Biodegradation of recalcitrant compounds and phthalates by a consortium of *Liometopum apiculatum* gut microbiota

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Abstract

Liometopum apiculatum ants species are widely distributed in arid and semi-arid ecosystems where there is a relative food shortage. They have established an ecological balance involving symbiotic interactions, which have allowed them to survive through mechanisms that are still unknown. The aim of this study was to explore the metabolic potential of isolated bacteria from *L. apiculatum* using substrate assimilation and enzymatic activity assay. Results revealed a complex bacteria consortium belonging to Proteobacteria, Firmicutes, and Actinobacteria phylum. These symbionts showed activities associated with biopolymers degradation, suggesting their important role in nutrients release stored in plant-derived material. By other hand, some microorganisms had a high capacity for degradation of phthalate esters. These results not only revealed the important contribution of the symbionts in *L. apiculatum* ants feeding habits, but also they showed a promising source of enzymes with high potential for biotechnological applications such as lignocellulosic biomass hydrolysis and bioremediation.

Introduction

Modern biotechnological approaches have focused on developing sustainable processes based on the use of renewable energy source and bioremediation of contaminated ecosystems (Pandey et al., 2008; Kothari et al., 2010; Wang et al., 2019). Wastes from industry and agriculture can be converted through microbiological reactions in energy forms such as biohydrogen, biogas, and bioethanol (Kothari et al., 2010). Moreover, microorganisms can be used to degrade contaminants that represent a potential risk for humans and environmental health such as phthalate esters (PAEs), which are ubiquitous contaminants because of their wide usage in the industry to produce plastic materials and pesticides (Lu et al., 2009; Benjamin et al., 2017). In fact, they have been found in human tissues and body fluids, and their presence is associated with detrimental health effects. PAEs work as endocrine disruptors and are linked with developmental and reproductive toxicity in humans and animals (Katsikantami et al., 2016). Therefore, the scientific community have focused on the search for effective methods for remove phthalates from the contaminated environment, being the microbial degradation a promising approach as a result of its high efficiency and cost-effectiveness (Zhao et al., 2016; Zhao et al., 2018). Nonetheless, all these bioprocesses depend basically on metabolically versatile bacteria that carry out the biodegradation of several compounds, including some highly recalcitrant and toxic (Díaz, 2004; Pandey et al., 2008). Thus, there is a constant interest in the identification of new microbial resources, mainly those thriving in adverse environments, as they have developed diverse strategies to obtain energy (Díaz, 2004).

Symbiotic bacteria are important in the evolutionary adaptations of the insects, as well as in the responses to environmental stresses (Warnecke et al., 2007; Shi et al., 2013; Xia et al., 2017). Studies have suggested that one of the main roles carried out by insect's symbionts is linked to the decomposition of recalcitrant compounds and xenobiotics through the production of several enzymes. For instance, pectinolytic enzymes derived from microorganisms have been identified in guts of honey bees (*Apis mellifera*), enzymes that participate in the breakdown of pollen walls (Engel and Moran, 2013). In termite's species, bacterial enzymes that hydrolyse cellulose and xylan have been identified, indicating

that microorganisms help in degradation of wood (Warnecke et al., 2007). Microorganisms from the mountain pine beetle (*Dendroctonus ponderosae*) have been associated with catabolism of aromatic-, sulfonate-, and nitroaromatic compounds (Adams et al., 2013). It has also been reported that bacteria isolated from Diamondback moth (*Plutella xylostella*) are able of degrading phenolic compounds (Xia et al., 2017). The presence of bacteria that degrade organophosphorous compounds in symbionts of bean bug (*Riptortus pedestris*), was related with the insecticide resistance of its host (Kikuchi et al., 2012). All these findings suggest that microorganisms are involved in detoxification of plant toxic compounds and soils chemical contaminants, which contributes to the survival of many insects to unfavourable environments. Although insect microbial consortia have become of major interest, since it represents a potential source of valuable enzymes for biotechnology applications, the information of many insect symbionts remains to be revealed.

Liometopum apiculatum is a species of ant in the subfamily Dolichoderinae, it is known as escamolera ant and is an important species of edible insect that contributes to the food supply for humans (Ramos-Elorduy, 2006). *L. apiculatum* is distributed in arid and semi-arid ecosystems where a relative food shortage can be appreciated when compared with tropical ecosystems. However, the escamolera ant has established an ecological balance to survive, through diverse symbiotic interactions with other insects, plants, and microorganisms (González-Escobar et al., 2018). *L. apiculatum* ant has been described as an omnivorous insect because of the versatility of its feeding habits. Its diet has been associated with honeydew produced by insects, floral nectar, seeds, pollen, fruits, plants, insect's pupae, crustaceans, annelids, mollusks, and animal droppings (Hoey-Chamberlain et al., 2013). Enzymatic activities such as cysteine and serine proteases, and chitinases have been observed in *L. apiculatum* (Castillo Andrade et al., 2015; Huerta-Ocampo et al., 2018). In others insects, these enzymes have been associated with activity of gut symbionts, establishing an important contribution in food digestion and protection against harmful compounds for the host (Visôto et al., 2009; Pilon et al., 2013). However, there is limited information in *L. apiculatum* regarding the capacity to process the wide range of organic matter. Therefore, the aim of the present work was to investigate the capacities of ant's microbial symbionts on the degradation of lignocellulosic biomass and toxic compounds such as phthalates. Results will help not only to understand the functional contribution of *L. apiculatum* gut microbiota in its biological processes, but also to generate information that will be useful in future studies in biomass and bioremediation degradation processes.

