other hand, it has been demonstrated that CBDs binding to crystalline cellulose shares similar rigid backbone structures, allowing correct positioning of the side chains required for the substrate recognition and binding (Nidetzky & Claeyssens, 1994; Linder & Teeri, 1997). This situation explains the diversity of enzymes found in microbial enzymatic

compounds as well as the synergism found between endo and exoglucanases or between exoglucanases alone (Nidetzky, et al., 1994)

Determination of cellulolytic activity in insects

As previously mentioned one of the keys to insect's successful diversification is their symbiosis with microorganisms that contributes in a variety of benefits, synthetizing essential substances, providing vitamins and aminoacids, recycling nitrogen-waste-compounds, and playing an important role in helping to degrade and assimilate food (Moran, et al., 2003). In fact, most of the interaction between insects and microorganisms takes place in insect's gut and the amount of microorganisms living in their digestive tracts may easily outnumber their own cells as it has been suggested in multicellular organism hosting beneficial microbes (Dillon & Dillon, 2004).

However, analyzing insect symbiotic microbiome, particularly that of their digestive tract has gained increasing scientific interest, since the presence of cellulolytic enzymes indicates potential ability to degrade the crystalline cellulose (Martin & Martin, 1978; Cazemier et al., 1997; Mika et al., 2013). Hence, the salivary glands, the intestinal tissue and the digestive fluids are normally used to identify cellulolytic activity in insects (Martin, 1983; Cazemier et al., 1997). In this sense, evidence demonstrates that the cellulolytic activity of insect microbiota depends on the host species, the analyzed tissue and the method used for the analysis rather than the substrate in which the tests are conducted (Martin, 1983).

Additionally, it has been reported that product inhibition may interfere in the hydrolysis rate of both endoglucanases (EG) and cellobiohydrolases (CBH) (Väljamäe, et al., 2001; Jing, et al., 2009), but this limitation may be overcome by the addition to the reaction of β-glucosidase, during the degradation of cellobiose into glucose (Willis et al., 2010). In many cases the degradation of cellulose is measured using two assays: 3,5-dinitrosalicylic acid (DNSA) (Nidetzky, et al., 1994) or tetrazolium blue (Jue & Lipke, 1985), which are able to detect the reducing sugars during the degradation of cellulose, particularly with the addition of reagents such as sodium tartrate, increasing sensitivity and reducing reaction time.

However, initially DNSA assay appeared susceptible to interference from pure or pre-treated cellulose, particularly when compared to HPLC or YSI (Yellow Springs Instruments) assays, sought to determine the release of specific reducing sugars (Rivers, et al., 1984; Schwald, et al., 1988). Nevertheless, DNSA assay is perfectly applicable to

preliminary assays intended to determine specific and non-specific cellulolytic activity of microbial samples, from the digestive tract of insects, measured spectrophotometrically (Gupta, et al., 2012)

The type of substrates is another point of concern in quantifying the degradation of cellulose. Traditionally, filter papers (FP) (Xiao, et al., 2004) and modified or crystalline cellulose (Suen et al., 2010) have been used to test the cellulolytic activity. But, as non-purified substrates such as lignocellulosic biomass is gaining increasing attention, the conventional assays offer little precision, since the presence of lignin and other substances, present in vegetable tissues, interfere with saccharification process (Zhang & Lynd, 2004) In this case, previously studied methods include iodometric procedures like those described by Somogyi (1952), for samples containing no less than 10 μg of glucose. Colorimetric techniques have also been suggested for samples down to 5 μg of glucose as reported by Nelson (1944), as well as the oxidase/peroxidase method (Breuil & Saddler, 1985).

On the other hand, the filter paper assay has been demonstrated susceptible to glucose and cellobiose accumulation and therefore to the end-product-inhibition, particularly at higher substrate concentrations (Breuil, et al., 1992). This leads to consider that accurate measurement of the hydrolytic enzymatic compounds must not rely on FP assay alone (Chan, et al., 1989). Additional assays intended to detect cellulolytic activity on insect intestinal samples include spectrophotometry, based on the release of p-nitrophenolate (Terra, et al., 1979; Chipoulet & Chararas, 1985; Gijzen, et al., 1994; Ferreira et al., 2001; Marana, et al., 2004; Yapi Assoi Yapi, et al., 2009) or methylumbelliferyl (Marana, et al., 2001). Chromatography techniques have also been described to determine the amount of sugar produced during the degradation of cellulose either by HPLC or thin layer chromatography (Adams & Drew, 1965; Berlin, et al., 2007; Chundawat, et al., 2008).

The inherent complexity of the cellulolytic activity demands also a diversity of substrates to test, either specific or non-specific enzymatic activity from insect samples. Therefore, a variety of oligo- and polysaccharides have been used with this scope, depending on the targeted area of the intestine and the type of assay required. This also allows better comprehension of the digestion of cellulose in insects (Tokuda, et al., 2005). Normally, the substrates employed are filter paper (FP), microcrystalline cellulose (MCC) and cotton. These substrates are used to test the presence of a complete cellulolytic activity, which means the involvement of EG (endoglucanase), CBH (cellobiohydrolases) and β-glucosidase (Willis et al., 2010)

Studies have demonstrated the evidence of EG and β-glucosidase activity of intestinal samples of insects, but deficient activity of CBH. In fact, it has been suggested that CBH activity is carried out mostly by the endosymbiont microbiota rather than the intestine itself (Martin, 1983; Scrivener & Slaytor, 1994).

Nonspecific cellulolytic activity is commonly evaluated on carboxymethylcellulose (CMC) or modified cellulose, as they are easily degradable by EGs. In fact CMC has been broadly used to test and screen cellulolytic activity on microorganisms (Gijzen et al., 1994; Zverlov, et al., 2003; Ni, et al., 2007; Rehman et al., 2009). These substrates are intended to provide CMC or other forms of cellulose as the sole source of carbon, allowing qualitative or semi-quantitative determination of enzymatic activity, either in enriched or non-enriched media (Delalibera, et al., 2005).

However, despite the fact that these assays are not suitable to measure cellulolytic activity as the releasing of reducing sugars, they provide a practical procedure to evaluate cellulolytic activity among the intestinal microbiota in insects, since CMC and other modified celluloses are easily degradable by EGs (Oppert et al., 2010). Additionally, Congo Red staining has also been used to detect the microbial cellulolytic activity. In this case, the presence of cleared halos surrounding the incubated colonies allows detection of positive activity (Teather & Wood, 1982b). Comparison between the diameters of the colonies and cleared halos also allows a to estimate and compare cellulolytic activity among different samples. (Rinke et al., 2011)

As aforementioned, there are several procedures intended to test the cellulolytic activity. The specificity of the assays depends on their purpose. Hence, non-specific semi-quantitative assays help to screen the cellulolytic activity among microbial communities, whereas specific assays are generally intended to quantify enzymatic activity on either isolated or non-purified enzymatic compounds. In this sense, the β-glucosidase activity is generally measured with the oxidase/peroxidase method (Ferreira & Terra, 1983; Zinkler & Götze, 1987; Marana et al., 2000; Yapi Assoi Yapi et al., 2009). Hydrolysis of p-nitrophenol by glycosidic derivatives (NP β-glycosides) or is also used to quantify the enzymatic activity (Ferreira et al., 2001) as well as the fluorescence of methyl-umbrilliferil (MU) (Marana et al., 2001). Table 2 provides a list of different taxonomic groups of insects in which the cellulolytic activity has been previously studied as well as the specific substrate and the methodology used for the analysis.

Table 2. Taxonomy, life stage and diet of insects studied for their enzymatic activity (Willis et al., 2010).

Order (family)	Species	Substrate	Detection method	Purification	Size (kDa)	Reference
(Termitidae)	*Nasutitermes takasagoensis*	CMC	TB, Zymogram	SEC	47	Tokuda & Watanabe, 2007; Tokuda *et al.*, 1997
	Odontotermes formosanus	CMC	AC	AEC	80	Mo *et al.*, 2004; Yang *et al.*, 2004
Lepidoptera (Papilionidae)	*Parnassius apollo* ssp. *frankenbergeri*	CMC	DNSA	NP	NP	Nakonieczny *et al.*, 2006
(Saturniidae)	*Philosamia ricini*	CMC	AC	NP	NP	Pant & Ramana, 1989
Orthoptera (Acrididae)	*Schistocerca gregaria*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
(Gryllidae)	*Acheata domesticus*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
	Teleogryllus emma	CMC	DNSA	TAC	47	Kim *et al.*, 2008
Plecoptera (Peltoperlidae)	*Peltoperla arcuata*	CMC	AC	NP	NP	Walters & Smock, 1991
(Pternoarcidae)	*Allonarcys proteus*	CMC	AC	NP	NP	Walters & Smock, 1991
Phasmatodea (Phasmatidae)	*Eurycantha calcarata*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
Trichoptera (Limnephilidae)	*Pycnopsyche* spp.	CMC	AC	NP	NP	Walters & Smock, 1991
Thysanura (Lepismatidae)	*Thermobia domestica*	CMC	GOP	NP	NP	Zinkler & Gotze, 1987
	Ctenolepisma lineata	RC	AC	NP	NP	Lasker & Giese, 1956

Abs, Absorbance; AEC, anion exchange chromatography; AC, alkaline copper; AP, agarose plates; CA, cellulose azure; CMC, carboxymethylcellulose; DNSA, dinitrosalicylic acid; HIC, hydrophobic interaction chromatography; NP, not provided; OBRH, ostazin brilliant red hydroxyethyl-cellulose; RC, regenerated cellulose; SEC, size exclusion chromatography; TAC, tag affinity chromatography; TB, Tetrazolium blue.

Rhynchophorus palmarum (Linnaeus, 1758)

The normal lifecycle of *Rhynchophorus palmarum* also known as the Black Palm Weevil (BPW) last around 120 days, though it has been reported that in sugar cane substrate under controlled conditions it may last between 158-170 days (Restrepo et al., 1982). This lifecycle comprises an egg stage (3,5 days) a larval stage that last around 60,5 days, a nymph stage (16 days) and an adult stage which last around 40 days (Mexzón et al., 1994) Table 3 provides taxonomic information of *R. palmarum*.

Table 3. *Rhynchophorus palmarum* taxonomy

Taxonomic Classification of *Rhynchophorus palmarum*	
Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Coleoptera
Family	Curculionidae
Genus	*Rhynchophorus*
Species	*R. palmarum*
Binomial name	*Rhynchophorus palmarum* (Linnaeus, 1758)

Eggs are ovoid and pale white, with a creased surface. The average length of an egg is 2,40 mm and 0,8 mm wide (Figure 9). The size of the eggs remains constant until the terminal incubation period; when the development of the first instar larva slightly increases its diameter (Hagley, 1965).

Figure 9. *Rhynchophorus palmarum* eggs

The larvae of *R. palmarum* are eruciform and legless, with a dark-brown strongly chitinous head; provided with a masticatory apparatus consisting of two mandibles that allow them to bore on vegetable tissue. During the initial instars larvae look pale white and their heads look glossily reddish. Gradually their skin color changes to a more yellowish resemblance with a markedly wrinkled surface and their head turns dark brown as well (Figure 10).

Figure 10. *Rhynchophorus palmarum* larva.

The thorax is divided into three sections. The first section is large, muscled and easily-recognizable. It is surrounded by a semi rigid ring immediately behind the head. The other two sections are more cramped and hardly distinguishable from above. The abdominal sections are also muscled and provided with creases that facilitate the locomotion. The terminal aft section is flat and rigid. Tactile setae are also distributed along the larval skin. However, they are particularly visible on the aft portion (Mexzón et al., 1994).

Early-born larvae are around 2,40-2,65 mm long; 0,94 mm wide and 1,9 mg heavy. During the larval stage, individuals gradually increase their size and weight to a significant extent. Some authors have described 9 to 13 larval maturation instars. At the last instar larvae reach around 57-76 mm length; and around 6,24 g weight (Restrepo et al., 1982; Mexzón et al., 1994).

Prior to pupation, larvae decrease their feeding activity and begin to form their cocoons with the leftover strong vegetable fibbers densely woven (Figure 11). The cocoons are gradually formed surrounding the larvae from the bottom up

Figure 11. *Rhynchophorus palmarum* cocoon and pupa.

Pupation begins once the larva completely encloses its cocoon. Internally, the pupa is covered by a soft cuticle, that gradually turns more rigid, with an average size of 7–9 cm long and 3–4 cm in diameter.

The adults of the Black Palm Weevil are large and robust beetles measuring circa 30-50 mm long and 12-14 mm wide. They are covered by a black, strong chitinous cuticle and have the characteristic elytra, common to all Coleoptera. Initially, the cuticle is glossy, turning gradually dull until the end of their lifecycle.

The rostrum of the Black Palm Weevils is small and curved. Eyes are ovoid and large, allocated at the top of the rostrum. The rostrum is dominated by a distinctive slightly curved proboscis that allocates a pair of laterally erected antennae at the base of the rostrum. The adult *R. palmarum* individuals have sexual dimorphism. Females differ from males which possess a distinctive bunch of hairs in the terminal portion of the proboscis (Figure 12).
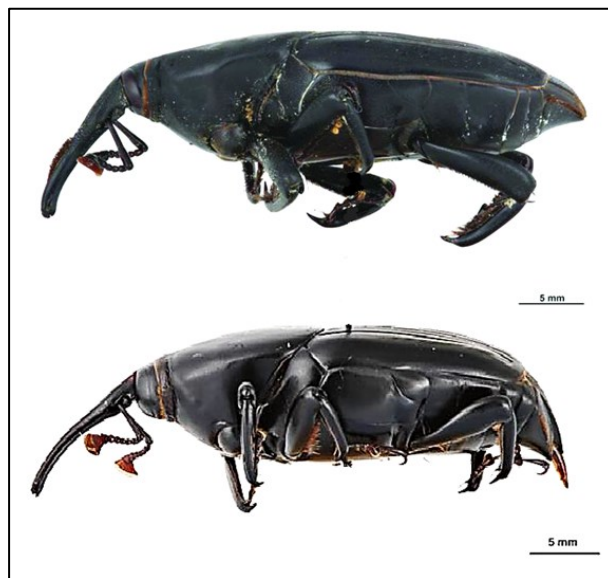
Figure 12. Adult Black Palm Weevils male (above) and female (below)

Ecology and importance of the BPW (*Rhynchophorus palmarum)*

The Black Palm Weevil (BPW) is a "snout beetle" belonging to the Curculionidae family, which has been reported to feed and reproduce on an estimated 35 diferent vegetable species, but most notably on palms of the Arecaceae family (Wattanapongsiri, 1966; Griffith, 1987; Magalhães, et al., 2008). The *Curculionidae* family is indeed the most diverse family of animals on Earth, with at least 60.000 known species of weevils (Howard, et al., 2001).

The oviposition occurs normally on the leaf axils or wounded tissues and there is a strong evidence suggesting that the volatile byproducts of fermentation such as ethyl acetate, isoamyl acetate, pentane, isopentanol and hexane, attract the adult BPW and stimulate their activity (Aldana et al., 2010). In fact, the use of fermenting stem sections is a common weevil trapping technique (Rochat, 1990; Hernández et al., 1992). The reproductive behavior of the BPW is also stimulated by a male aggregation pheromone (E)-6-methyl-2-hepten-4-ol better known as Rhynchophorol, first described by Rochat et al., (1991). BPW females have been described to lay their eggs in holes made with their rostrum and normally a female may oviposit 120-150 eggs in the course of 30 days. The oviposition takes place even while females feeding (Giblin-Davis et al., 1996).

The adult BPW's are reported to cover a distance of 1,6 Km in a single day, flying at average speed of 6 m/s (Hagley, 1965; Griffith, 1987; Hernández et al., 1992). Their preferred habitat includes the palm leaf axils, in which they can be found hidden, particularly in summer days and on basal stem proximities in which they are found either feeding on palm wounds close to the ground, or in the fibbers surrounding young basal petioles (Griffith, 1987), They also prefer to hide in leaf litter or buried in the soil (Hagley, 1965).

On the other hand, the weevils such as *R. palmarum*, are common vectors of palm pathogens, such as the red ring disease nematode *Bursaphelenchus cocophilus* Cobb. (Oehlschlager, et al., 2002; Giblin-Davis, et al., 2006). This has led to consider BPW as a major pest in commercial palm plantations, particularly oil palm *Elaeis guineensis* Jacq. that has been introduced in South American tropical forests in large monocultures, causing important losses (Sánchez & Cerda, 1993).

