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Physiological groups of heterotrophic community bacteria present at River Actopan, Chachalacas barrier, Veracruz

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ABSTRACT

The aim of this study was to determine physiological groups of heterotrophic community bacteria in River Actopan mouth, Chachalacas, Veracruz, Mexico. Water samples were collected in sterile jars in three zones of river: zone A (ÚrsuloGalván bridge), zone B (La Loma de San Rafael) and zone C (La Bocana). The water samples were analyzed through specific culture mediums for heterotrophic bacteria groups that degrade collagen, cellulose, chitin, starch and hydrocarbons. Species were identified by conventional biochemical tests, identification strips API 20E and 20NE and for the case of bacteria that degrade hydrocarbons it was used the sequencing of RNA gen 16S. The results indicate that in zone A, bacteria that use collagen and starch as source of carbon were most abundant (105.6 cfumL^{-1} and 100 cfumL^{-1} respectively), in zone B, collagen and chitin bacteria obtained 71.6 cfumL^{-1} and 60 cfumL^{-1}, respectively. While in zone C, higher values were shown in gelatinases and cellulose degradative bacteria with 109 cfumL^{-1} and 106 cfumL^{-1} respectively, although it was also observed that this zone has more abundance in oil degrading bacteria with respect to zone A and B.

1. Introduction

Estuaries and coastal lagoons have an important ecological function for a large quantity of species that inhabit these environments (Martínez-Viloria et al., 2006). Nevertheless, they are very fragile and sensitive ecosystems that present series of gradients or ecotones that are linked together, through flow of matter and energy (Mendoza, 2010). Its main pressures are due to human activities, since they receive pollutants from urban areas, livestock and agricultural activities (Cárdenas and Ezcurra, 2004). Household wastes have high percentages of microorganisms of fecal origin, that cause illness that generate high mortality and morbidity percentages in population, while agroindustry wastes as fertilizers, insecticides and hydrocarbons extraction affect negatively water quality. Chachalacas, Veracruz isn't the exception, because it is affected by wastewater discharge of Cardel-National Park zone, the excessive use of agrochemicals in agriculture and industrial contamination that favors the sugar industry, has modified biodiversity in the zone, mainly in structural and functional composition of bacterial communities that develop and function as main indicator of ecosystems health because they are characterized by a high physiological activity and quick response to environmental changes (Bastardo, 2007). Specifically, heterotrophic bacteria are important in the transformation and recirculation of nutrients, but their increase also can mean an alteration in any coastal system environment, which allow the detection of unfavorable conditions before affectation can be evidenced in organisms of superior trophic system (Defeo, 2003; Miravet, 2003). That is why, this study has the aim to characterize physiological groups of heterotrophic bacteria in River Actopan mouth, Chachalacas, Veracruz.

2. Materials and methods

The study took place in River Actopanlocated in Chachalacas barrier in ÚrsuloGalván town, Veracruz, Mexico, during May 2016. Water samples were collected in three zones: zone A (ÚrsuloGalván bridge), zone B (La Loma of San Rafael) and zone C (La Bocana) considering industrial, agricultural, livestock, tourism and household contaminant inputs to the system, samples were extracted at different depths (surface, medium, bottom), and different lengths west (R3), center (R1), and east (R2).

2.1. Sample collection

Water samples were analyzed through specific culture mediums for each heterotrophic group as follows: for those who hydrolyze collagen (gelatinase), it was inoculated 1 mL of tube sample with liquid medium enriched with grenetin and collagen at 2%. Samples were incubated during 24 hours at room temperature and test was positive by observing medium liquefaction. For cellulite bacteria isolation, it was prepared tubes with enriched broth with microcrystalline cellulose, iron chloride (0.1 M), and it was added cigarette paper without gum of 5 cm length, the sample was inoculated and left for 15 days at 30°C to observe if there was bacterial metabolism when degrading paper strip. Starch degrading bacteria were detected by sample inoculation in nutritive agar plates enriched with soluble starch and incubated during 24 hours, after that period, it was counted and confirmed the bacterial group by adding a Lugol drop, considering positive bacteria that formed a translucent halo around the colony. Chitin degradative bacteria were inoculated in agar plates with peptone, chitin and yeast extract, and were maintained in incubation during five days, growth was considered by presence of holes in medium or by clear zones around colonies in growth. The hydrocarbon clasts were incubated during two months in oil enriched medium, after this period, it was extracted 1 mL and inoculated in Zobel agar plates with oil added with streptomycin and tetracycline (50 g L^{-1}), the plates were incubated during seven days at 25°C.