Materials And Methods

2.1. Chemicals

Corn core xylan and alkali lignin were purchased from TCI America (Portland, OR, USA), soluble starch from Jalmek (Monterrey, NL, México), and Carboxymethylcellulose (CMC, sodium salt) from Sigma-Aldrich (St. Louis, MO, USA). Diethyl phthalate (DEP), di-n-butyl phthalate (DBP), benzyl butyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP), and diisononyl phthalate (DINP) with purity >99% were

purchased from Sigma-Aldrich. Stock solutions (10 g/L) of DEP, DBP, BBP, DEHP, and DINP were prepared in methanol. All chemicals and solvents used were of analytical reagent grade.

2.2. Bacteria isolation and identification

Fifty complete larvae (18-25 days of age) and fifty adult ants (60-70 days of age) were collected from three different colonies at Pocitos Charcas, San Luis Potosí, Mexico. Samples were surface-sterilized with washes of 70% ethanol solution, 15% sodium hypochlorite solution (5.25% stock solution), and sterile water. Adult ants were dissected as reported by Kautz et al. (2013) using a stereomicroscope Discovery V8 (Carl-Zeiss Vision Inc., San Diego, CA, USA). Larvae and ant's gut were macerated, homogenized, and serially diluted (10^{-3} to 10^{-6}) for plating on different growth media: Brain Heart Infusion (BHI), Blood Agar Base (BAB), Sabouraud Dextrose Agar (SDA), and Man Rogosa Sharpe (MRS). Culture media were incubated at 35 °C under aerobic, anaerobic, and microaerophilic conditions using GasPak EZ systems (Becton, Dickinson and Company, Sparks, MD, USA). Bacteria were recovered after 7 days and up to 1 month. Pure cultures were obtained by continuous transference on BHI medium, taking into account their colony morphology and cell morphology by Gram staining. Bacterial isolates were stored to their preservation in 30% glycerol in BHI media at -80 °C.

Isolated microorganisms were identified by Matrix Assisted Laser Desorption/ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, GE) using a standard method according to the manufacturer Standard Operating Procedure (Gekenidis et al., 2014). The data acquisition was carried out with AutoFlex III Mass Spectrometer and MALDI-Biotyper 3.0 software (Bruker) with library version 3.3.1.0 (4613 entries). Each sample was measured in duplicate and was correctly identified using Bruker Daltonics cut-off scores values.

2.3. Enzymatic activities screening by API® Microsystems

Bacterial enzyme activities were assayed with API® 20NE and API® ZYM microsystems (BioMérieux, Marcy l'Etoile, France). API® ZYM microsystem contains dehydrated chromogenic substrates for 20 enzymatic reactions involved in the breakdown of peptides, phosphomonoesters, lipids, and polysaccharides, including chitin, cellulose, starch, and galactans. API® 20NE was used to evaluate NO₃ reduction, indole production from tryptophan, L-arginine dihydrolase activity, fermentation of glucose, urease activity, hydrolysis of gelatin, and growth on 12 different organic carbon compounds. Pure cultures were grown on BHI broth at 35 °C for 24 h (bacteria) and 72 h (actinobacteria). Cultures (10 mL) were harvested by centrifugation at 1,935×g for 15 min at 4 °C. Bacterial pellets were washed twice with sterile phosphate-buffered saline (PBS) pH 7.4 and re-suspended in 3 mL of sterile saline solution (0.85%). Two cell suspensions concentration were obtained, one with OD₆₀₀ of 0.4 that was used for API® ZYM measurements and the second with an OD₆₀₀ of 0.07 was used for API® 20NE.

Tests were carried out and examined according to the manufacturer's instructions. Bacterial isolates were examined at least in triplicates with independent subcultures.

2.4. Qualitative tests of enzymatic hydrolysis of lignocellulosic substrates

Peroxidase activity using pyrogallol was carried out on colonies grown on BHI medium using 30 µL of 0.4% (v/v) hydrogen peroxide and 0.1% pyrogallol in water. Congo red decolourization assay was carried out on M1 plates containing 0.1 g/L yeast extract, 40 nM glycerol, and 50 mg/L CR. Microorganisms were inoculated and incubated at 35 °C for 7 days (Falade et al., 2017).