In Ecuador, the oil palm was first introduced as a commercial culture during the 1940's in the Coastal Region and since the late 1950's in the northern Amazon (Figure 13). As of 2012, a total of 2.6 million hectares of oil palm are distributed among the mentioned regions, from which an estimated 32.300 hectares are spread across Sucumbios and Orellana provinces in the Ecuadorian Amazon (INEC, 2012).

Figure 13. Oil palm plantations in northern Ecuador.

Although there is no specific information regarding the losses of oil palm culture related to BPW infestation in Ecuador, it has been reported and trapped in commercial plantations. On the other hand, the particularly fastidious effects of BPW infestation are not endemic of tropical regions, as human activities have caused dispersion of BPW and other known weevils, with ecological and economic implications (EPPO, 2005).

This situation means that most of the research on BPW is focused on controlling or depleting its population, either by trapping adults or deploying entomopathogens on infested commercial plantations. Nonetheless, in rural areas or tropical forest, the interaction of *R. palmarum* and human activities is different. In fact, native Amazon populations consider BPW larvae as food and its consumption has also spread to urban areas in the Amazon provinces of Ecuador (Figure 14) (Sancho, et al., 2015).

Figure 14. Roasted "Chontacuro" a typical dish of the Ecuadorian Amazon.

This fact supports the approach of studying insects from the perspective of entomophagy and the potential contribution of insects, normally regarded as pests, to industrial applications. In this case, the evidence demonstrates that *R. palmarum* larvae represent an excellent source of highly digestive proteins, vitamins and non-saturated fatty acids (Table 4, Table 5) (Sancho, et al., 2015).

Table 4. Nutritional content of *R. palmarum* larvae.

PARAMETER	CONTENT
Moisture	59.60%
Ash	0.66%
Total Fat	30.23%
Protein	9.49%
Calories	310.11 kcal/100g
Calcium	2.50 mg/100g
Magnesium	25 mg/100g
Vitamin A	1677.50 IU/100g
Vitamin E	10.20 IU/100g

Table 5. Lipid content on *R. palmarum* larvae

PARAMETER	CONTENT
Saturated	36.80%
Monounsaturated	60.40%
Polyunsaturated	1.46%
Palmitic acid	28.01%
Oleic acid	59.20%
Linoleic acid	0.32%

In addition, a better comprehension of the role of the insects in different ecosystems, has contributed to support research from the perspective of their potential benefits to human activities and one of these emerging approaches is the study of the degradation of lignocellulosic biomass by insects, particularly weevils of the Curculionidae family, suggesting the perspective of isolating a wide range of enzymes, from laccases to

glycosidases, either from their digestive fluid or the endosymbiont microbiota (Chipoulet & Chararas, 1985;Yapi Assoi Yapi et al., 2009; Huang, et al., 2012b; Rojas-Jiménez & Hernández, 2015).

Thielaviopsis ethacetica (Went, 1893)

Thielaviopsis ethacetica is part of a wide group of fungal pathogens. The genus *Thielaviopsis* (*Ceratocystis*) is often regarded as one of the most economically important, due to the prejudice they cause to agriculture. Nonetheless, it is a widely diversified genus comprising different species, among which are not only plant pathogens, but also significant insect symbionts and even timber-decaying fungi. Taxonomic classification of *T. ethacetica* is presented in Table 6.

Table 6. Taxonomic classification of *T. ethacetica*

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Sordariomycetes
Subclass:	Hypocremycetidae
Order:	Microascales
Family:	Ceratocystidae
Genus:	*Thielaviopsis*
Species:	***T. ethacetica***

The genus *Ceratocystis* was first named in 1890, when fungal specimens were isolated from potato cultivars suffering from a devastating black-rot infection. The first species to be identified was *Ceratocystis paradoxa*. However, some other alike fungi were isolated in the following years, sharing the same morphological characteristics, and were incorrectly designated as *C. paradoxa*. This was the case of *Thielaviopsis ethacectica*. This species was until recently considered a synonym of *Ceratocystis paradoxa* (Mbenoun et al., 2015).

Thielaviopsis ethacetica was originally described as the type species for a new genus, *Thielaviopsis*, which was first identified in Java by Went, after an outbreak of sett rot in sugarcane, in 1893. However, as abovementioned, due to its similarities, it was later included in the *Ceratocystis. paradoxa* (de Seynes) complex, and often referred as synonym of this (Mbenoun, et al., 2014).

Similarities among members of *Ceratocystis* family are result of convergent evolution linked to an insect-associated ecology. Morphologically, these fungi have usually dark-based ascomata, from which long-stem ostiolar hyphae emerge. Ascospores are found tightly bound to hyphae, arranged in slimmy-like masses (Figure 34). The asexual states of *Ceratocystis* spp. is roughly similar, characterized by a typical tubular-conidiogenous cell structure. These cells, are branched in their apices, producing chains of rectangular-shaped conidia and sometimes a secondary chain of conidia, associated to the main chains (de Beer, et al., 2014).
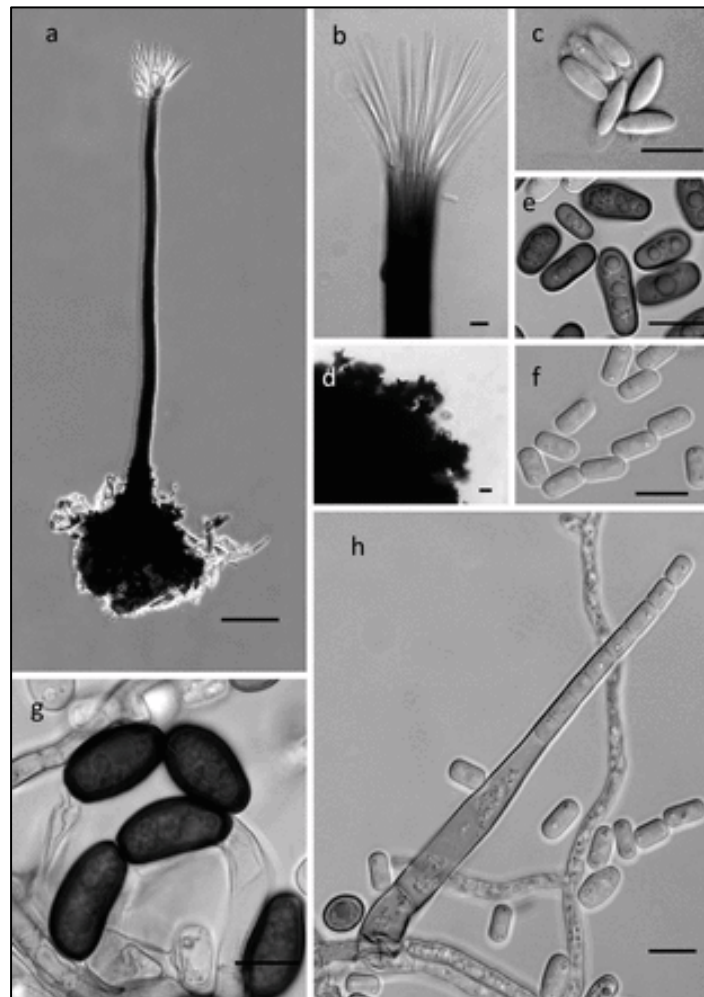
Figure 15. Sexual and asexual structures of *T. ethacetica* (Mbenoun et al., 2014). (a) Perithecium with globose base; (b) Divergent ostiolar hyphae; (c) Ellipsoidal ascospores invested in mucous sheaths; (d) Digitate ornamentations on perithecial base; (e) Oblong secondary conidia; (f) Cylindrical primary conidia; (g) Thick-walled aleurioconidia in short chains; (h) Flask-shaped phialidic conidiophore. Bars: (a) = 100 μm, (b) = 10 μm.

OBJECTIVE

The emerging worldwide interest in finding novel sources of fermentable sugars, as well as the means to obtain them from a renewable basis, has been translated into an ever growing research effort focused in achieving viable procedures to render lignocellulosic biomass a plausible substrate that can be transformed in biofuels, biogas, biohydrogen and other eventual industrial derivatives.

From this point of view, it is extremely important to depolymerize the highly packaged and recalcitrant structure of lignocellulosic biomass. In addition, traditional pretreatment methods, mostly relying on concentrated acids or alkalis, is being reconsidered as unsustainable. However, the use of microorganisms as well as microbial-derived enzymes is gaining increasing interest.

On the other hand, insects are natural degraders of lignocellulosic biomass and Coleoptera is one of the most specialized insect orders adapted to naturally feed on lignocellulose, mostly in their larval instars.

Among the most known Coleoptera is the Black Palm Weevil *Rhynchophorus palmarum* which is distributed in tropical and subtropical areas of Central and South America and Africa. However the genus *Rhynchophorus* has indeed a negative standing, since these weevils are responsible for transmitting diseases associated to nematodes and other phytopathogens.

On the other hand, the role of insects must be studied not only from the perspective of their negative effects to human activities, but from the potential benefits to humans as well. Indigenous populations, such as Kichwa, Shuar and Waorani in the Ecuadorian Amazon, have long coexisted with insects, in a very close relationship that involves economic, nutritional and even cultural aspects. It is known that these indigenous people consider *R. palmarum* larvae as food, with healthy benefits, particularly in helping to relieve respiratory diseases.

Evidence supports the fact that *R. palmarum* larvae are promising sources of poly-unsaturated fatty acids, vitamins, as well as highly digestive proteins; with notable contents of essential aminoacids. Additionally, it must be considered that the biomass, from which these larvae feed on, is not rich enough in terms of nutrient value. Hence, the ability to render biomass into biologically available sugars plays an essential role in the metabolism of these larvae.

This situation, indeed, opens a pathway to study the Black Palm Weevil as a potential source of cellulolytic enzymes. In fact, it has been demonstrated the presence of at least one

β-glucosidase in the digestive fluid of *R. palmarum* larvae. Nevertheless, it remains unclear whether these enzymes are associated to their intestinal microbiota, since these insect-borne symbionts are responsible for most of the lignocellulosic degradation carried out by insects.

The scope of this research is to emphasize the prospect of studying *Rhynchophorus palmarum* larvae as a source of cellulolytic enzymes that shall eventually be applicable at industrial aims. In this sense, the approaches considered in this research are focused on elucidating the fundaments for the eventual use of the enzymatic complex of *T. ethacetica* for the saccharification of lignocellulose or the use of this ascomycete, alone or in combination with other microorganisms, for the degradation of lignocellulosic biomass. In all these cases, further studies are required to deepen this line of research from the perspective of developing the means that ultimately render renewable energy sources competitive.

MATERIALS AND METHODS

General overview

This research has been performed in a cooperation frame with Universidad Estatal Amazónica of Puyo, Ecuador. Experiments were carried out at Microbiology Laboratory at Universidad Estatal Amazónica as well as in the Biotransformation and Biocatalysis Laboratory at the University of Ferrara, Italy.

The aim of this research is to isolate cellulolytic enzymes from the endosymbiont microbiota living in the digestive tract of *Rhynchophorus palmarum* larvae. Research was divided in several stages, schematically represented on Figure 16. Despite the fact that this research is focused exclusively on *R. palmarum* larvae, the impossibility of transporting *R. palmarum* larvae from Ecuador to Italy, due to phytosanitary restrictions made it necessary that microbial isolation protocol, as well as cellulolytic activity protocol were first assayed with *R. ferrugineus* larvae and then replied with *R. palmarum* larvae in Ecuador.
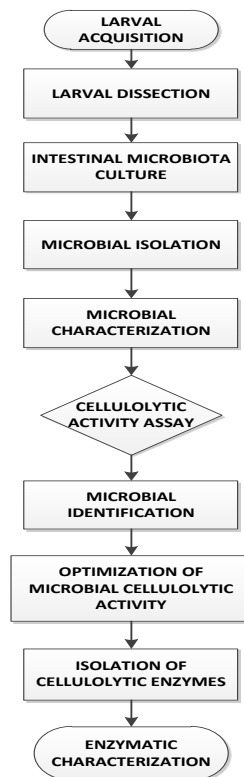
Figure 16. Schematic representation of research stages

Reagents

Reagents and microbiological substrates, used in this research, were purchased from Sigma-Aldrich (USA), Fluka (USA), Lickson (Italy) Merck Millipore (Germany). Table 7 provides information of reagents used in this research.

Table 7. Reagent information

REAGENT	DESCRIPTION	ACRONYM
Tryptic Soy Broth (Sigma-Aldrich)	Casein peptone (pancreatic), 17 g/L Dipotassium hydrogen phosphate, 2.5 g/L Glucose, 2.5 g/L Sodium chloride, 5 g/L Soy peptone (papain digest.), 3.0 g/L	TSB
Potato Dextrose Broth (Fluka)	Potato extract, 4.0 g/L Dextrose, 20.0 g/L Agar, 15.0 g/L	PDB
Carboxymethilcellulose (Sigma-Aldrich)	Sodium Salt, Low Viscosity (25 - 75 mPas, 2% H_2O, 25°C)	CMC
Yeast Extract (Sigma-Aldrich)	For microbiology use	YE
3-5 Dinitrosalicylic Acid (Sigma)	3,5-Dinitro-2-hydroxybenzoic acid (228.12 g/mol)	DNSA
Phenol (Sigma-Aldrich)	For molecular biology, ≥99% GC	-
Rochelle salt (Sigma-Aldrich)	Sodium-potassium tartrate (tetrahydrate) ACS reagent, 99%	-
Sodium thiosulfate (Sigma-Aldrich)	Soudium thiosulfate pentahydrate ReagentPlus®, 99%	-
Ammonium sulfate (Sigma-Aldrich)	ReagentPlus®, ≥99.0%	-
Congo Red (Sigma-Aldrich)	Analytical Standard, ≥97%	-
Gram Color Staining Kit (Merck Millipore)	Gram Color Kit 111885: Crystal violet solution Lugol's solution stabilized Decolorization solution Safranin Solution	-
Sodium Acetate (Sigma-Aldrich)	Anhydrous, ReagentPlus®, ≥99.0%	-
Sodium phosphate monobasic (Sigma)	BioUltra, for molecular biology, ≥99.0%	-
Diethanolamine (Sigma-Aldrich)	Reagent grade, ≥98.0%	-
Violet Red Bile Lactose	Yeast Extract 3.0 g	VRB

Agar + Neutralizier (Lickson)	Enzymatic Digest of Gelatin 7.0 g Bile Salts Mixture 1.5 g Lactose 10 g Sodium Chloride 5.0 g Neutral Red 0.03 g Crystal Violet 0.002 g Agar 15.0 g	
Manitol Salt Agar (Lickson)	Lab-Lemco' powder 1.0 g Peptone 10.0 g Mannitol 10.0g Sodium chloride 75.0 g Phenol red 0.025g Agar 15.0 g	MSA
Rose-Bengal CAF Agar + Neutralizier (Lickson)	Mycological peptone 5.0 g Glucose 10.0 g Dipotassium phosphate 1.0 g Magnesium sulphate 0.5 g Rose-Bengal 0.05g Agar 15.5 g	RBA

* Contact plates used exclusively for selective culture of *Rhynchophorus ferrugineus* microbial samples.

Instruments

The instrumentation and equipment used in this research depends on requirements of the different stages. Table 8 provides information of the instrumentation used in this research.

Table 8. Instrumentation and equipment information

INSTRUMENT	MODEL	MANUFACTURER	DESCRIPTION
Laminar flow cabinet	HS9	Heraeus (Hanau, Germany)	Required for microbial isolation and specific procedures; demanding sterile conditions.
Incubation jar	AnaeroJar AG0025	Oxoid™ (Hampshire, United Kingdom)	Microbial incubation jar, required for anaerobic or microaerophillic culturing.
Anaerobic generation system	AnaeroGen AN25US	Oxoid™ (Hampshire, United Kingdom)	Self-contained gas generation system, required for strictly anaerobic culturing.
Microaerophillic generation system	CampyGen CN025A	Oxoid™ (Hampshire, United Kingdom)	Self-contained gas generation system, required for microaerophillic culturing.
Optical Microscope	Eclipse E200	Nikon (China)	For observation and identification of microbial strains.
Stub metalizer	S150	Sputter Edwards (England)	For stub metallizing required for Scanning Electron Microscopy
Scanning Electron Microscope (SEM)	EVO40	Zeiss (Germany)	For observation and characterization of microbial isolates.
Centrifuge	5415 D	Eppendorf (Germany)	Microcentrifuge required for obtaining cellulolytic crude extracts.
Rotary incubator	SKI4	Argolab (Italy)	For liquid-based microbial culturing and enzymatic reactions
Plate heating stirrer	ARE F20520162	VELP Scientifica (Italy)	For heating/stirring during DNS assay
UV-Visible Spectrophotometer	UV 1601	Shimadzu Europe GmbH (Germany)	For determination of reducing sugars with DNS method
Tissue culture flasks	1100-025	Iwaki (Japan)	70 mL /25 cm^2 surface, untreated tissue culture

			flasks with two position cap, for microbial liquid culture.