For heterotrophic bacteria quantification and characterization, it was proceeded with plates lectures after incubation, obtaining forming colony quantification units per milliliter (cfu^{1}) using a colony counter of SOL-BAT brand model Q-20. Subsequently, through successive inoculations it were obtained pure strains, and it was made Gram stain to observe cellular morphology in an optical microscope Olympus BZ600. For identification of degradative bacterial groups, it was made conventional biochemical tests and it was used API20E and 20NE system, confirming with the Apiweb™ Biomerieux program, nevertheless, in hydrocarbon degrading bacteria case, identification was made through sequencing of gen 16S of RNA. For the isolation of DNA, it was used the extraction system of DNA genomic-Wizard Genomic DNA Purification Kit (PROMEGA™), following manufacturer's instructions. With isolated DNA, it was made gen 16S of rRNA amplification, using the system for PCR "Flexi" of PROMEGA™ and universal primers 8 forward (5'-AGACTTTGATCATGGCTCAG-3') and 1492 reverse (5'-

TACGGCTACCTTGTTACGACTT-3'). PCR reactions were made in a thermocycler Amplitron II Thermolyne Barnstead International. PCR products were purified with ExoSAP-IT® system, following manufacturer's instructions and obtained products were sent to sequencing system Macrogen Korea. Obtained sequences were interpreted with BLAST program and were compared with the global base sequences (GENEBANK) to obtain phylogenetic relations. Regarding to physicochemical parameters that were considered such as temperature (water and environmental), dissolved oxygen, depth, salinity, nitrites, nitrates and ammonium; were analyzed with a multiparametric brand HANNA.

3. Results and discussion

3.1. Bacterial abundance per zone

Regarding to abundance of different heterotrophic groups, it was obtained that in zone A, bacteria that use collagen and starch as carbon source, showed values of 105.6 cfumL^{-1} and 100 cfumL^{-1} respectively, while the group with lowest abundance were bacteria that use cellulose with 6 cfumL^{-1} (Fig. 1). Variance analysis showed significant differences between the different bacterial groups with a value of p=0.02. In zone B, highest abundance was collagen and chitin degradative bacteria with 71.6 cfumL^{-1} and 60 cfumL^{-1} respectively. The bacteria with lowest abundance were chitinolytic (50.6 cfumL^{-1}). In this case, there wasn't significant difference due to a value of p=0.20, according to variance analysis (Fig. 1).

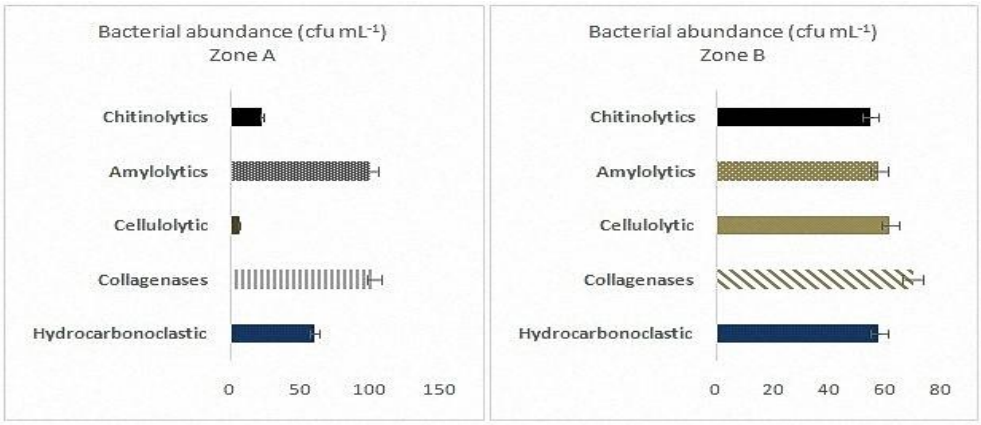

Fig. 1. Bacterial abundance for each analyzed physiological group for zone A and B.

Zone C, also presented higher abundance in collagenases and cellulolytic groups with values of 109 cfumL^{-1} and 106 cfumL^{-1} respectively. This zone also presented higher abundance in oil degradative bacteria compared to zone A and B. The variance analysis indicated significant differences between groups by obtaining value of p=0.03 (Fig. 2).

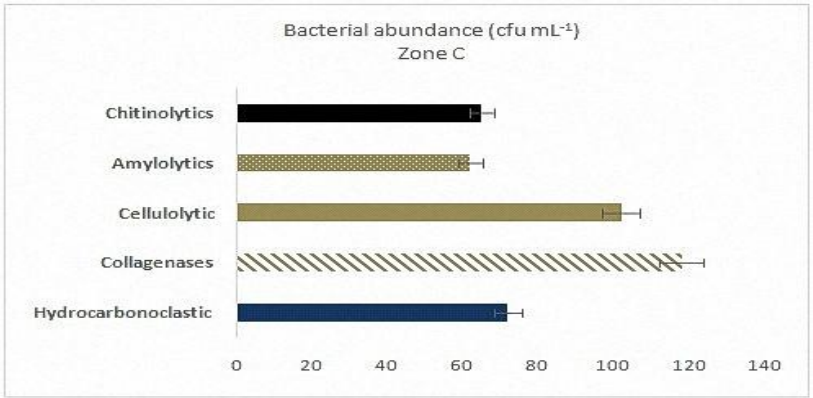

Fig. 2. Bacterial abundance for each analyzed physiological group for zone C.