Amylase, xylanase, and carboxymethylcellulase (CMCase) activities were evaluated by their corresponding polysaccharide hydrolysis in medium (M) containing: 10 g/L polysaccharide (starch, xylan, or CMC); 15 g/L agar; 1.0 g/L peptone; 4.0 g/L (NH₄)₂SO₄; 0.5 g/L NaCl; 2.0 K₂HPO₄; 1.0 g/L CaCl₂·2H₂O; 0.5 g/L MgSO₄·7H₂O. Pure bacterial cultures (20 µL) were plated on solid medium and incubated for 3 days at 35 °C. Plates were stained with 4% iodine solution to detect starch hydrolysis, 1% Congo Red (CR) solution to detect xylan and CMC hydrolysis (Pinheiro et al., 2015; Afrisham et al., 2016).

Ligninolytic activities were assessed through degradation of guaiacol (0.1%) or lignin (0.1%) in a solid medium (M1) containing: 15 g/L agar; 4.55 g/L K₂HPO₄; 0.53 g/L KH₂PO₄; 0.5 g/L NaCl, 0.5 g/L NH₄NO₃, 0.5 g/L MgSO₄·7H₂O, and 0.1 mg/L CaCl₂. Pure bacterial cultures (20 µL) were inoculated, and incubated for 7 days at 35 °C. Guaiacol and lignin hydrolysis were revealed by an iodine solution (4%).

2.5. Enzymatic activity measurements

Amylase, xylanase, and CMCase activities were determined by measuring the reducing sugars released during the enzymatic hydrolysis of specific polysaccharides using the dinitrosalicylic acid (DNS) method (Miller, 1959). Isolated microorganisms were inoculated in batch cultures containing 20 mL of medium M1 supplemented with 1.0% of the corresponding polysaccharide. Cultures were incubated at 35 °C for 5 days in a shaker at 150 rpm. Standard reaction mixtures containing 100 µL of cell-free culture supernatant and 100 µL of specific substrates (1% starch, 1% xylan, or 0.5% crystalline cellulose) in 100 mM sodium citrate buffer (pH 5.0) were carried out at 50 °C for 30 min. One-hundred microliters of reaction mixture were mixed with 200 µL of DNS reagent and subsequently incubated for 20 min at 95°C. Samples were allowed to cool and absorbance was measured at 540 nm using in a Multiskan microplate spectrophotometer. A calibration curve for the different sugars released (glucose, xylose, and maltose) was prepared using different concentrations from 0.2 to 1.0 mg/L (Pinheiro et al., 2017). The extracellular protein concentration in the cell-free culture was determined by using the Protein Assay reagent (Bio-Rad, Hercules, CA, USA), using BSA as standard. All experiments were carried out at least by triplicates.

2.6. Phthalates tolerance assay on plates

Phthalate tolerance assays were carried out in solid minimal media containing each PAEs at different concentrations: 0, 500, and 1000 mg/L. Tween 20 (500 mg/L) was used to ensure the homogeneity of DEHP solution (Xu et al., 2017). The mineral salt medium (MSM) was used as a basal media contained: 0.02 g/L CaCl_2 , 2.0 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.16 g/L MgSO_4 , 4.5 g/L KH_2PO_4 , 5.8 g/L K_2HPO_4 , 15 g/L agar and 1 mL/L trace elements solution which contains: 0.015 g/L $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 0.00036 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.00034 g/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0007 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0002 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0002 g/L Na_2SeO_3 , 0.010 g/L MgSO_4 , 0.023 g/L ZnCl_2 , 0.030 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. Bacterial isolates (2 μL) were plated on solid media and incubated for 3 days at 25 °C. Microorganisms that were able to grow in PAEs at concentration of 1000 mg/L were selected as phthalate-tolerant bacteria and were used in bacterial consortium tests.

2.7. Phthalates degradation test

The microbial degradation capacity on DEP, DBP, BBP, and DINP, which are compounds with different alkyl chain lengths (**Supplementary Table S1**) was evaluated as follows: the bacterial consortium enrichment was inoculated into 25 mL MSM medium containing 500 g/L of each PAEs individually, as the only carbon source and energy. Non-inoculated medium was used as a negative control. The cultures were incubated at 31 °C, substrate utilization was assessed by microbial growth by measuring the OD_{600} every 12 h until 72 h. All experiments were carried out in triplicates.

2.8. Bacterial consortium enrichment and kinetics of DEHP degradation

Once selected the phthalate-tolerant bacteria, they were used to prepare the bacterial consortium. The enrichment procedure for bacteria consortium was following reported protocols with some modifications (Wu et al., 2010). Cultures were established in a 50 mL Erlenmeyer flask containing 25 mL sterilized MSM supplemented with DEHP (100 mg/L). Subsequently the culture, at OD_{600} of 0.1, was serially transferred to fresh medium, each time containing a higher concentration of DEHP (250, 500, 600, 750, and 1000 mg/L) and incubated at 31 °C under constant agitation (180 rpm) for 5 days. The last enrichment culture was selected to carry out the tests. Bacterial cells were harvested by centrifugation and washed two times with phosphate buffer (pH 7.0). Bacterial suspension with an OD_{600} of 0.1 was used to inoculate 25 mL fresh MSM medium supplemented with DEHP (500 g/L) as a sole carbon source at pH 6.8. Tween 20 (500 mg/L) was added for enhance the solubility (Jin et al., 2015; Xu et al., 2017){Jin, 2015 #3}. Non-inoculated medium was used as an experimental control. The cultures were incubated at 31 °C for 7 days. Biomass growth was monitored by measuring the OD_{600} every 12 h. All experiments were carried out in triplicates.