Preparation of microbial culture substrates

Tryptic Soy Agar (TSA)

Tryptic Soy Agar was prepared in 1.0 L Schott Flasks; by dissolving 30.0 g TSA and 15.0 g Agar in 1000 mL distilled water. This mixture was heated in plate stirrer at 100°C until Agar was completely dissolved. pH was adjusted to 7.1-7.2 and the mixture was sterilized at 121°C for 15 minutes.

After sterilization 20.0 mL TSA were poured on Petri plates under sterile conditions. TSA plates were sealed once agar had solidified and stored at 4°C.

Tryptic Soy Broth (TSB)

Tryptic Soy Broth is prepared in 1.0 L Schott Flasks; by dissolving 30.0 g TSB in 1000 mL distilled water. This mixture was stirred until completely dissolved. pH was adjusted to 7.1-7.2 prior to sterilization at 121°C for 15 minutes. TSB was used only for liquid-base culture, and as inoculum for cellulolytic activity assay.

Potato Dextrose Agar (PDA)

Potato Dextrose Agar was prepared in 1.0 L Schott Flasks; by dissolving 24.0 g PDB and 15.0 g Agar in 1000 mL distilled water. The mixture was stirred and 50 mM Acetate buffer were added. pH was adjusted to 5.0.

After sterilization, 20.0 mL PDA were poured onto Petri plates. PDA plates were sealed and stored at 4°C.

Potato Dextrose Broth (PDB)

Potato Dextrose Broth is prepared similarly to PDA but it is a non agarized substrate. PDB was used for liquid based culture as well as inoculum for cellulolytic activity assay.

Cellulolytic Activity Substrates (M1a/b)

For determination of cellulolytic activity a plate based protocol was established. Carboxymethylcellulose (CMC), low viscosity sodium salt, was used as primary carbon source substrates, in conjunction with Yeast Extract, serving as a minimal medium, providing essential nutrients for microbial replication.

CMC based substrates, was prepared based on specifications of Delalibera et al., (2005), and were labeled M1a (agarized) and M1b (non-agarized). The composition of these substrates is detailed in Table 9.

A modification to Delalibera et al., (2005) substrates, was the use of buffers to regulate pH for specific microbial growing conditions when necessary. pH was adjusted in these substrates with the addition of 50 mM phosphate, acetate or diethanolamine buffers, when required.

Table 9. Composition of carboxymethylcellulose-based substrates

ITEM	M1a (Agarized)	M1b (Non-agarized)
Carboxymethylcellulose Low Viscosity Sodium Salt	5.0 g/L	5.0 g/L
Yeast Extract	0.20 g/L	0.20 g/L
Agar	15.0 g/L	-
Sodium Phosphate Monobasic[1]	6.90 g/L	6.90 g/L
Sodium Acetate[2]	4.10 g/L	4.10 g/L
Diethanolamine[3]	5.25 g/L	5.25 g/L

[1] Concentration adjusted to 50mM phosphate buffer pH 6.0 – 8.0

[2] Concentration adjusted to 50mM acetate buffer pH 4.0 – 5.5

[3] Concentration adjusted to 50mM diethanolamine buffer pH 8.5 – 10.0

Larval acquisition and conformation of experimental units

Rhynchophorus ferrugineus larvae

Rhynchophorus ferrugineus is a quarantine phytophagous included in A2 list of EPPO (European and Mediterranean Plant Protection Organization) due to palm infestations occurred in the Mediterranean area since the early 1990s and it is controlled by specific phytosanitary measures in Italy to avoid its diffusion (Decr. 9/11/2007, GU n. 37 del 13/02/2008; Decr. 7/02/2011, G.U. n.36 del 14/02/2011).

Five *R. ferrugineus* larvae were recovered from *Phoenix dactylifera* palm tissues, after a controlled cutting down at Imperia, Italy. These larvae were preserved in their natural feeding substrates to avoid changes in the composition of intestinal microbiota.

Rhynchophorus palmarum larvae

Rhynchophorus palmarum larvae were purchased at the local native-food markets of Francisco de Orellana (Orellana Province, Ecuador). Despite this city is 150 Km north of Puyo, *R. palmarum* larvae are more abundant in northern Ecuadorian Amazon and are sold at the typical food markets at a much lower price. A total of 150 larvae were purchased and kept in their natural feeding substrates consisting of "Morete" palm fibber (*Mauritia flexuosa*).

Once arrived in Puyo, larvae were disposed over a large plastic basket for size and weight selection. Dead or wounded individuals were not considered as part of the experiments and were discarded.

Larval selection was done by determining larval weight, length and width. Weight was measured with a Sartorius Extend ED2201; 2200 × 0.1 g balance. Length and width were measured with a calibrometer. The selection criteria were established attending to eighth and ninth larval instar described by Mexzón et al., (1994) (Table 10).

Table 10. Larval weight and length selection criteria

PARAMETER	SELECTION CRITERIA
Weight	6.47 g – 10.23 g
Length	66.0 mm – 76.0 mm
Width	10.0 mm – 15.0 mm

A total of 120 individual larvae were organized into experimental units, consisting of five lar|vae. The purpose of experimental units was to assure uniformity in the composition of intestinal microbiota once larvae were dissected. Experimental units were formed with randomly-selected larvae, which were then deposited into 1.0 L plastic jars, with holes over its surface to allow air diffusion. In addition, larvae were left with enough palm fibbers for covering, feeding and to avoid cannibalism. Plastic jars containing experimental units were moistened daily to avoid larval dehydration and stress.

Eventually, wounded or dead larvae, found on each experimental unit, were replaced by new larvae acquired at the local food markets from Puyo, respecting the selection criteria shown on Table 10. A total of 24 experimental units were formed and prepared for cellulolytic microbial screening.

Larval dissection

Rhynchophorus ferrugineus

A preliminary microbial isolation assay was established to determine the specificity of microbial strains to inhabit in a particular portion of the digestive tract of *R. ferrugineus* larvae. Three major sections of larval guts were considered: foregut, midgut and reargut. On the other hand, this assay was to develop a standard dissection and microbial selection protocol to be replied on *R. palmarum* larvae in Ecuador.

Dissection was carried out under sterile conditions and each larva was dissected separately to avoid crossed contamination.

Initially, *R. ferrugineus* larvae were deposited into 500 mL beakers and disinfected by immersion on anhydrous ethanol for 60 seconds, after which larvae were rinsed twice in sterile water to remove ethanol.

Disinfected larvae were transferred into a sterilized beaker and placed over a dish containing ice to immobilize them. Once immobilized, individual larvae were placed onto a Petri plate for dissection.

A transversal incision was initially done on the first thorax segment, to separate the larval head, followed by a longitudinal incision on the larval skin along the thorax, leaving the abdominal section exposed. A final incision on the last abdominal segment was practiced to separate the tail from the larvae.

Adipose tissue surrounding the digestive tract was carefully removed. Digestive tubes, attached to head and tail were then separated and placed in another Petri dish and then flooded with saline solution (0.9 % NaCl). Finally, larval head and tail were removed, leaving only the digestive tube which was transferred to a separate Petri dish and cut into three portions. The first third of the tube was the foregut and the remaining portions were midgut and reargut, respectively (Figure 17).

Larval foreguts, midguts and rearguts from each experimental unit were placed into separate sterile vials, containing 5.0 mL saline solution and vortexed for 30 seconds. Vials were then sealed, labeled and stored at 4°C.
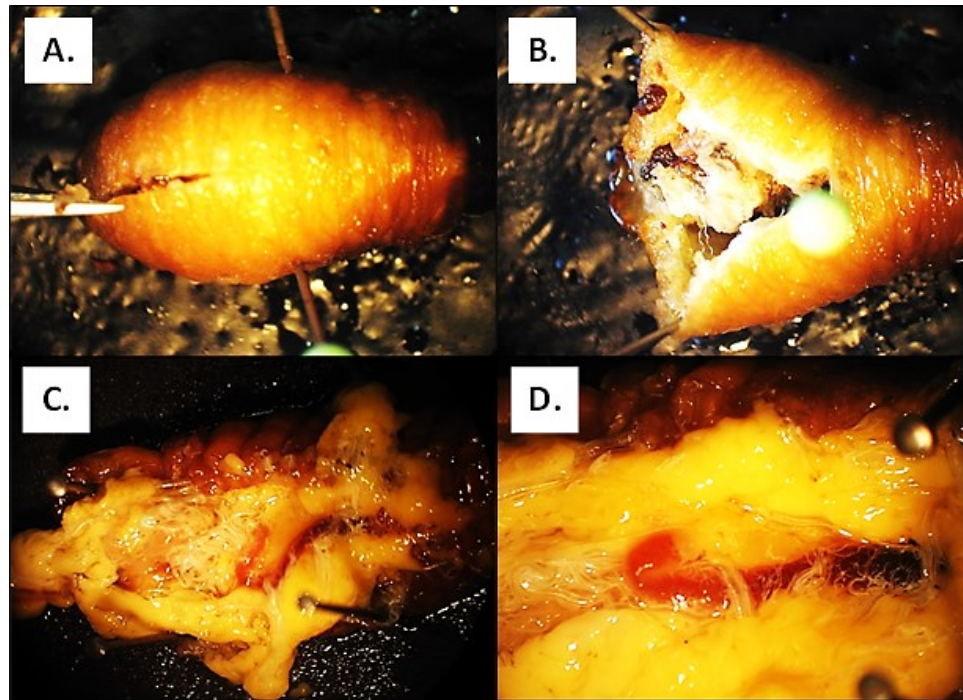
Figure 17. *Rhynchophorus ferrugineus* dissection.

A = Abdominal incision, B = Abdominal cavity exposed, C = Digestive tube surrounded by adipose tissue; D = Detailed intestine

Rhynchophorus palmarum

Larval dissection was carried out following the same protocol established for *R. ferrugineus* larvae. Initially, *R. palmarum* larvae were separated from the jars, containing the experimental units and transferred into a non-sterile 500 mL beaker that was placed into a 1000 mL basket; containing ice, to immobilize larvae.

Once immobilized, larvae were transferred to the laminar flow cabinet and dissected following the specifications of the aforementioned larval dissection protocol. However, digestive tubes were not divided into foregut midgut and reargut, as in *R. ferrugineus* dissection, since no evidence was found to support the fact that microbial isolates inhabit in a specific portion of the intestine.

R. palmarum digestive tubes were placed in vials, containing 10 mL of sterile saline solution, and vortexed for 120 seconds, disrupting the intestinal tissue, to obtain an intestinal suspension, which is the basis for microbial culturing. Vials containing intestinal suspension were then sealed and stored at 4°C (Figure 18). This protocol was observed for every experimental unit.
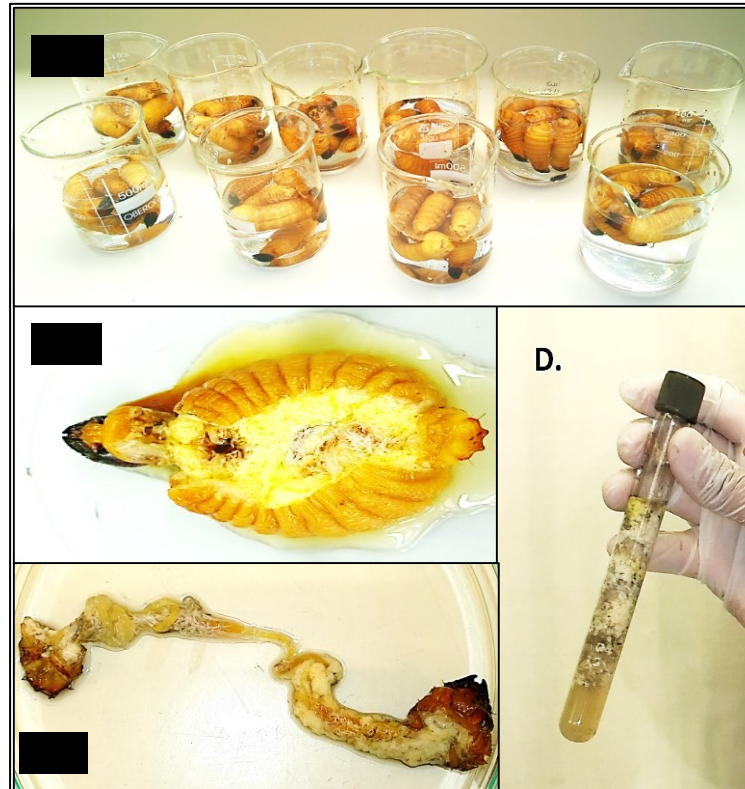
Figure 18. Detail of dissection process: A = Disinfection of experimental Units,

B = Abdominal incisions, C = Larval digestive tube removal,

D = Intestinal suspension.

Microbial isolation from *R. ferrugineus* digestive tract

As *R. ferrugineus* larvae were purchased exclusively for development of the research protocols to be applied in *R. palmarum* larvae, microbial isolation on *R. ferrugineus* did not include low-oxygen atmosphere conditions. Only aerobic cultivable bacteria were considered in this assay.

Three vortexed fluid samples (100 µL) of *R. ferrugineus* digestive tract (foregut, midgut and reargut, respectively) were seminated onto TSA plates and incubated at 30°C for seven days. Each gut segment culture had a replica.

Subsequent isolation was carried out from the original foregut, midgut and reargut plates, by disseminating TSA plates with individual isolates, until reaching pure cultures. The resulting microbial isolates were labeled as indicated in Table 11. The same codification was used for *R. palmarum* isolates and is also included herein.

Table 11. Experimental codification of microbial isolates from *R. ferrugineus* and *R. palmarum*

Origin	Microbial Category	Isolation Conditions	Isolate Number	Id Code
Rhynchophorus ferrugineus larvae (**RF**)	Bacterial (**b**)	Aerobic (**EC**)	001 002 etc.	**RFbEC-001**
Rhynchophorus palmarum larvae (**RP**)	Bacterial (**b**) Fungal/Yeast (**m**)	Aerobic (**EC**) Microaerophilic (**ED**) Anaerobic (**AN**)	001 002 etc.	**RPbEC-001** **RPmEC-001** **RPbED-001** **RPmED-001** **RPbAN-001** **RPmAN-001**

Microbial isolation from *R. palmarum* digestive tract

Since there was no specific information regarding the composition of the intestinal microbiota of *R. palmarum*, two separate approaches were considered: isolation of bacteria and isolation of fungi or yeast. Bacteria were the first targeted microorganisms, after which fungi and yeast were studied. Twelve experimental units, each consisting of five larvae (*See Larval acquisition*), were destined for bacterial isolation and the remaining for fungal/yeast isolation.