2.9. Analysis of DEHP and identification of its metabolites

The DEHP residue and its metabolites were analysed from cultures containing DEHP (500 mg/L). Non-inoculated medium containing 500 mg/L of DEHP was used as a control. The samples were obtained from the culture media at intervals of 24 h. Cell-free supernatants were obtained by centrifugation and metabolites were extracted with dichloromethane (Zhang et al., 2018; Li et al., 2019). Briefly, a half volume of dichloromethane was added directly to each sample and mixed vigorously for 2 min. The aqueous and organic phase was separated by centrifugation at 5000 rpm for 10 min. The dichloromethane phase was extracted and evaporated to dryness. The residue was dissolved in 1 mL of methanol and metabolites were identified by gas chromatography-mass spectrometry (GC/MS) 7820A/5977E System (Agilent Technologies, Santa Clara, California, USA). The following detection conditions of GC/MS were used according to Rastkari et al. (2018): an HP-5 MS column (30 m x 250 µm x 0.25 µm) with helium as a carrier gas at a flow rate of 1.0 mL/min. The column temperature was increased from 50 °C hold for 1 min to 280 °C at 30 °C/min, followed by a second ramp to 300 °C (hold for 4 min) at 15 °C/min. The injector temperature was set at 250 °C in splitless mode. MS detector was operated under Electron Impact Ionization at 70 eV using the SIM and Scan mode at 45-300 amu. The metabolites identified by mass spectrometry analysis were matched with authentic standard compounds from National Institute of Standards and Technology (NIST) library database.

2.10. Statistical analyses

Data obtained were expressed as mean values of triplicates. One-way analysis of variance (ANOVA) at 95% of confidence level and Tukey's multiple comparison tests were carried out to determine the significant differences. Analyses were carried out using GraphPad Prism v 5.01 (GraphPad Software, La Jolla CA, USA).

Results And Discussion

3.1. Isolation and identification of bacteria from *L. apiculatum* larvae and adult ants guts by culturomics approach

Insects gut microbiota play a fundamental role in regulating the insect's metabolism by producing several enzymes capable of hydrolysing the food they consume (Warnecke et al., 2007; Engel and Moran, 2013). Furthermore, it has been reported that some of those microorganisms can degrade xenobiotic compounds such as pesticides, conferring to insects with mechanisms of resistance and survival (Gressel, 2018). Therefore, the functional diversity of cultivable bacterial community associated with *L. apiculatum* ant was analysed.

A total of 67 bacterial colonies were isolated from larvae and 46 from adult ants gut microbiota. All isolated bacteria were found to be facultative anaerobes. According to their growth characteristics, colony

morphology, and cell morphology by Gram staining, unique colonies from larvae (36) and guts from adult ants (23), were selected for MALDI-TOF-MS identification. Biotyping (genotyping) was carried out by comparison of experimental and reference mass spectra using a pattern-matching algorithm (**Figure 1**). Identified microorganisms belonged to Firmicutes, Proteobacteria, and Actinobacteria phyla. Firmicutes and Proteobacteria were mostly present in larvae, while Actinobacteria were characteristic in guts of adult ant (**Table 1**). Moreover, *Macrococcus caseolyticus*, *Enterobacter asburiae*, *Pantoea agglomerans*, and *Kocuria palustris* were detected only in larvae, while *Exiguobacterium* sp., *Rhodococcus* sp., *Microbacterium hydrocarbonoxydans*, and *Dietzia natronolimnaea* were specific of guts of adult ants. A wide range of bacterial enzymatic activities that contribute not only in the recalcitrant compounds hydrolysis but also in xenobiotic degradation was observed in *L. apiculatum* gut microbiota.