In addition, low oxygen concentrations were also considered in this assay, resulting in parallel screening under aerobic, microaerophillic (5% O_2) and strictly anaerobic conditions ($O_2 < 1\%$). Figure 19 illustrates the procedure followed for microbial isolation. This protocol was followed for both bacterial and fungal/yeast isolation under aerobic, anaerobic and microaerophillic conditions.
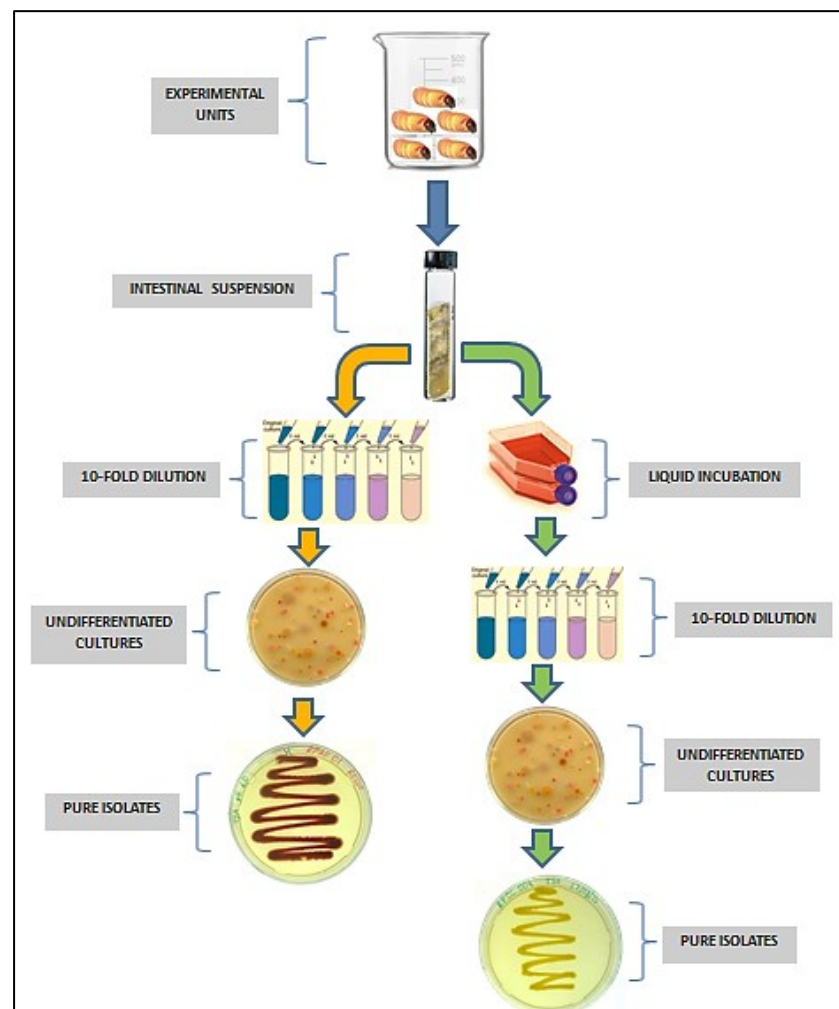

Figure 19. Microbial isolation procedure

Obtaining of undifferentiated microbial cultures

Undifferentiated cultures were obtained from intestinal suspension, consisting of the resulting vortexed fluid from five *R. palmarum* larval guts, collected after dissection (*See dissection of R. palmarum larvae*).

For bacterial isolation, plate based TSA as well as liquid based TSB cultures were considered. Intestinal suspension was ten-fold diluted and 100 µL of 10^{-10} dilution were seminated onto TSA plates and distributed over the plate with a Drigalski spatula. Plates were incubated at 30°C for seven days.

The reason for diluting intestinal suspension, prior to inoculation, was to facilitate microbial isolation, since seminating intestinal suspension directly on the plates, as it was done for *R. ferrugineus*, made difficult to separate individual colonies, due to a dense microbial growth, resulting in tightly overlapped colonies.

Liquid based cultures were prepared by inoculating 100 µL of intestinal suspension in Iwaki 25 cm^2 tissue culture flasks with 30 mL TSB. These flasks were incubated in a reciprocal shaker incubator at 120 rpm and 30°C for seven days. Samples of liquid based cultures were serially diluted as in plate culturing. 100 µL of 10^{-10} dilution were inoculated in TSA plates and incubated at 30°C for seven days.

Isolation of fungi and yeast was done in a similar way to bacterial isolation described above, using PDA for plate based isolation and PDB for liquid based isolation. In addition, both liquid based and plate based cultures had a replica. As result, a total of 288 undifferentiated cultures were produced from intestinal suspension stocks (12 for bacteria and 12 for fungi/yeast) over an 18-month period, as indicated in Figure 20.
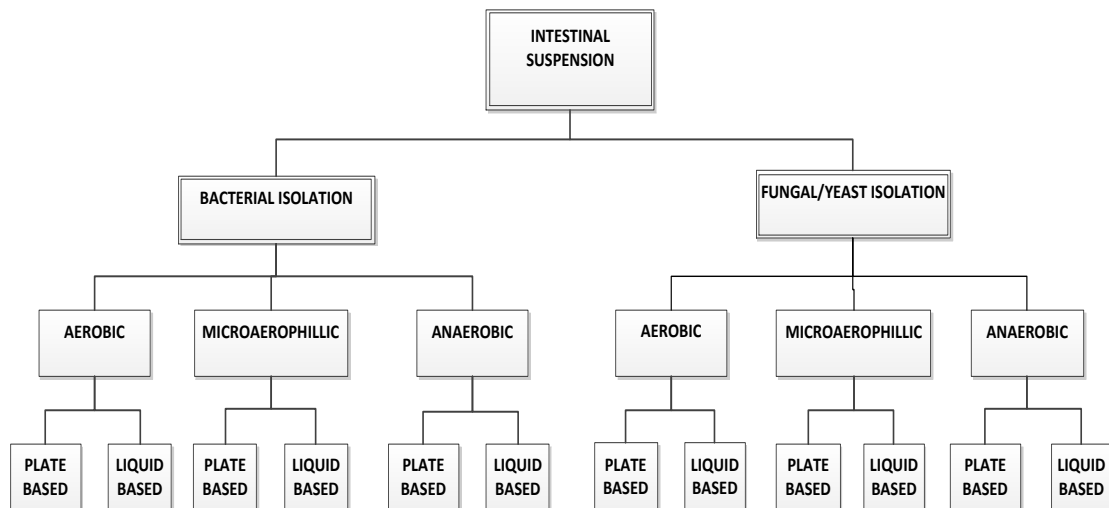
Figure 20. Schematic representation of undifferentiated culture obtaining

Incubation in low-oxygen atmosphere conditions

Oxoid CAMPYGEN and ANAEROGEN, self-containing atmosphere generation systems were used to produce microaerophillic (5% O_2) as well as strictly anaerobic (<1% O_2) conditions, respectively. These systems were used in conjunction with an 2.5 L Oxoid AnaeroJar, to incubate undifferentiated microbial cultures (*See Table 8*).

For plate based incubation in low-oxygen conditions, TSA as well as PDA plates were previously prepared, by opening small holes around the lateral surface of the plate, with a metal rod heated with a Bunsen lamp, prior to microbial inoculation. These holes were made under sterile conditions and their purpose was to allow gas circulation during incubation in the anaerobic jar.

Once holes were made, TSA and PDA plates were seminated with 100 µL of 10^{-10} dilution of intestinal liquid, disseminated over the plate with a Drigalski spatula, as aforementioned, and placed inside the anaerobic jar.

Additionally, Iwaki flasks containing TSB and PDB were placed horizontally, together with PDA and TSA plates, with their curved nose pointing upwards. The caps were partially closed, to allow sufficient gas diffusion.

Sachets containing gas generation systems were open under sterile conditions, and closed inside the jar. The whole process was controlled to not exceed 30 seconds, as recommended by the manufacturer. Samples were then incubated, without agitation, at 30°C for seven days (Figure 21).

Figure 21. Detail of incubation in aerobic and low-oxygen conditions

Obtaining of pure microbial isolates

Individual microbial isolates were obtained by successively reseminating cultures, until satisfactory isolation was achieved. Once undifferentiated plate cultures were incubated, individual colonies were separated, attending to morphological characteristics (color, shape and type of growth). These isolates were pin-point separated with a sterile spatula and seminated into either PDA or TSA plates, as required.

For microbial isolation from liquid-based PDB and TSB undifferentiated cultures, an intermediate step was required. Samples of inoculated PDB and TSB were ten-fold diluted and 100 µL of these dilutions were seminated onto PDA and TSA plates to obtain plate-based undifferentiated cultures from which individual colonies would be isolated (*See Figure 19*). These plates were incubated at 30°C for seven days, either in aerobic or microaerophillic/anaerobic conditions.

As abovementioned, 288 undifferentiated cultures were obtained throughout the microbial isolation phase of this research. However, to facilitate discrimination and selection of individual isolates, only those cultures showing evident colony dimorphism were selected. Additionally, when two or more plates showed coincidence in their cultures, only those plates with very differentiable colonies were retained and the rest were discarded. This procedure in fact eliminated, for some strains, the necessity of further plate culturing to consider them pure isolates.

In addition, microbial isolates were code-labeled, as indicated in Table 11.

Isolation procedure resulted in a total of three aerobic bacteria from *R. ferrugineus* larval digestive tract and fourteen isolates (bacteria and fungi/yeast) obtained from *R. palmarum*. This information is presented in Table 12.

Table 12. Results of microbial isolation from the digestive tract of *R. ferrugineus* and *R. palmarum* larvae.

Origin	Group	Id Code
Rhynchophorus ferrugineus	Aerobic Bacteria	**RFbEC-001** **RFbEC-002** **RFbEC-003**
Rhynchophorus palmarum	Aerobic Bacteria	**RPbEC-001** **RPbEC-002** **RPbEC-003** **RPbEC-004** **RPbEC-005** **RPbEC-006** **RPbEC-007** **RPbEC-008**
	Microaerophillic Bacteria	**RPbED-001**
	Anaerobic Bacteria	**RPbAN-001**
	Aerobic Fungi/yeast	**RPmEC-001** **RPmEC-002** **RPmEC-003**
	Microaerophillic fungi/yeast	**RPmED-001**

Microbial identification and characterization

Identification and characterization of microbial isolates from R. ferrugineus larvae

Microbial isolates from *R. ferrugineus* were observed under Scanning Electron Microscopy (SEM). For this purpose, microbial samples were deposited in Eppendorf tubes and washed with sodium cacodylate buffer (0.125 M sodium cacodylate + 12% saccharose). Microbial samples were then treated with fixative (0.125 M sodium cacodylate + 12% saccharose + 2.5% glutaraldehyde) and passed through alcohol scale folding (50, 60, 70, 80, 90 and 100% V/V). Samples were finally centrifuged at 6000 RPM for 10 minutes and supernatant was removed. Precipitate was then collected, placed in stubs for metallization and observed through a SEM (Figure 22).
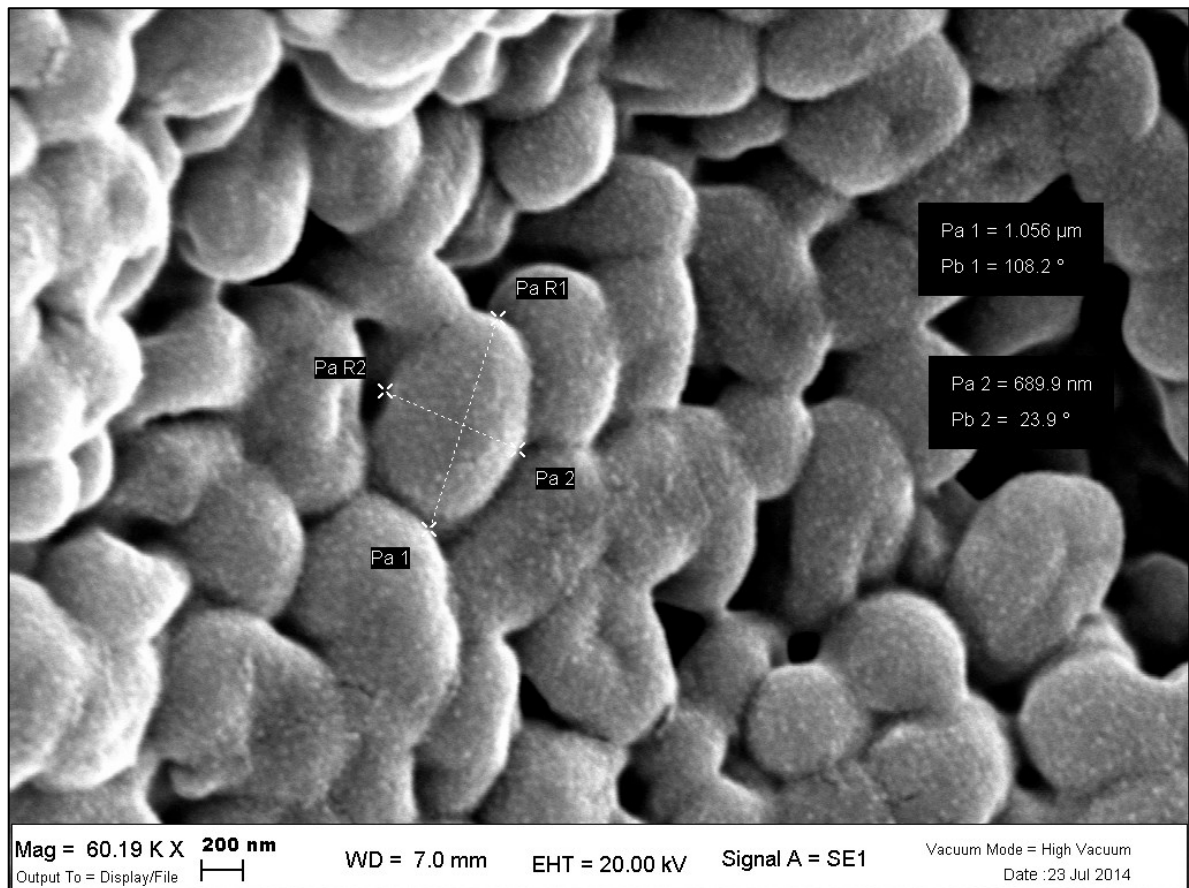

Figure 22. Microbial observation under SEM

Additionally, a Gram staining was carried out to the isolates. This procedure was performed as follows:

- Under sterile conditions, microbial samples were spread on microscopy slides. A water drop was added and microbial samples were emulsified with a sterile lance and left to air dry. Once dried slides were flamed out with a Bunsen lamp.

- Once flamed, slides were flooded with crystal violet solution, for one minute, allowed to dry and washed with distilled water to remove the excess violet dye.

- Slides were then flooded with Lugol's solution (Gram Iodine) for one minute and washed with distilled water.

- Slides were left to dry and washed with ethanol 95% V/V for discoloration, until no violet color was washed away. Slides were then allowed to dry.

- Slides were finally flooded with Safranin, as contrast colour, for 30 seconds and washed with distilled water. Once dry slides were observed under optical to evaluate the Gram category of microbial isolates.

In addition to Gram staining, microbial isolates were cultivated in selective contact plates (4x5 Ø = 65 mm) containing selective media for *Staphylococcus* (MSA), Coliforms (VRB) and Mold/yeast (RBA). Samples of each isolate were disseminated separately on each medium contact plate and incubated for five days at 30°C (Figure 23). Isolates from *R. ferrugineus* larvae were found to be Gram negative and VRB positive. On the other hand, no difference was found, concerning the distribution and eventual selectivity of microbial strains to inhabit in a specific tram of the digestive tube.

Figure 23. Selective media contact plates (From left to right) Mannitol Salt Agar, Rose Bengal Agar and Violet Red Bile Agar

The results of microbial identification were listed in Table 13.

Table 13. Microbial isolates from *R. ferrugineus* larvae

Strain	Colony description	Overall shape	Gram test	Isolation conditions
RFbEC-001	Transparent yellow colonies with defined borders and globular growth	Bacillus	Gram (-)	Aerobic
RFbEC-002	White round colonies with defined borders	Bacillus	Gram (-)	Aerobic
RFbEC-003	Red colonies with defined borders and globular growth	Bacillus	Gram (-)	Aerobic

Identification and characterization of microbial isolates from R. palmarum larvae

Microbial colonies were differentiated attending to morphological parameters. Initially, color was the primary reference to identify individual colonies. Regarding color, bacterial isolates were found to exhibit a marked dimorphism. However, when individual isolates had the same color; i.e. white, secondary identification through growth dimorphism was necessary.

Most bacterial isolates were found to have a globular growth. However, some colonies exhibited a branched growth, which was decisive criterion if color dimorphism was not conclusive. On the other hand, when colony growth did not allowed sufficient criteria for differentiation, a tertiary step required Gram staining as means of successfully resolving morphological discrepancy in the differentiation of microbial isolates.

Regarding fungal/yeast isolates, morphological differentiation required less effort, since morphological dimorphism allowed sufficient criteria for establishing pure isolates. Additionally, the use of buffered PDA/PDB on a pH range of 5.0 - 5.1, reduced the possibility of misidentification, since bacteria generally grow on a nearly-neutral pH.