3.2. *Proteases, phosphatases, carboxyl esterases*

Proteolytic activities associated with leucine aminopeptidase (LAP) and valine aminopeptidase (VAP) were observed mainly in microorganisms belonging to Proteobacteria and Actinobacteria (**Figure 2A**). LAP showed the highest catalytic activity with 40 nanomoles of hydrolyzed substrate, while VAP catalytic activity was 20 nanomoles. Cystine aminopeptidase (CAP) and α -chymotrypsin (CTR) activities were observed in few isolated bacteria with low catalytic activity. By other hand, API® 20NE system showed that a wide range of microorganisms were able to hydrolyse gelatin (GEL), which is a complex protein and enzymatic activities associated with protein turnover such as hydrolysis of arginine (ADH), tryptophan (TRP), nitrate reduction (NR), and urea (URE) were detected in some microorganisms (**Supplementary Figure S1A**). These results are in agreement with Castillo-Andrade et al. (2015), who reported that *L. apiculatum* larvae have high proteolytic activity. Proteases are central enzymes that participate in food digestion, breaking-down proteins into amino acids that insects use for growth and development (Terra and Ferreira, 2012). However, recent studies have reported that proteolytic enzymes from insect's gut bacteria possess other functions in the host beyond of their basic food digestion activity. In the velvet bean caterpillar (*Anticarsia gemmatilis*) several symbionts synthesize serine- and cysteine aminopeptidase involved in protection not only against plants proteases inhibitors (PI), but also against synthetic PI (insecticides) (Pilon et al., 2013). Indeed, proteases produced by *Bacillus* sp. YP1 from cotton bollworm (*Helicoverpa armigera*) degrade PI present in seeds (Shinde et al., 2012). Proteolytic enzymes have also been linked with multifunctional activities such as lipases, arylesterase, thioesterase, and phospholipases (Akoh et al., 2004).

Enzymatic activities associated with phosphatases and carboxyl esterases were widely observed in all bacterial population with highest activity in Proteobacteria phyla (**Figure 2B and 2C**). Additionally, assimilation of capric acid (CAP), phenylacetic acid (PAC), and adipic acid (ADI) was detected in Proteobacteria phyla (**Supplementary Figure S1B**). Those activities are associated with releasing and assimilation of esters of different molecular weights

3.3. Complex polysaccharide-degrading enzymes

Complex polysaccharide-degrading enzymes were observed in microorganisms isolated from *L. apiculatum*. β -galactosidase (β -Gal) activity, associated with xyloglucan side-chains breakdown, showed different catalytic activity from 20 to 40 nanomoles. Most microorganisms displayed a high enzymatic hydrolysis from 30 to 40 nanomoles in α -Glucosidase (α -Glu) and β -Glucosidase (β -Glu), which is linked with starch and cellulose breakdown, respectively, while β -N-acetylglucosaminidase (β -Nag) activity, associated with chitin breakdown, was observed in fewer numbers of bacteria (**Figure 2D**). The activity of β -Glu and β -Gal were confirmed by hydrolysis of 7-hydroxycoumarin 6- β -glucopyranoside (ESC, esculin) and 4-Nitrophenyl β -glucopyranoside (PNPG), respectively (**Supplementary Figure S1C**). On the other hand, most microorganisms assimilated different monosaccharides and organic acids (**Supplementary Figure S1B-C**).

The enzymatic activity associated with the degradation and assimilation of starch, CMC, and xylan was evaluated by plaque assays and afterwards by batch cultures. *Exiguobacterium* sp., *Acinetobacter johnsonii*, *Pantoea agglomerans*, *Serratia marcescens*, *Serratia rubidaea*, *Enterobacter asburiae*, *Serratia ficaria*, and *Bacillus simplex* displayed clear halos on starch containing plates (**Figure 3A-H**). However, only *Exiguobacterium* sp and *Bacillus simplex* showed a high specific activity for starch degradation, indicating not only a high production of amylolytic enzymes but also higher total activity on substrate (**Figure 4A**). On the other hand, *Bacillus pumillus*, *Acinetobacter johnsonii*, *Pantoea agglomerans*, *Enterobacter asburiae* showed cellulolytic activity on plate (**Figure 3I-L**), from these *A. johnsonii* and *B. pumillus* showed the highest specific activity (**Figure 4B**). *Dietzia natronolimnaea*, *Acinetobacter johnsonii*, *Staphylococcus warneri*, and *Bacillus pumillus* exhibited xylanolytic activity on plate (**Figure 3M-O**), while *A. johnsonii* and *B. pumillus* showed the highest activity for xylan degradation (**Figure 4C**).

These results seem to be directly linked with feeding behaviour of the *L. apiculatum* adult ant, since these insects have been specialized for the collection of different food of plant origin (Hoey-Chamberlain et al., 2013). Therefore, they require several enzymes to digest their diet. It has been reported that in many xylophagous- and detritophages- insects, enzymes from symbiotic bacteria such as cellulases, xylanases, and amylases assist in the degradation of the plant-derived materials to release fermentable simple carbohydrates (Warnecke et al., 2007; Rogers and Doran-peterson, 2010; Fabryová et al., 2018).

It is known that most of the main provision of protein collected by ants is to feed the growing larvae, while worker ants depend basically on a carbohydrate-rich diet to obtain energy (Cassill et al., 2005). Nonetheless, larvae have an essential contribution in the colony feeding; they degrade solid food pellets that are consumed by other members of the colony (Medina et al., 2007; Lee et al., 2008). In this context, *L. apiculatum* larvae seem to support with hydrolysis of plant cell wall polysaccharides by Proteobacteria that are present in their microbiota. Moreover, in guts of adult ants were also detected microorganisms that contribute with plant material degradation. These data suggest that both larvae and adult ants harbour microorganism competent for recalcitrant compounds breakdown, which could play a key role

not only in the monosaccharide production from plant materials, but also with sugars fermentation for energy supply in the *L. apiculatum* ants.

3.4. Oxidation and assimilation of aromatic compounds

Ligninolytic activity of microorganisms isolated from *L. apicultum* was evaluated by growth on the lignin monomer (guaiacol) and lignin polymer. *Bacillus simplex*, *Exiguobacterium* sp., *Rhodococcus* sp, *Kocuria palustris*, *Microbacterium hydrocarbonoxydans*, *Acinetobacter johnsonii*, *Dietzia natronolimnaea*, *Serratia marcescens*, *Staphylococcus warneri*, *Serratia rubidaea*, *Serratia ficaria*, *Pseudomonas graminis*, *Pantoea agglomerans*, *Arthrobacter crystallopoietes*, *Bacillus pumilus*, and *Enterobacter asburiae* exhibited cell growth that depended on lignin assimilation and guaiacol, which were named as ligninolytic microorganisms (**Supplementary Figure S2 and S3**). On the other hand, the oxidation of aromatic compounds by lignin peroxidase activity was evaluated using pyrogallol and congo red. Most of ligninolytic microorganisms showed the ability to oxidize pyrogallol and grow on congo red medium; however, only *Pseudomonas graminis* and *Bacillus simplex* showed hydrolysis halos around colonies on congo red medium (**Supplementary Figure S4**).

Some insects are exposed to terpenes, phenolic compounds, and even xenobiotics such as pesticides (Kikuchi et al., 2012; Xia et al., 2017). For this reason, certain insect's symbionts have developed enzymatic systems to catabolize toxic substances either for employment them as a carbon source or to reduce their toxicity (Warnecke et al., 2007; Kikuchi et al., 2012; Dang et al., 2017; Xia et al., 2017). For instance, in termite symbionts have been found several detoxifying enzymatic systems, including oxidoreductases requiring oxygen for lignin degradation and xenobiotic compounds (Sethi et al., 2013). In symbionts from bed bug, it has reported the presence of esterases and oxidases, which contribute for host insecticide resistance (Lilly et al., 2016).

3.5. *L. apiculatum* microorganisms are able to use phthalate esters as sole carbon source and energy

Symbiont-mediated mechanisms not only have provided insights how insect cope against toxic compounds but also have become an emerging tool for remediation of environmental contaminants. In this sense, the capacity of *L. apiculatum* isolated strains from both singly colonies and mixed culture consortium were used to evaluate the capacity to degrade phthalates esters (PAEs). Results showed that 11 microorganisms could use different PAEs as an only source of carbon and energy (**Supplementary Figure S5**). *Serratia ficaria*, *Enterobacter asburiae*, *Pantoea agglomerans*, *Acinetobacter johnsonii*, *Serratia rubidaea*, *Serratia marcescens*, *Staphylococcus warneri*, and *Microbacterium hydrocarbonoxydans* were able to grow on five phthalate esters tested at high concentrations (up to 1000 mg/L). While *Pseudomonas graminis* grew only in DEP, BBP, DEHP; *Rhodococcus* and *Bacillus simplex* grew only BBP, DEHP, and DNP). All phthalates-tolerant bacteria showed a colour change from pigmented trails to pale

and a decrease in growth in solid medium compared with the control (**Supplementary Figure S5**), which may be related with the characteristics of degradation products. These results show that microorganisms could use different phthalates as a carbon source to support cell growth.

These eleven strains were integrated as a consortium and were on medium containing DEHP (500 mg/L) as a sole carbon source and energy. The concentration of DEHP in the medium decreased sharply, when the highest cell biomass was reached during the first 72 h (**Figure 5A**). However, the highest removal (88%) was observed in the stationary phase after 150 h (**Figure 5B**). These results indicate a highly efficient degradation of DEHP by microbial activities. In addition, a substrate utilization test using the same bacterial consortium was carried out in order to evaluate the ability degradation of the others phthalates (DEP, DBP, BBP, and DINP) as a sole source of carbon and energy (**Figure 6**). The results showed that bacterial consortium grew using phthalates of long and short alkyl-chains. The present results are comparable with reports that evaluated phthalates degradation using complex consortiums of microorganisms from sludge. Li et al. (2018) reported that using a consortium composed by *Gordonia* sp. (54.93%), *Rhodococcus* sp. (9.92%), *Achromobacter* sp. (8.47%), *Microbacterium* sp. (0.08%), and *Cellulomonas* sp. (0.01%), a high removal (93.84%) of DEHP (1000 mg/L) was observed. Similarly, Wang et al. (2017) found a DBP degradation ratio of 97.6% to concentration of 1000 mg/L using a consortium integrated by *Brucella* sp. (62.78%) and *Sinobacter* sp. (14.83%). He et al. (2013) also observed a degradation rate of DEP (82.96%) and DBP (93.83%) to concentrations of 1200 mg/L using a consortium composed by *Gordonia* sp., *Achromobacter* sp., and *Burkholderia* sp. Then, it seems that microbial consortiums can act as complex metabolic networks for degrade different phthalates.