A Merck Gram Staining Kit was used in this assay. The protocol for Gram staining was the same as followed for *Rhynchophorus ferrugineus* isolates. A predominance of Gram negative bacteria was found in the digestive tube of *Rhynchophorus palmarum* larvae.

Bacterial isolates were also observed under Scanning Electron Microscope. For that purpose, samples of each isolate were treated as indicated previously for *R. ferrugineus* larvae. Results of the identification and characterization procedures are listed on Table 14.

Table 14. Characterization of microbial isolates from *Rhynchophorus palmarum* larvae.

Strain	Colony description	Overall shape	Gram test	Isolation conditions
RPbEC-001	Pale yellow colonies with defined borders and branched growth	Bacillus	Gram (-)	Aerobic
RPbEC-002	Pale-red round colonies with undefined borders and branched growth	Bacillus	Gram (-)	Aerobic
RPbEC-003	Orange colonies with multiple-point growth	Bacillus	Gram (-)	Aerobic
RPbEC-004	Yellow colonies with undefined borders and round growth	Bacillus	Gram (-)	Aerobic
RPbEC-005	White colonies with defined borders and round growth	Bacillus	Gram (+)	Aerobic
RPbEC-006	Transparent yellow colonies with undefined borders and round growth	Bacillus	Gram (+)	Aerobic
RPbEC-007	Red colonies with defined borders and round growth	Bacillus	Gram (-)	Aerobic
RPbEC-008	Pale white colonies with green halos and undefined borders	Bacillus	Gram (-)	Aerobic
RPbED-001	Pale white colonies with defined borders and globular growth	Bacillus	Gram (-)	Micro-Aerophillic
RPbAN-001	Transparent yellow colonies with rugged borders and branched growth	Bacillus	Gram (-)	Anaerobic
RPmEC-001	Black amorphous colonies with hyphal mycelia and annular growth	Mold	-	Aerobic
RPmEC-002	Pale white colonies with undefined borders	Yeast	-	Aerobic
RPmEC-003	Yellow colonies with rigid borders and linear growth	Yeast	-	Aerobic
RPmED-001	Transparent yellow colonies with rugged borders and round growth	Yeast	-	Micro-aerophillic

Cellulolytic activity assay

Cellulolytic activity of R. ferrugineus microbial isolates

Cellulolytic activity of microbial isolates was evaluated with carboxymethylcellulose (CMC) acting as sole source of carbon in M1a substrate, which was prepared in accordance to specifications of Delalibera et al., (2005) (*See Preparation of Microbial Culture Substrates*).

In this preliminary assay pH of M1a medium was standardized to 7.0; with the addition of 50 mM of phosphate buffer. The principle of this assay is that obligate cellulolytic microorganisms necessarily release cellulolytic enzymes, in order to survive in a medium, where bioavailability of carbon is restricted to a derivative of cellulose.

Liquid inoculum was prepared from plate microbial stocks by seminating samples of each isolate in tissue culture flasks containing 30 mL of TSB. The inoculum was incubated for seven days at 30°C in a reciprocal shaker. Samples of the inoculum were then serially diluted 10^0, 10^{-1}, 10^{-2} and 10^{-3}. 50.0 µL of these dilutions were seminated onto M1a plates and incubated at 30°C for seven days (Figure 24).
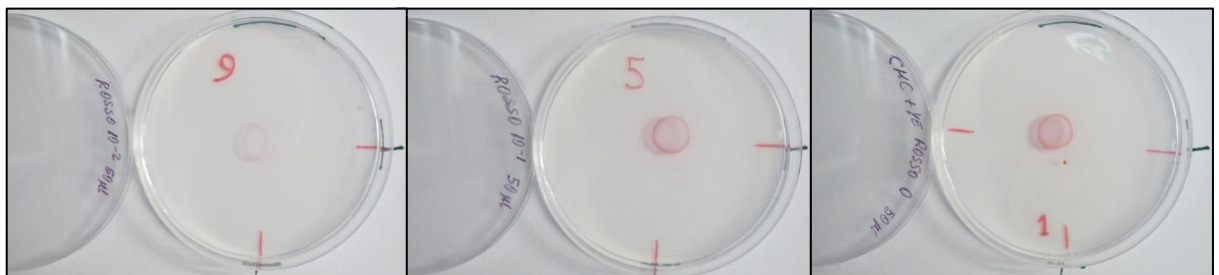

Figure 24. Microbial isolate dilutions cultivated on M1a plates.

Cellulolytic activity was determined by staining with Congo Red, as described by Teather & Wood, (1982a). This assay is based on the premise that amino ends of Congo Red bond to contiguous β(1-4) D-glucopyranosil units of non-depolymerized CMC, resulting in a stable glucan-dye complex.

Plates were flooded with Congo Red (1.0 mg/mL) for 30 minutes. After removing the excess of Congo Red, plates were washed with 1 M NaCl solution for 15 minutes and allowed to dry. The presence of cleared halos surrounding the microbial colonies indicated positive cellulolytic activity (Figure 25).
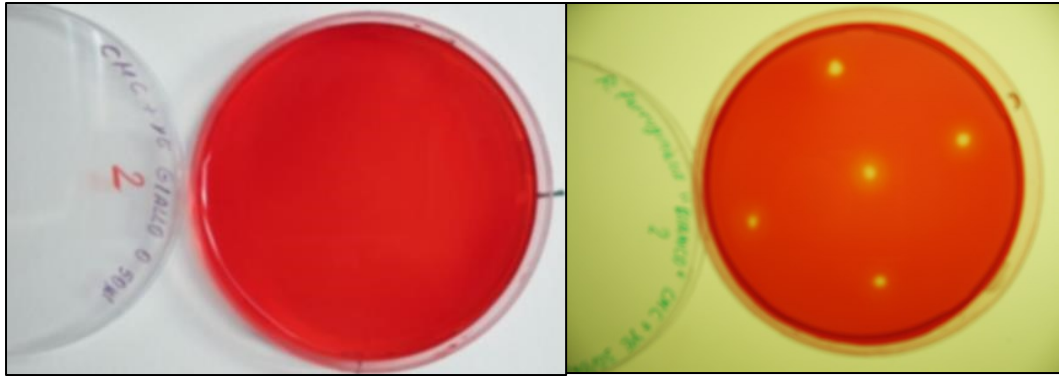
Figure 25. Congo Red staining. Negative (left) and positive (right) cellulolytic activity

The estimation of cellulolytic activity was done as Hankin & Anagnostakis, (1975). The presence of cleared halos surrounding the inoculated colonies revealed positive cellulolytic activity. Enzymatic activity (E_A) is represented as a quotient of halo diameter (*Hd*) and colony diameter (*Cd*):

$$E_A = \frac{Hd}{Cd}$$

Enzymatic activity was categorized as High, Medium, Low or None, according to E_A ratio values (Table 15):

- High: $E_A \geq 1.50$
- Medium: $1.50 > E_A > 1.10$
- Low : $1.10 > E_A > 1.00$
- None: $E_A = 0.00$ (*Hd* = 0)

Table 15. Cellulolytic activity of *R. ferrugineus* microbial isolates, expressed as quotient of halo diameter and colony diameter

STRAIN	ENZYM. ACTIVITY (E_A 10^0)	ENZYM. ACTIVITY (E_A 10^{-1})	ENZYM. ACTIVITY (E_A 10^{-2})	ENZYM. ACTIVITY (E_A 10^{-3})	CATEGORY
RFbEC-001	0.00	0.00	0.00	0.00	NONE
RFbEC-002	1.03	1.07	1.00	1.02	LOW
RFbEC-003	0.00	0.00	0.00	0.00	NONE

Cellulolytic Activity Assay of R. palmarum microbial isolates

Cellulolytic activity of *R. palmarum* isolates was evaluated with a similar protocol as presented in the precedent paragraph. However, due to logistical issues, this assay was carried out in two stages. Bacterial strains were first assayed, since they represented a large number of the isolates, and fungal/yeast strains were tested at last.

Microbial inoculum was prepared as indicated as for *R. ferrugineus* isolates. PDB was used for bacterial inoculants and TSB for fungal/yeast inoculants. Nonetheless, no serial dilutions were done, aiming to obtain more visible and consistent microbial colonies, prior to Congo Red staining. Additionally M1a pH was adjusted to 7.0-7.2 for bacterial cellulolytic assays and 5.0-5.2 for fungal/yeast cellulolytic assay.

100 µL of microbial inoculum were seminated in M1a plates and incubated at 30°C for 7 days. To facilitate detection of cellulolytic activity, microbial inoculants were deposited in the centre of the plate. Microaerophillic as well as anaerobic strains were incubated in an Oxoid AnaeroJar, with CampyGen and AnaeroGen self-contained gas generating systems, respectively. Cellulolytic activity was determined with Congo Red staining (*see Microbial identification*). Figure 26 shows an example of cellulolytic activity of microbial isolates from *R. palmarum* larvae.

Plate-based cellulolytic activity assays were conducted in triplicate for all isolates and the enzymatic activity was quantified as a quotient between the diameter of the observed halos and the diameter of the colonies themselves
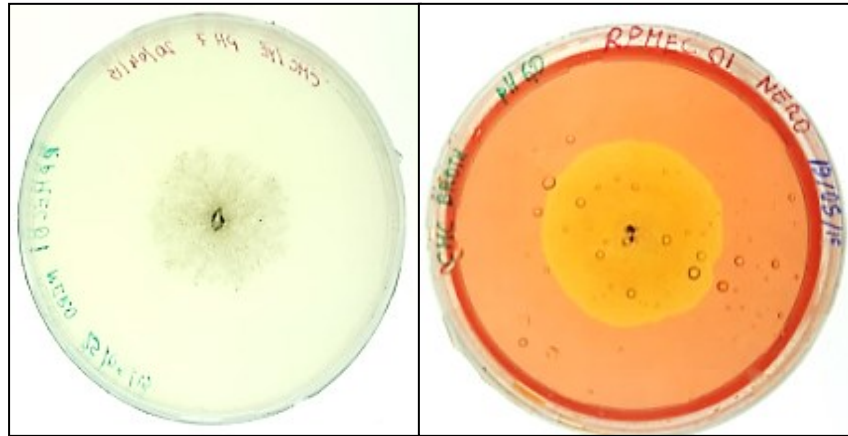
Figure 26. Determination of cellulolytic activity by Congo Red staining

. Enzymatic activity (*EA*) was also categorized as High, Medium, Low or None, according to *EA* ratio values (*see Microbial identification*) (Table 16).

Table 16. Cellulolytic Activity of microbial isolates

STRAIN	Colony Diameter [$\bar{X}$] (mm)	Colony Diameter [σ] (mm)	Halo Diameter [$\bar{X}$] (mm)	Halo Diameter [σ] (mm)	*EA*	REFERENCE
RPbEC-001	10.33	0.58	0.00	0.00	0.00	None
RPbEC-002	8.67	1.53	0.00	0.00	0.00	None
RPbEC-003	8.33	0.58	0.00	0.00	0.00	None
RPbED-004	9.33	0.58	0.00	0.00	0.00	None
RPbEC-005	8.33	1.15	0.00	0.00	0.00	None
RPbEC-006	10.00	1.00	0.00	0.00	0.00	None
RPbEC-007	9.33	1.53	0.00	0.00	0.00	None
RPbEC-008	8.33	0.58	0.00	0.00	0.00	None
RPbED-009	9.67	0.58	0.00	0.00	0.00	None
RPbAN-010	10.33	0.58	0.00	0.00	0.00	None
RPmEC-001	38.67	1.53	59.67	2.08	1.54	High
RPmEC-002	9.33	1.15	0.00	0.00	0.00	None
RPmEC-003	18.67	1.53	21.67	1.53	1.16	Medium
RPmED-004	8.33	1.53	8.67	2.08	1.04	Low

Microbial selection and identification

Once results of preliminary cellulolytic activity assay were complete, RPmEC-001 was selected for isolation of cellulolytic enzymes. Additionally, samples of RPmEC-001 were sent to the Leibniz-Institut DSMZ GmbH; in Germany for DNA sequencing as well as identification through morphological characterization.

Gene identification was made through 18S rDNA Internal Transcriber Spacer (ITS) and rDNA Large Subunit (LSU). Comparison of assembled rDNA ITS sequences was performed with GenBank and MycoID databases.

Cellulolytic activity of *Thielaviopsis ethacetica* Went (MB 239913) as a function of the cultivation time

T. ethacetica was inoculated in 250 mL flasks containing 100 mL of M1b medium adjusted to pH 6.0 and incubated at 30°C and 120 rpm under aerobic conditions in a reciprocal shaker for 14 days. Every 24 hours, 1.0 mL samples were withdrawn and treated at 4°C with ammonium sulfate (0.697 g/mL; 100% saturation) in order to precipitate all the proteins. The samples were centrifuged at 10000 rpm for 10 minutes and the precipitates were stored at 4°C without removing supernatant.

The cellulolytic activity of the precipitates was evaluated by diluting each of them, after removing of the supernatant, into 10 mL of 1% carboxymethylcellulose (CMC) solution (W/V) in 50 mM phosphate buffer, pH 6.0, and incubating the mixtures (enzymatic reactions) in sealed vials at 30°C and 120 rpm for 72 hours. At regular intervals (3 hours, 24 hours and 72 hours) the presence of reducing sugars was assayed as response of cellulolytic activity.

Reducing sugars were determined with the dinitrosalicylic acid (DNS) method as described by Miller (1959). DNS reagent was prepared by dissolving dinitrosalicylic acid (0.2 g, 0.88 mmol) phenol (0.04 g, 0.42 mmol), sodium thiosulfate (0.01 g, 0.04 mmol) and sodium-potassium tartrate (4.0 g, 14.2 mmol) in 10 mL of NaOH (2% W/V). Distilled water was added to this solution to a final volume of 20 mL.

For determination of reducing sugars, 2.0 mL of DNSA reagent were added to 500 µL of enzymatic reaction sample and 500 µL distilled water. This mixture was heated in boiling

water for 5.0 minutes and left to cool at room temperature. The absorbance of these samples was measured at 540 nm in a Shimadzu UV-1601 spectrophotometer.

Influence of pH and temperature on the production of cellulolytic enzymes by *T. ethacetica*

Incubation factors, temperature and pH of the medium, were assayed as modifiers of cellulolytic enzymes obtainable by *T. ethacetica* cultures. Experimental temperature interval was considered from 20°C to 40°C and pH interval was established from 4.0 to 8.0. Cultures of 100 mL were started with *T. ethacetica* inoculum. These cultures were grown for seven days in M1b medium with pH adjusted to the proper value and at the temperature described in Table 17.

Table 17. Influence of pH and temperature on the production of cellulolytic enzymes by *T. ethacetica*

RUN	BLOCK	FACTOR 1 A: pH	FACTOR 2 B: Temperature (°C)	RESPONSE 1 ABSORBANCE (540 nm)	RESPONSE 2 REDUCING SUGARS (g/L glucose)
1	Block 1	7.0	30.0	0.223	1.111
2	Block 1	4.0	20.0	0.816	2.809
3	Block 1	4.0	20.0	0.815	2.806
4	Block 1	4.0	40.0	0.003	0.481
5	Block 1	7.0	40.0	0.082	0.707
6	Block 1	5.5	20.0	0.442	1.738
7	Block 1	5.5	30.0	0.597	2.182
8	Block 1	7.0	40.0	0.079	0.698
9	Block 1	5.5	40.0	0.380	1.560
10	Block 1	7.0	20.0	0.005	0.486
11	Block 1	4.0	30.0	0.623	2.256
12	Block 1	7.0	20.0	0.004	0.483

Analytical combinations of pH and temperature were generated with Design-Expert Version 6.0.1 (Stat-Ease, Inc. USA) software. After seven days the samples of cultures (1.0 mL) were treated with ammonium sulphate (0.697 g) and the precipitates were used to catalyze enzymatic hydrolysis of a 1% CMC solution in 50 mM phosphate buffer at pH 6.0. After 72 hours the samples (0.5 mL) of the reaction were treated with DNS reagent as previously described.

A D-OPTIMAL + RESPONSE SURFACE statistical design was applied to process experimental data and Absorbance at 540 nm, as well as Reducing Sugars (g/mL), were selected as experimental responses.