The identification of the DEHP degradation intermediaries of the bacterial consortium was evaluated for 7 days. To explore the degradation pathways of DEHP by the bacterial consortium, the metabolites were determined by GC/MS. At the beginning of the experiment (**Figure 7A**), only the presence of a peak corresponding to DEHP (RT: 9.39 min) was detected. During the biodegradation process, the presence of DEHP decreased and at the same time more peaks appeared (**Figure 7B-D**), the identified compounds were: methylhexyl phthalate (MEHP; RT: 7.84 min), 2-ethylhexanol (2-EH; RT: 3.53 min), dibutyl phthalate (DBP; RT: 7.61 min), diethyl phthalate (DEP; RT: 6.26 min), dimethyl phthalate (DMP; RT: 5.68 min), to determine the authenticities of the compounds were compared with the NIST database (**Supplementary Figure S6**). It was also detected that de concentration of these compounds decreased gradually through the biodegradation process, which means that the compounds were not accumulated and therefore degraded by the consortium. On the other hand, none of the metabolites were detected in the control sample, only DEHP was detected. Nevertheless, based on presence of metabolites in the degradation process the main metabolic pathway was de-esterification, which is consistent with several studies reported (Zhao et al., 2016; Li et al., 2019). The bacteria consortium members from *L. apiculatum* with a high esterase activity and able to catabolized DEHP were *Rhodococcus* sp., *Serratia marcescens*, *Serratia rubidaea*, and *Staphylococcus warneri* (**Figure 2C**). Previous studies showed that the *Gordonia* sp. and *Acinetobacter* sp. strains can degrade phthalic acid esters through the β -oxidation degradation pathway (Xu et al., 2017). Nonetheless, a deeper investigation is required not only to establish the pathway of phthalates degradation but to improve the degradation efficiency as well. This data showed that the

consortium has the ability to transform DEHP by two different pathways: de-esterification and β -oxidation as shown in **Figure 8**.

Conclusions

In conclusion, our results showed that metabolic potential of *L. apiculatum* isolated bacteria such as *Exiguobacterium* sp., *Acinetobacter johnsonii*, *Pantoea agglomerans*, *Serratia (marcescens, rubidaea, and ficaria)*, *Enterobacter asburiae*, *Bacillus (simplex and pumillus)*, *Dietzia natronolimnaea* and *Staphylococcus warneri* are associated with the production of cellulases, amylases, xylanases, and ligninases, By other hand, microorganisms such as *Exiguobacterium* sp, *Acinetobacter johnsonii*, *Rhodococcus* sp., *Microbacterium hydrocarbonoxydans*, *Arthrobacter crystallopoietes*, and *Dietzia natronolimnaea* showed with high ability to degrade aromatic compounds such as phthalates. The metabolic versatility of *L. apiculatum* symbionts showed a promising source of enzymes for biotechnological applications such as biomass hydrolysis and bioremediation.

Declarations

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding with the publication of this paper.

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AVAILABILITY OF DATA

All generated data during this study is included in this published article (and its additional files). The spectral data have been deposited in the PeptideAtlas (<http://www.peptideatlas.org/PASS/PASS01383>). Request for materials should be addressed to the corresponding author.

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Tables

Table 1. Identified microorganisms in *L. apiculatum* (adult ants and larvae) by MALDI-Biotyping and Gram stain.

Microorganism	BioTyper (score)*	Gram stain	No. of isolates	
			Larvae	Adult ants
Firmicutes				
<i>Exiguobacterium</i> sp.	1.95	+	ND	2
<i>Staphylococcus warneri</i>	1.83	+	1	1
<i>Macrococcus caseolyticus</i>	1.93	+	3	ND
<i>Bacillus simplex</i>	2.05	+	3	1
<i>Bacillus pumilus</i>	1.98	+	3	1
<i>Weissella viridescens</i>	2.25	+	3	1
<i>Lactococcus garvieae</i>	2.17	+	3	2
Proteobacteria				
<i>Enterobacter asburiae</i>	2.20	-	2	ND
<i>Pantoea agglomerans</i>	2.34	-	2	ND
<i>Serratia ficaria</i>	2.40	-	3	1
<i>Serratia rubidaea</i>	2.17	-	2	1
<i>Serratia marcescens</i>	2.36	-	2	1
<i>Acinetobacter haemolyticus</i>	1.82	-	3	1
<i>Acinetobacter johnsonii</i>	2.40	-	2	2
<i>Pseudomonas graminis</i>	1.80	-	1	2
Actinobacteria				
<i>Kocuria palustris</i>	2.18	+	2	ND
<i>Rhodococcus</i> sp.	1.80	+	ND	1
<i>Microbacterium hydrocarbonoxydans</i>	2.17	+	ND	2
<i>Arthrobacter crystallopoietes</i>	1.80	+	1	2
<i>Dietzia natronolimnaea</i>	1.93	+	ND	2

*Score value of identification results after comparison of the generated spectrum with the BioTyper software. Identification with values ≥ 1.8 was considered as correct genus identification.