For determination of reducing sugars, a glucose calibration curve was plotted, for 0.5, 1.0, 1.5, 2.0 and 2.5 g/L. The absorbance was measured at 540 nm with the DNS method, as indicated in Figure 27.
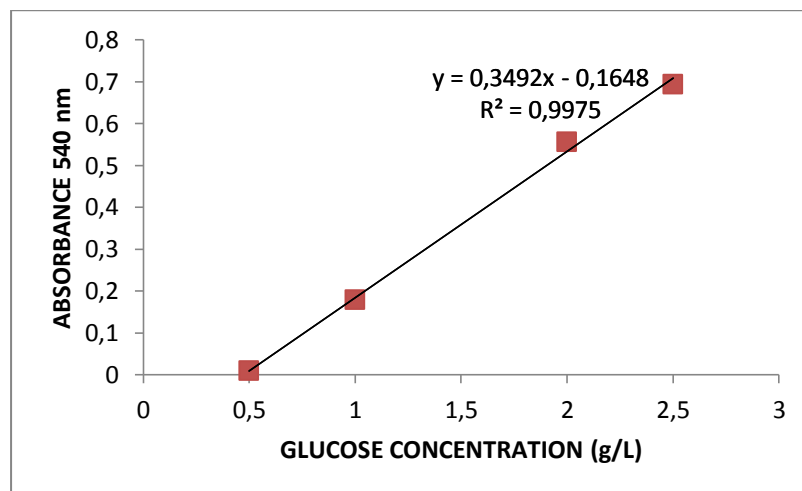

Figure 27. Calibration curve for determination of reducing sugars

Preparation of cellulolytic crude extract from *T. ethacetica*

T. ethacetica was cultivated for four days at 20°C in 250 mL flasks containing 100 mL of M1Bmedium adjusted to pH 4.0. Samples of 1.0 mL of culture were treated with ammonium Sulfate (697 mg/mL) and centrifuged at 10.000 rpm for 10 minutes. Resulted precipitates were stored at 4°C and constituted the enzymatic crude extract stocks from *T. ethacetica* (Figure 28).
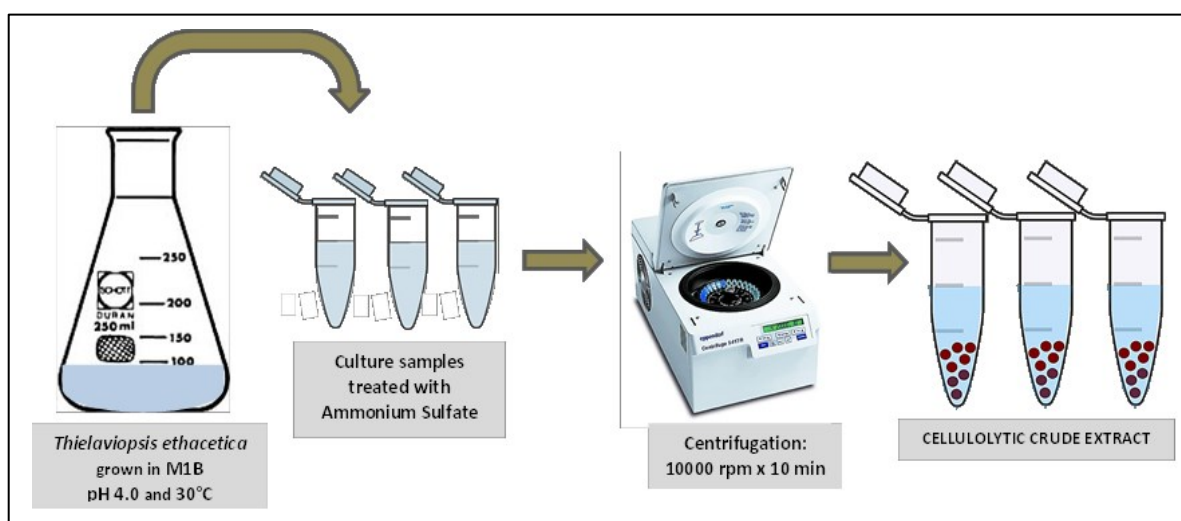

Figure 28. Obtaining of *Thielaviopsis ethacetica* cellulolytic crude extract

Determination of the optimum catalytic pH of the T. ethacetica enzymatic crude extract

Optimal pH was determined by measuring release of reducing sugars, from 1% carboxymethylcellulose solution treated with enzymatic crude extract stocks from *T. ethacetica* on a pH range from 4.0 to 10.0.

Reaction mixture was prepared by dissolving 1% (W/V) of carboxymethylcellulose low viscosity sodium salt in 10.0 mL of 50 mM buffer solution at the proper pH, using as buffering agent the compounds indicated on Table 18.

One stock of enzymatic crude extract from *T. ethacetica* obtained as above reported was added to the mixture and the reactions were maintained at 30°C and 120 rpm on a reciprocal shaker incubator. Release of reducing sugars was measured with DNS method at 0, 3, 6 and 24 hours of reaction.

Table 18. Buffering agents adopted for the determination of optimal pH.

Buffer	Reaction pH
Sodium Acetate	4.0
	4.5
	5.0
	5.5
Sodium Phosphate Monobasic	6.0
	6.5
	7.0
	7.5
	8.0
Diethanolamine	8.5
	9.0
	9.5
	10.0

Determination of the optimal catalytic temperature of the T. ethacetica enzymatic crude extract

Optimal temperature was determined by measuring the release of reducing sugars from 1% (W/V) CMC solution in 50 mM Acetate at pH 4.5 treated with *T. ethacetica* enzymatic crude extract in a temperature range from 30°C to 80°C.

Tubes containing 10.0 mL of CMC solution were preheated to specific temperatures and then set to reaction with one stock of *T. ethacetica* enzymatic crude extract. Cellulolytic activity, expressed as release of reducing sugars, assayed with DNS method, was measured after 1 and 3 hours of reaction.

Stability of the enzymatic crude extract under optimized pH and temperature conditions

Enzymatic stability under optimal reaction conditions was deduced by the residual activity of the enzymatic crude extract set in the presence of the substrate at the optimal pH and temperature for different time (primary reaction). Three equivalent tubes, each containing 10.0 mL of 1% w/v CMC in 50 mM acetate buffer at pH 4.5 were preheated to 45°C and set to reaction each of one with a stock of enzyme crude extract, in a reciprocal shaker incubator at 120 rpm.

After 0, 1, 3, 5, 7, 24, 48 and 72 hours 1.0 mL of reaction mixture was used to catalyze the hydrolysis on 1.0 mL of 1% (w/v) CMC in 50 mM acetate buffer at pH 4.5, for 60 minutes at 45°C (secondary reaction). After that, a sample of the secondary reaction was analyzed by DNS method.

A baseline correction was necessary to subtract the reducing sugars already present in the sample of the primary reaction used to catalyze the secondary reaction. For this purpose, samples of the primary reaction (1 mL) taken at 0, 1, 3, 5, 7, 24, 48 and 72 hours and diluted with 1.0 mL of 1% CMC solution in 50 mM acetate buffer at pH 4.5 were immediately analyzed to determine the blank absorbance (B_{act}).

Variation of catalytic activity (Δ_{act}) was calculated by subtracting absorbance of the blank (B_{act}) from that of the catalyzed samples (C_A).

RESULTS AND DISCUSSION

The aim of this research was the isolation and characterization of cellulolytic enzymes from the digestive tract of *Rhynchophorus palmarum* larvae. Similar studies demonstrated the presence of such enzymes in the digestive fluid of these larvae, but their origin was not defined. In addition, as far as evidence supports, insect gut microbiome is a promising source of cellulolytic enzymes and other biomolecules of growing importance for biotechnological applications. This study was set in a cooperation frame between the University of Ferrara and Universidad Estatal Amazónica in Ecuador. The experimental stage of this research consisted of two approaches. Initially, cellulolytic microorganisms were isolated from the digestive tract of *R. palmarum* larvae and then the resulting cellulase-producer strains were cultivated to obtain and characterize their cellulolytic enzymes.

Additionally, as *R. palmarum* is listed among the EPPO's insect control and restriction list, and the mobilization of Amazon forest resources is penalized by the Ecuadorian government, it was decided to perform the experimental stages both in Italy and Ecuador. In this sense, it was decided to develop an experimental protocol in which *Rhynchophorus ferrugineus* was used as test bed, particularly for larval dissection and microbial isolation, which were latter replied in Ecuador. This protocol involved different approaches, including low-oxygen incubation atmospheres, as well as selective substrates, for fungi and bacteria, in order to guarantee the maximum isolated cellulolytic strains as possible. This protocol was elaborated in cooperation with Dr. Marilena Leis, from the Department of Life Science and Biotechnology of the University of Ferrara.

To develop the microbial isolation protocol, five exemplars of *R. ferrugineus* larvae were obtained from infested palms, downed at Imperia, Italy, and their dissected intestines were collected, in order to obtain aerobic cellulase-producing microorganisms, from which cellulolytic enzymes would be isolated. Nor anaerobic or microaerophillic strains were isolated in this case, since *R. ferrugineus* was not the scope of this research.

A total of three aerobic strains were isolated from *R. ferrugineus*. Initially, the digestive tract of these larvae was divided into foregut, midgut and reargut to evaluate the specificity of microorganisms to grow in a particular portion of the larval intestine. However, no evidence was found, indicating any difference in the number or type of isolates, in relation to the intestine segment from which they were isolated. As result, microbial isolation protocol on *R. palmarum* larvae did not include segmentation of the digestive tract.

On the other hand, microbial isolates of *R. ferrugineus* were characterized by observation under optical microscopy as well as through Scanning Electron Microscopy (SEM) and

Gram staining. A further characterization included also culture on selective media for coliform (Violet Red Bile), staphylococcus (Mannitol Salt Agar) and fungi/yeast (Rose Bengal Agar). As result, it was determined that these strains corresponded to VRB-positive, Gram negative, *Bacillus*. Once characterized, cellulolytic activity of *R. ferrugineus* microbial isolates was determined by Congo Red staining, after incubation in Delalibera, et al., (2005) M1a modified medium, consisting of carboxymethylcellulose (CMC) (5 g/L), yeast extract (0,2 g/L) and agar (15 g/L) . Results of microbial characterization and cellulolytic activity of *R. ferrugineus* are presented in Table 19.

Table 19. Characterization and cellulolytic activity of microbial isolates from *R. ferrugineus* larvae

Strain	Colony description	Overall shape	Gram test	Isolation conditions	Cellulolytic activity
RFbEC-001	Transparent yellow colonies with defined borders and globular growth	*Bacillus*	Gram (-)	Aerobic	Negative
RFbEC-002	White round colonies with defined borders	*Bacillus*	Gram (-)	Aerobic	Negative
RFbEC-003	Red colonies with defined borders and globular growth	*Bacillus*	Gram (-)	Aerobic	Negative

Once developmental stages were completed for *R. ferrugineus* larvae, the resulting protocols were implemented in Ecuador for *R. palmarum* larvae, with certain modifications. Due to the relative abundance of these larvae, which is widely consumed as a typical food of indigenous populations of Ecuadorian Amazon, a total of 120 larvae were assessed for cellulolytic microbial screening. These larvae were organized in experimental units, in order to ensure uniformity and significance of the obtained experimental data.

After dissection, larval intestines were placed together, with 10.0 mL distilled water and vortexed. The resulting intestinal suspension was ten-fold diluted and seminated onto Tryptic Soy Agar (TSA) and Potato Dextrose Agar (PDA) plates; as well as in Tryptic Soy

Broth (TSB) and Potato Dextrose Broth (PDB) for liquid incubation, under aerobic, microaerophillic (5% O_2) and strictly anaerobic (<1% O_2) conditions. As result, a total of 288 undifferentiated cultures were obtained, from which individual colonies were isolated. These isolates were characterized, following the same protocol developed for *R. ferrugineus*, except for the incubation on selective media, which was not considered in this case. As result, a total of 15 microbial isolates were identified from *R. palmarum* digestive tract. These isolates are constituted mostly by Gram negative bacteria (11 isolates) and, in less proportion, by fungi and yeast (4 isolates). This situation has also been observed in similar research, intended for other lignocellulose-feeding insects, particularly in the Curculionidae family.

The exact composition of *R. palmarum* larval microbiota, however, remains undefined, since this research was not focused on microbial DNA identification of the entire intestinal community. Nonetheless, the isolation protocol was developed on the basis that cellulolytic microorganisms, living in the digestive tube of insects, may include either aerobic, obligate-anaerobic or microaerophillic bacterial and fungal/yeast strains.

Regarding incubation under low-oxygen conditions, it was found that most of the intestinal isolates were aerobic, despite the fact that two isolates were microaerophillic and one was strictly anaerobic. This situation supports the fact that most of the intestinal microbiota is assimilated by lignocellulose-feeding insects, from the surrounding environment, not necessarily selecting specifically-evolved endosymbionts, but including a large number of opportunistic strains.

Microbial isolates from *R. palmarum* larval intestine were incubated in M1a, to determine their cellulolytic activity after staining with Congo Red. Results demonstrated that only fungal and yeast strains had cellulolytic activity, whereas no cellulolytic bacteria were identified. The absence of cellulolytic bacteria, among the intestinal isolates, indicates that most of these act as commensals and do not play a significant role in lignocellulose degradation.

On the other hand, evidence demonstrates that the composition and characteristics of cellulolytic microbiota in insects differs between species of the same taxonomic order and are closely related to their type of diet. For instance, Hu, et al., (2014) report the presence of at least ten bacterial strains involved in cellulolytic degradation of wood borer Coleoptera *Dendrococcus armandi*, while Rinke et al., (2011) report only five bacterial cellulolytic strains and four filamentous fungi, inhabiting in the digestive tube of the sugar

cane weevil *Sphenophorus levis*. However, in this study, only a filamentous fungus and two yeasts had cellulolytic activity, whereas no cellulolytic bacteria were found on the digestive tract of *R. palmarum* larvae.

In addition, the fermenting nature of decaying palm fibbers, from which *R. palmarum* larvae feed, poses an ideal environment for the development of saprophytic fungi, more efficient in degradation of such low-lignified substrates, than bacteria. This explains the scarcity of cellulolytic bacteria in the digestive tract of *R. palmarum* larvae.

Besides, cellulolytic activity of fungal/yeast isolates was quantified as quotient of halo diameter versus colony diameter, as reported by Hankin & Anagnostakis, (1975). As result, it was observed that fungal and yeast isolates differed in their ability to degrade CMC, under the same assay conditions. The isolated black rot fungus RPmEC-001 showed the maximum cellulolytic activity and was therefore selected for production and characterization of cellulolytic enzymes.

Samples of RPmEC-001 were sent to the Leibniz Institut DSMZ GmbH; in Germany for morphological identification as well as DNA sequencing through 18S rDNA Internal Transcriber Spacer (ITS) and rDNA Large Subunit (LSU). Comparison of assembled rDNA ITS sequences was performed with GenBank and MycoID databases (Figure 29).
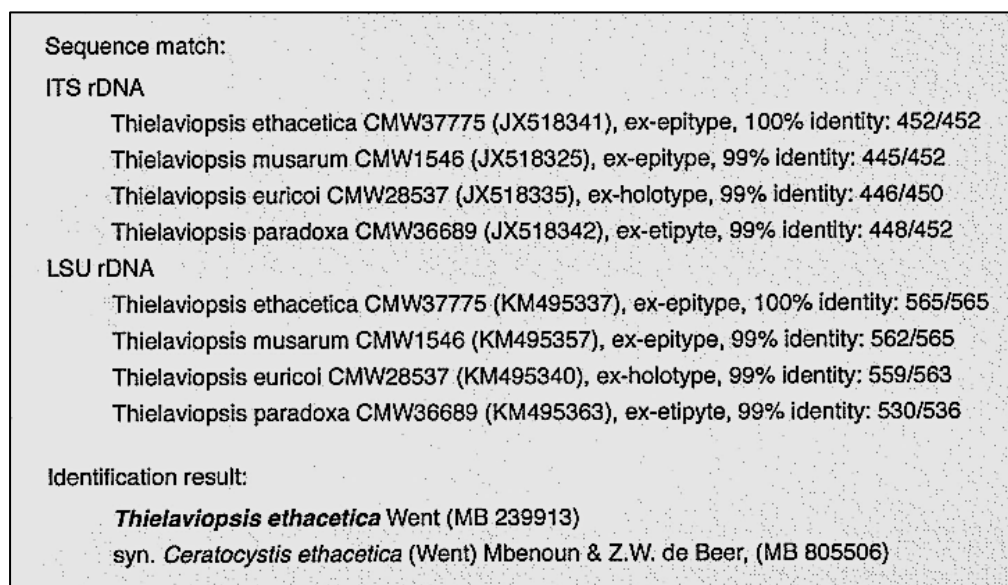
```
Sequence match:
ITS rDNA
      Thielaviopsis ethacetica CMW37775 (JX518341), ex-epitype, 100% identity: 452/452
      Thielaviopsis musarum CMW1546 (JX518325), ex-epitype, 99% identity: 445/452
      Thielaviopsis euricoi CMW28537 (JX518335), ex-holotype, 99% identity: 446/450
      Thielaviopsis paradoxa CMW36689 (JX518342), ex-etipyte, 99% identity: 448/452
LSU rDNA
      Thielaviopsis ethacetica CMW37775 (KM495337), ex-epitype, 100% identity: 565/565
      Thielaviopsis musarum CMW1546 (KM495357), ex-epitype, 99% identity: 562/565
      Thielaviopsis euricoi CMW28537 (KM495340), ex-holotype, 99% identity: 559/563
      Thielaviopsis paradoxa CMW36689 (KM495363), ex-etipyte, 99% identity: 530/536

Identification result:
      Thielaviopsis ethacetica Went (MB 239913)
      syn. Ceratocystis ethacetica (Went) Mbenoun & Z.W. de Beer, (MB 805506)
```

Figure 29. Results of 18S ITS/LSU DNA sequence matching of RPMEC-001, identified as *Thielaviopsis ethacetica* Went (MB 239913)

The presence of *Thielaviopsis* spp. in the digestive tube of *R. palmarum* is already known. Indeed, *Thielaviopsis* (*Ceratocystis*) *paradoxa* is frequently reported in literature as a palm phytopathogen, with Curculionidae weevils as their principal vectors. More recently, the presence of *T. paradoxa* was confirmed in the digestive tube of adult *R. palmarum* specimens. However, as this research demonstrates, larval instars of this weevil are also hosts of *Thielaviopsis ethacetica*. It appears that once *Thielaviopsis* spp. colonize the digestive tube of larval *R. palmarum* their presence remains throughout the entire lifecycle, excluding perhaps the egg stage of the Black Palm Weevil.

Once results of DNA identification were available; a protocol for extracting and characterizing cellulolytic enzymes from *T. ethacetica* was established. This protocol was developed in cooperation with Dr. Pier Paolo Giovannini from the Department of Chemical and Pharmaceutical Science of the University of Ferrara.

Initially, *T. ethacetica* was cultivated in 100 mL of Delalibera et al., (2005) M1b modified medium, consisting of CMC (5 g/L) and yeast extract (0,2 g/L), at 30°C for fourteen days. During incubation period, 1.0 mL samples of *T. ethacetica* culture were daily withdrawn, treated with ammonium sulfate (697 mg/mL) and centrifuged at 10000 rpm, to obtain a protein precipitate, which constituted its enzymatic crude extract.

Each of these enzymatic precipitates was set to reaction with 10 mL of 1% CMC reagent in 50 mM phosphate buffer at pH 6.0. Reaction mixture was incubated at 30°C and 120 rpm for 72 hours. At regular intervals (3 hours, 24 hours and 72 hours) the presence of reducing sugars was assayed as response of cellulolytic activity, with DNS method.

As result it was observed that *T. ethacetica* enzymatic crude extract showed no cellulolytic activity until the third day of incubation, reaching a maximum activity at 7-8 days, after which it remained almost constant until fourteenth incubation day, as seen in Figure 30.
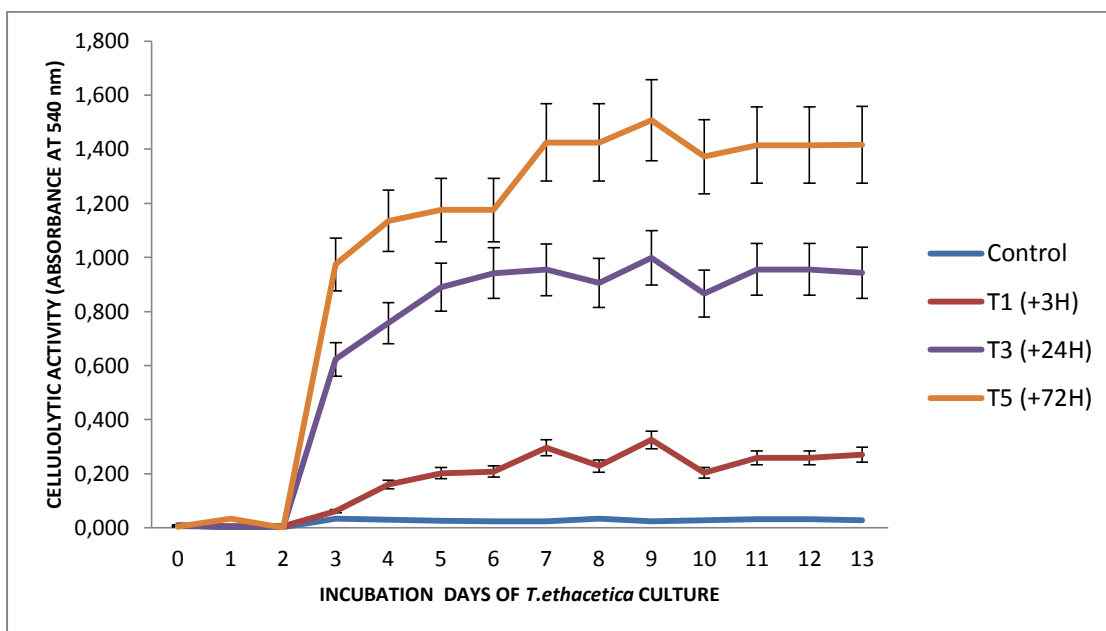
Figure 30. Cellulolytic activity of proteins precipitated from *T. ethacetica* culture, as function of the incubation time.

As aforementioned, the collected data of preliminary cellulolytic activity assay allowed establishing the basis for further extraction of cellulases from *T. ethacetica* culture. However, the relation between incubation conditions and cellulolytic expression of *T. ethacetica* was not elucidated.

In this sense, an experiment was set to evaluate incubation temperature and pH of the medium, as modifying factors of cellulolytic activity of enzymes obtained from *T. ethacetica* cultures. Experimental temperature interval was considered from 20°C to 40°C and pH interval was established from 4.0 to 8.0.

A D-OPTIMAL + SURFACE RESPONSE statistical design was chosen for this assay and analytical combinations of interacting factors were generated with Design-Expert Version 6.0.1 (Stat-Ease, Inc. USA) software. The design of this experiment was made in collaboration with Dr. David Sancho Aguilera from the department of Earth Sciences of Universidad Estatal Amazónica.

Cellulolytic activity, as absorbance at 540 nm, and reducing sugars, as glucose (g/L), were the assayed experimental responses. Enzymatic reactions were performed with the abovementioned enzymatic and substrate concentrations.

As result, it was found that maximum cellulolytic activity of *T. ethacetica*, as well as the highest amount of reducing sugars released by enzymatic hydrolysis of CMC, was reached in cultures incubated at pH 4.0 and 20°C (Figures 31 and 32).
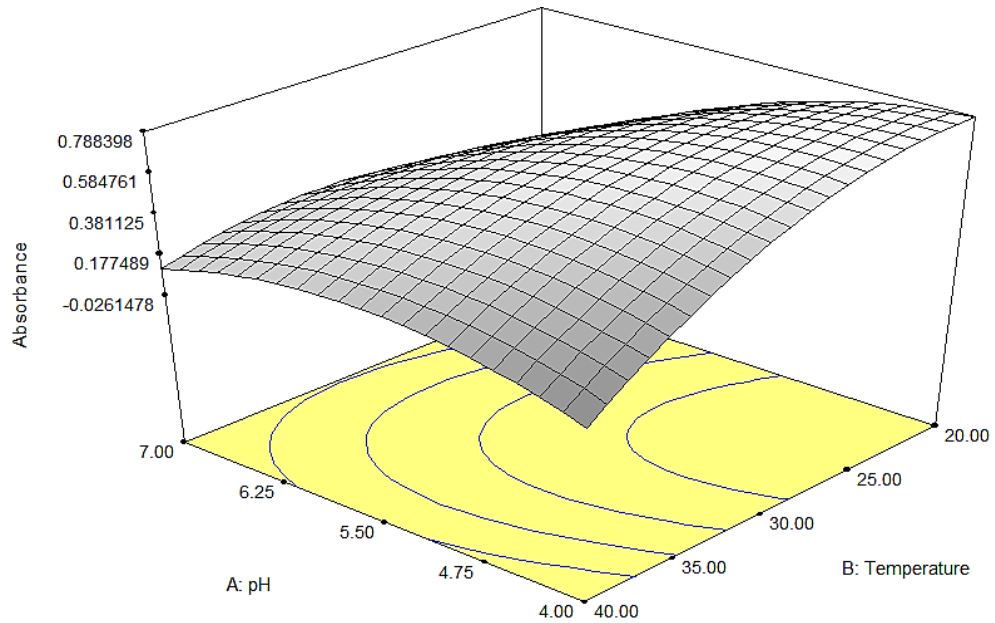

Figure 31. Response surface of cellulolytic activity (absorbance at 540 nm) of *T. ethacetica* precipitated proteins as function of incubation pH and temperature.
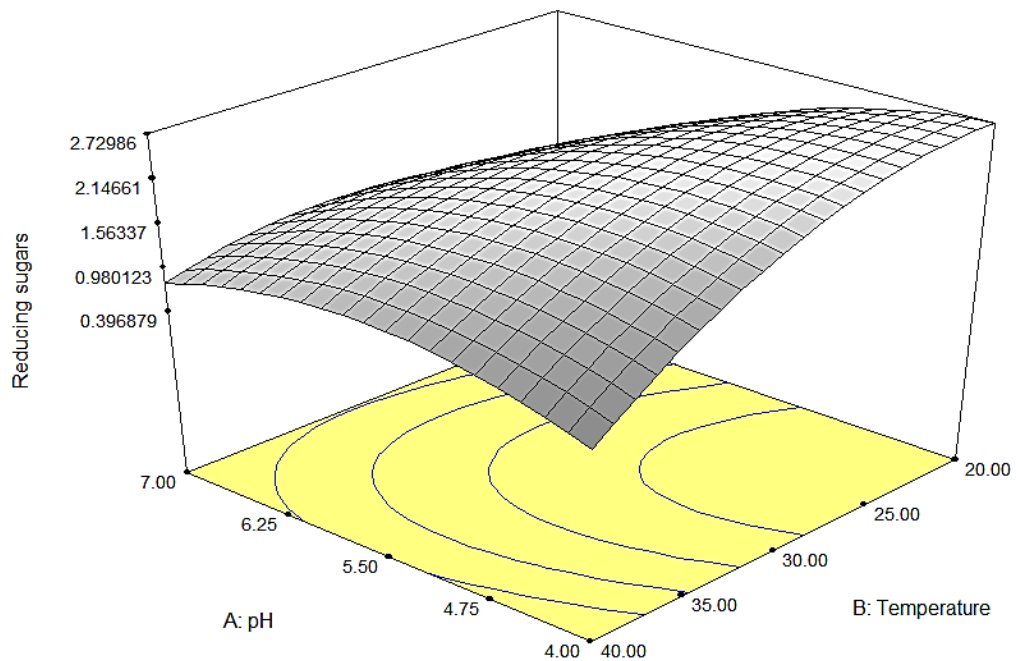

Figure 32. Response surface of reducing sugars, as glucose (g/L), of *T. ethacetica* precipitated proteins as function of incubation pH and temperature.

This fact clearly supports evidence indicating that cellulase production is closely related to mycelial growth, as indicated by Youatt, (1958). In addition, it is well known that incubation factors, such as temperature and pH have a direct impact on cellulase production of filamentous fungi, as demonstrated by Eriksen & Goksöyr, (1976). Moreover, optimized incubation conditions (pH 4.0 and 20°C) visibly reduced the time, in which cellulolytic activity was initially recorded, to the first 48 hours of incubation (Figure 33).
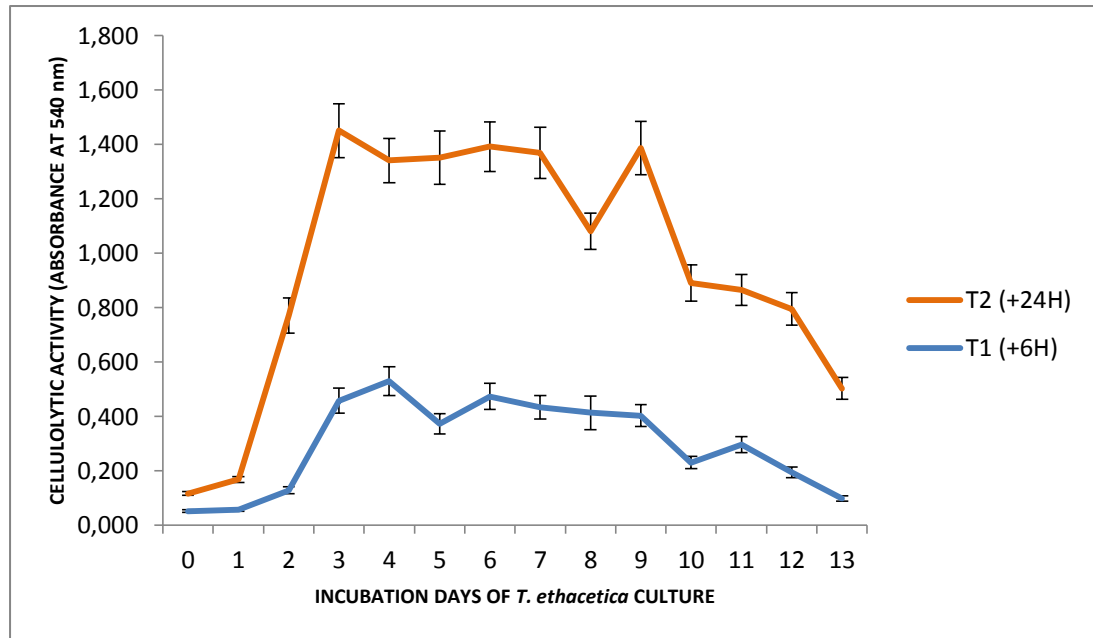

Figure 33. Cellulolytic activity of *T. ethacetica* in optimized conditions (pH 4.0 and 20°C). T1 and T2 represent enzymatic reactions of precipitated proteins from *T. ethacetica* culture; at 6 and 24 hours, respectively.

In addition, under optimized conditions, an increase in cellulolytic activity was registered in comparison to incubation at 30°C and pH 6.0. In these latter conditions, no significant reduction in cellulolytic activity was registered during incubation period. However, at pH 4.0 and 20°C, cellulolytic activity of the enzymatic crude extract of *T. ethacetica* decreases rapidly from the tenth incubation day on. This situation is seemingly attributed to a decrease in the availability of cellulose (CMC) in the incubation medium, suggesting that CMC is more rapidly hydrolyzed by this fungus under optimized conditions. In addition, cellulases expressed in the first period (from third to tenth day) probably become inactive.

Based on these results, subsequent culturing of *T. ethacetica* was carried out at pH 4.0 and 20°C, in order to maximize the amount of cellulases released by this fungus, which constituted the enzymatic stocks for further analysis.

Once the influence of incubation factors on cellulase production of *T. ethacetica* was established, optimal catalytic pH and temperature for the enzymatic crude extract catalyzed CMC hydrolysis were estabilished. Optimal pH was determined by measuring release of reducing sugars, with DNS method, from 1% carboxymethylcellulose solution; treated with enzymatic crude extract stocks from *T. ethacetica* on a pH range from 4.0 to 10.0.