Supplementary Files

Appendix A. Supplementary data

Supplementary data associated with this work can be found in online version

Supplementary Table S1. Name and molecular structure of phthalates used in this work.

Supplementary Figure S1. Enzymatic activities of isolated microorganisms from *Liometopum apiculatum* using API® 20NE.

Supplementary Figure S2. Lignin degradation by isolated strains from *Liometopum apiculatum*.

Supplementary Figure S3. Guaiacol degradation by isolated strains from *Liometopum apiculatum*.

Supplementary Figure S4. Qualitative determination of lignin peroxidase activity by isolated strains from *Liometopum apiculatum*.

Supplementary Figure S5. Isolated bacteria from *Liometopum apiculatum* tolerant to different phthalates esters (PAEs).

Supplementary Figures S6. GC/MS profile of metabolites produced from DEHP biodegradation by *Liometopum apiculatum* bacterial consortium.

Figures

Figure 2

Enzymatic activities of isolated bacteria from *Liometopum apiculatum*. Assays were carried out using API® ZYM. LAP=Leucine aminopeptidase, VAP=valine aminopeptidase, CAP=cystine aminopeptidase, CTR=α-chymotrypsin, ASBI-P phosphohydrolase, ACP=acid phosphatase, ALKP=alkaline phosphatase, Est C4-C12=esterase C4-C12, β-Gal=β-galactosidases, α-Glu=α-glucosidases, β-Glu=β-glucosidases, and β-Nag=β-N-acetylglucosaminidase. Colour gradient shows the catalytic activity from 0 to 40 nanomoles of hydrolysed substrate.

Figure 3

Hydrolysis in plaque of complex polymers. Isolated bacteria were grown on plates containing starch, CMC (carboxymethylcellulose) or xylan at 10 g/L. Plates were stained with 4% iodine solution to detect starch hydrolysis and 1% Congo Red solution to detect CMC and xylan hydrolysis. (A) *Exiguobacterium* sp.; (B, J, and N) *Acinetobacter johnsonii*; (C and K) *Pantoea agglomerans*; (D) *Serratia marcescens*; (E) *Serratia rubidaea*; (F and L) *Enterobacter asburia*; (G) *Serratia ficaria*; (H) *Bacillus simplex*; (I and O) *Bacillus pumilus*; (M) *Dietzia natronolimnaea*; (Ñ) *Staphylococcus warneri*.

Figure 4

Specific enzymatic activities of selected microorganisms from Supplementary Figure S3. (A) Amylolytic, (B) cellulolytic, and (C) xylanolytic activities were reported as U/mg protein. Comparisons were evaluated with one-way parametric analysis of variance (ANOVA) followed by multiple comparisons test. Similar letters indicate no significant differences at $P \leq 0.05$.

A)

B)

Figure 5

Growth of *Liometopum apiculatum* bacterial consortium and DEHP degradation kinetics. A) Cells were grown in minimal media without containing DEHP as the sole carbon and energy source. B) Degradation of DEHP by bacterial consortium. DEHP=di (2-ethylhexyl) phthalate.

Figure 6

Growth profile of *Liometopum apiculatum* bacterial consortium enriched with different phthalate. Phthalates were added to the MSM media at concentration of 500 mg/L. Bacteria was grown at 37 oC and the optical density (OD600) was registered at 72 h. DEP=Diethyl phthalate; DBP=di-n-butyl phthalate; BBP=benzylbutyl phthalate; DEHP=di-(2-ethylhexyl) phthalate; and DINP=diisononyl phthalate. Structural information of different phthalates is shown in Supplementary Table S1. Bars represent the mean values with the SD. Similar letters indicate no significant differences at $P \leq 0.05$.

Figure 7

GC-MS profile of phthalates degradation by *Liometopum apiculatum* bacterial consortium. Bacterial consortium was grown in minimal medium containing DEHP as a sole source of carbon and energy and was detected by GC-MS. A) At the beginning of the experiment, B) After 72 h, C) 120 h, and D) 168 h of bacterial growth. DEHP=di-(2-ethylhexyl) phthalate; MEHP=methylhexyl phthalate; 2-Ethylhexanol; DBP=di-n-butyl phthalate; DMP=dimethyl phthalate.

Figure 8

Proposed pathways of DEHP biodegradation by *Liometopum apiculatum* bacterial consortium based on detected metabolites from Figure 5. DEHP=Di-(2-ethylhexyl) phthalate; MEHP=methylhexyl phthalate; DBP=di-n-butyl phthalate; DEP=Diethyl phthalate; and DMP=dimethyl phthalate.

Supplementary Files

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