It was found that cellulolytic crude extract of *T. ethacetica* was more active on acidic pH. The maximum activity was registered at pH 4.5, with cellulolytic activity markedly decreasing from pH 5.5 to 7.0. Alkaline pH, from 8.0 to 10.0, caused complete inhibition of cellulolytic activity under assay conditions (Figure 34).
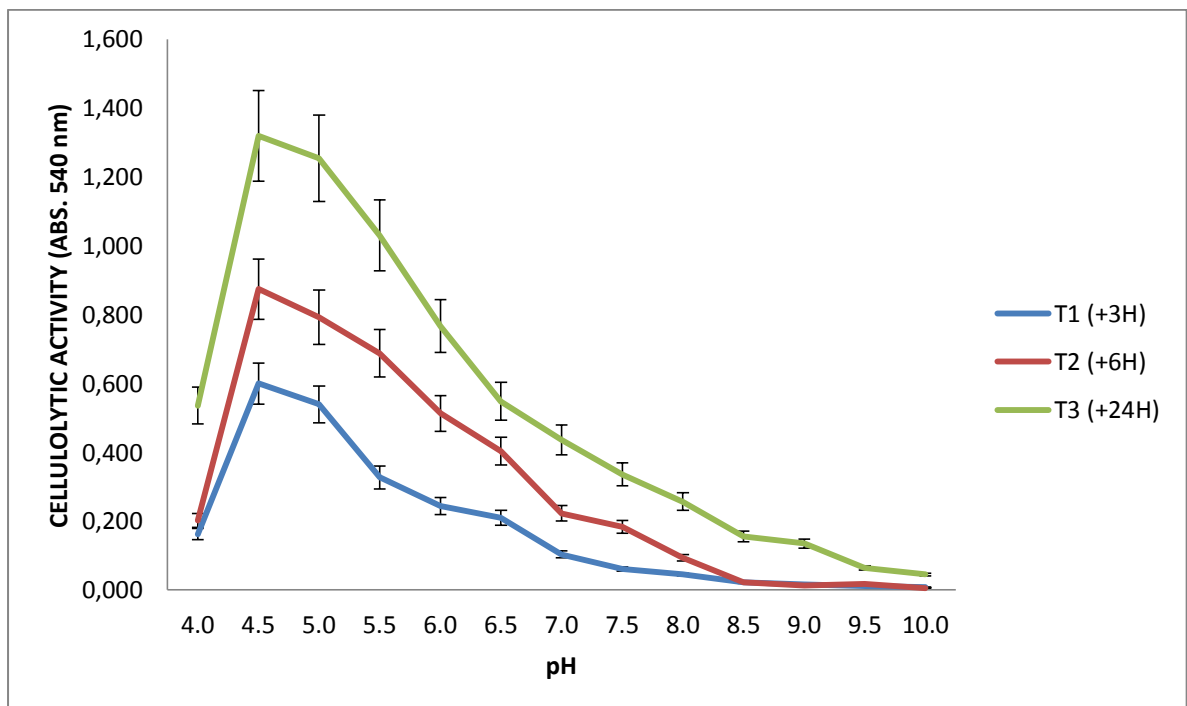

Figure 34. Cellulolytic activity as a function of the pH

Similar studies, carried out in *Chalara paradoxa* (the asexual form of *Thielaviopsis paradoxa*) showed a maximum activity, of an isolated β-glucosidase, on a pH range from 4.0 to 5.0. Comparable optimal pH has been described for other cellulase complexes from other filamentous fungi, such as *Neurospora crassa* (Yazdi, et al., 1990), *Aspergillus fumigatus* (Ximenes, et al., 1996) and *Aspergillus niger* (Arikan, et al., 2002).Regarding optimal catalytic temperature, it was determined by measuring the release of reducing sugars from 1% (W/V) CMC solution in 50 mM acetate at pH 4.5 treated with *T. ethacetica* enzymatic crude extract in a temperature range from 30°C to 80°C.

Tubes containing 10.0 mL of CMC solution were preheated to specific temperatures and then set to reaction with one stock of *T. ethacetica* enzymatic crude extract. Cellulolytic activity, expressed as release of reducing sugars, assayed with DNS method, was measured after 1 and 3 hours of reaction (Figure 35). T1 and T3 are the results of the reactions assayed after 3 and 24 h respectively.

The cellulolytic crude extract of *T. ethacetica* showed catalytic activity in temperatures ranging from 30°C to 70°C. Temperatures above 75°C caused complete inhibition of cellulolytic activity. The maximum activity was recorded at 45°C.
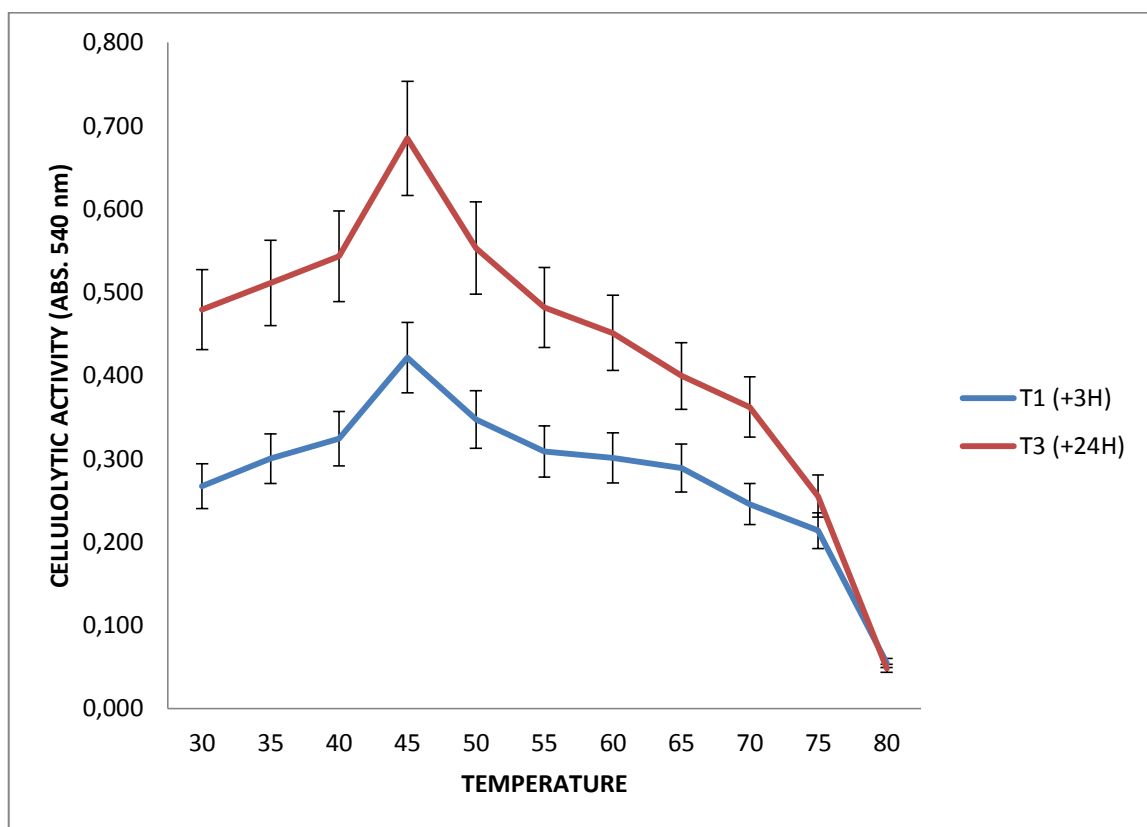

Figure 35. Cellulolytic activity as a function of the temperature

Comparable optimal pH and temperature have been described by other authors for isolated cellulolytic enzymes. Lucas, et al., (2000) reported a β-glucosidase from *Chalara paradoxa* with a maximum activity at pH 4.0−5.0 and 45 °C. A similarity in optimal pH and temperature is observed with an endoglucanase isolated from *C. paradoxa* by Lucas, et al., (2001). Additionally, Yapi Assoi Yapi, et al., (2009), isolated a specific β-glucosidase directly from the digestive fluid of *R. palmarum* larvae, which exhibited comparable maximum activity at pH 5.0 and 55°C, although the origin of this β-glucosidase was not

defined. This situation suggests that not only β-glucosidases, but also endoglucanases, may be included in the enzymatic complex of *T. ethacetica.*

An interesting fact, concerning temperature tolerances of *T. ethacetica* and its cellulolytic crude extract, is given by the potential applications of each one. Although *T. ethacetica* is a mesophilic fungus, its cellulolytic complex is able to tolerate temperatures that would be deleterious for most fungi. Therefore, the cellulolytic crude extract of *T. ethacetica* may be suitable for applications requiring obligate enzymatic pretreatment. On the other hand, biological pretreatment of lignocellulosic biomass, involving fungi, is perhaps the most promising application for *T. ethacetica*, since Solid State Fermentations involving mesophilic fungi, do not require an extra input of temperature, which results particularly appropriate for large-volume or room-temperature pretreatments.

Besides, as demonstrated by Bhat & Maheshwari, (1987) incubation conditions influence the differentiation of reproductive structures and the autolysis of hyphal cells, allowing the liberation of propagules, which renew growth immediately. This is compatible with what is observed on incubation of *T. ethacetica* under optimized conditions, which increased formation of fungal mycellium and therefore the liberation of comparably more cellulases, with respect to incubation on a different pH and temperature.

Once optimal enzymatic temperature and pH were determined, enzymatic stability under optimal reaction conditions was deduced by the residual activity of the enzymatic crude extract, after catalyzing a primary reaction, in CMC reagent, at pH 4.5 and 45°C. At specific timing, 1.0 mL of primary reaction mixture was used to catalyze a secondary reaction under the same conditions (*See Stability of the enzymatic crude extract under optimized pH and temperature*).

Cellulolytic activity of both primary and secondary reactions was assayed with DNS method. Results obtained from primary reaction constituted a baseline (B_{act}), that have been subtracted from the secondary-reaction activity (C_A), to obtain the variation of reducing sugars amount due to the catalytic activity (Δ_{act}), as presented in Table 20.

Table 20. Enzymatic stability of *T. ethacetica* cellulolytic crude extract

TIME	B_{act}	C_A	Δ_{act}
T	0.000	0.012	0.012
T + 1H	0.006	0.035	0.029
T + 3H	0.002	0.040	0.038
T + 5H	0.034	0.068	0.034
T + 7H	0.055	0.088	0.033
T + 24H	0.058	0.095	0.037
T+48H	0.088	0.125	0.041
T+72H	0.102	0.114	0.012

As result, it was observed that cellulolytic crude extract of *T. ethacetica* is active in the optimized reaction conditions, namely pH 4.5 and 45 °C for at least 72 hours. Nonetheless, enzymatic activity during the first three hours of reaction was less than expected, as seen in Figure 36. This situation could be attributed to a reversible denaturation of cellulolytic enzymes, during precipitation with ammonium sulfate, which could explains that catalytic activity is initially low until enzymes retake their active conformation.
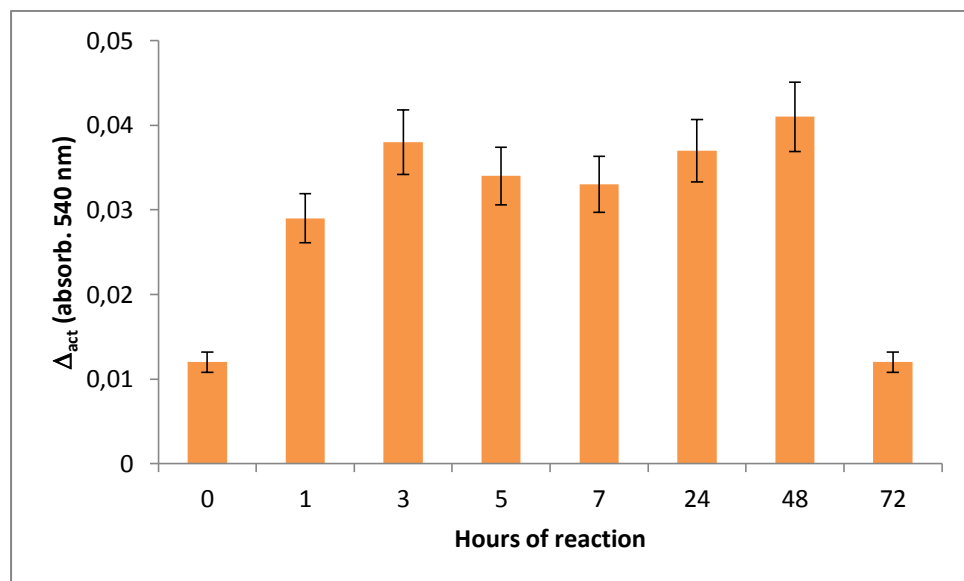

Figure 36. Enzymatic stability of *T. ethacetica* cellulolytic crude extract at optimized catalytic conditions (pH 4.5 and 45°C).

On the other hand, cellulolytic crude extract of *T. ethacetica* showed a stable activity for 48 hours; at optimal catalytic conditions, after which, enzymatic activity decreased. At 72 hours of reaction, a decrease of catalytic activity was observed and Δ_{act} value was similar to that obtained of reactions at T0, indicating that cellulolytic crude extract was losing stability.

CONCLUSIONS

The scope of this research was to isolate and characterize cellulolytic enzymes from the digestive tract of *Rhynchophorus palmarum* larvae, belonging to the Curculionidae, family which has been extensively studied, in order to control or eradicate the damages caused by larval and adult stages of this weevil in large-scale palm cultivations. Nonetheless, the relation between Ecuadorian Amazon populations and the Black Palm Weevil is entirely different, since indigenous populations include *R. palmarum* larvae in their diet, regarding these even healthy benefits.

This situation, added to the fact that insects are among the creatures which can effectively degrade and feed from lignocellulosic matrices, led to consider *R. palmarum* larvae as a potential source of cellulolytic enzymes. In this sense, endosymbiont microbiota, living in the intestinal walls of these larvae, was targeted as cellulase producers.

Additionally, although the entire composition of intestinal microbiota of *R. palmarum* larvae remains undefined, it was found that four fungi and yeast were the only isolated endosymbionts reporting cellulolytic activity. Among these microorganisms the ascomycete *Thielaviopsis ethacetica* MB 239913 was the most readily capable of hydrolyzing cellulose.

The presence of plant pathogens, belonging to *Thielaviopsis* (*Ceratocystis*) genus in the digestive tract of *R. palmarum* is well known, although this is the first time that *T. ethacetica*, as such, is identified among the intestinal community of larval Black Palm Weevil.

This fungus was studied in order to evaluate its ability to degrade carboxymethylcellulose (CMC) as sole source of carbon, as well as its cellulolytic response as function of incubation parameters. It was found that *T. ethacetica* expresses its maximum cellulolytic activity when incubated at pH 4.0 and 20°C. In these conditions, maximum enzymatic expression is reached after four days.

Regarding the cellulolytic complex of *T. ethacetica*, a cellulolytic crude extract was isolated through precipitation with ammonium sulfate of the protein released in the culture medium, obtained from cultures incubated at pH 4.0 and 20°C. The enzymatic extract was assayed to determine its optimal catalytic temperature and pH. It was demonstrated that enzymatic activity was higher at acid pH. The maximum activity was registered at pH 4.5. Nearly-neutral pH hampered cellulolytic activity and it was completely inhibited at alkaline pH from 8.0. Besides, the cellulolytic crude extract of *T. ethacetica* was capable of hydrolyzing CMC at temperatures from 30°C to 70°C. The maximum activity was registered at 45°C.

Temperatures above 75°C caused total inhibition of cellulolytic activity, under assay conditions.

Regarding the enzymatic stability of cellulolytic crude extract of *T. ethacetica* at optimal catalytic conditions (pH 4.5 and 45°C), it was established that enzymatic activity was stable for 48 hours at optimized conditions. Nonetheless it was found that precipitation with ammonium sulfate would probably reduce enzymatic activity, by denaturation of precipitated proteins, which resulted in cellulolytic activity lagging behind what is expected during the initial hours of reaction, until enzymes regain their active configuration.

On the other hand, evidence found in this research suggests that the cellulolytic complex of *T. ethacetica* would be formed by at least one endoglucanase and one β-glucosidase, based on similar results obtained from enzymatic characterization of cellulases either from other species of *Ceratocystis* genus or directly from the digestive fluid of *R. palmarum* larvae.

In addition, it has been demonstrated that *T. ethacetica* is a promising source of cellulolytic enzymes that could be eventually used for lignocellulosic pretreatment, such as Solid State Fermentation processes, in which degradation of lignocellulosic biomass would be accomplished by a combination *T. ethacetica* alone or in combination with thermotolerant fungi, in order to produce as much fermentable sugars as possible.

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