In Figure 3, it shows the general frequency percentage of the bacterial physiological groups in River Actopan mouth. Predominant heterotrophic group were hydrocarbon degrading bacteria with a 27%, followed by cellulolytic with 22%. The group lowest frequency was chitinolytic with a 13%.

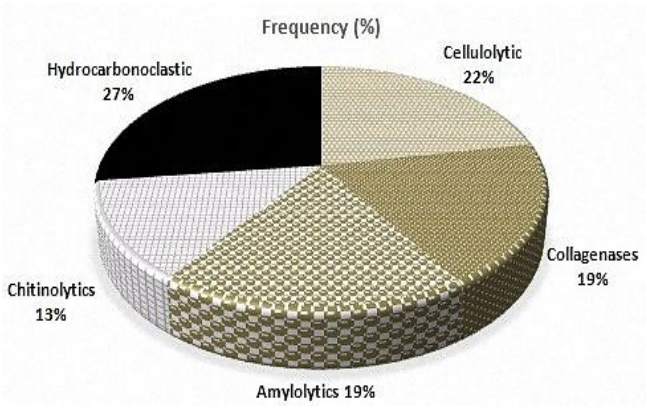

Fig. 3. Frequency percentage of physiological bacterial groups identified in River Actopan mouth.

3.2. Bacterial species identified by physiological group

Table 1 shows bacterial species which constitute isolated physiological groups in sample zones. It was obtained 37 strains totally, which belongs to 11 genders, which seven species belongs to amylolytic group, eight to cellulose degradation bacteria group, seven to keratolytic (*Serratia genus*), five to chitinolytic (*Bacillus genus*), and ten to hydrocarbons degradation bacteria.

Table 1
Bacterial identification with respect physiological analyzed groups.

Hydrocarbonoclastic	Amylolytic	Cellulolytic
Pseudomonas luteola	*Citrobacter freundii*	*Enterobacter gergoviae*
Pseudomona sp.	*Yersinia ruckeri*	*Erwina sp*
Pseudomonas cepacea	*Bacillus subtilis*	*Serratia ficaria*
Pseudoonas putida	*Erwina sp.*	*Serratia plymuthica*
Pseudomona aeruginosa	*Pseudomonas aeruginosa*	*Serratia fonticola*
Vibrio furnisii	*Pseudomonas luteola*	*Serratia sp*
Bacillus alcalophilus	*Providencia rettgeri*	*Pseudomonas sp*
Bacillus licheniformis	*Proteus miriabilis*	*Raoultella planticola*
Bacillus brevis,	*Providencia sp*	
Chromobacterium sp	*Enterobacter sp*	

Table 2
Physic and chemical analyzed parameters.

Zona	Reply	Temperature(°C)		Salinity	Dissolved oxygen	NO_2	NO_3	NH4
		Water	Environment	(g L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})
A	R1	27.3	29.0	0	11.8	0.16	15	2.9
	R2	27.8	29.0	0	11.5	0.16	15	2.9
	R3	28.4	29.0	0	11.8	0.16	15	2.9
B	R1	29.8	32.9	0	11.3	0.70	12	3.2
	R2	28.9	32.9	2	12.0	0.70	12	3.3
	R3	28.8	32.9	2	11.8	0.70	12	3.4
C	R1	28.4	31.0	3	13.1	0.21	16	3.5
	R2	28.4	31.0	3	13.7	0.21	16	3.5
	R3	28.6	31.0	3	13.9	0.21	16	3.5

Physic-chemical analyzed parameters were kept relatively constant in three sample zones. Water temperature vary between 27.3 to 29.8 °C, salinity between 0 to 3 g L^{-1}, dissolved oxygen between 11.8 to 13.0 mg L^{-1} values. Wide variations showed at nitrogen compounds. However, sample zones did not show significant differences between them (p=0.071) (Table 2).

The organic matter decomposition (OM) is one of environmental efficiency key processes, including continental and coastal aquatic ecosystems (Alvarez, 2005). The results obtained in this investigation shows that River Actopan mouth is characterized by a heterotrophic bacterial community that is carrying out its recycling function of all elements that are generated in this system produced by biological and anthropic waste products. With respect to total abundance of heterotrophic bacterial groups of each sampled area, variations were found according their location, since zone C that was closest to coast, had high abundance values of analyzed groups, compared to zone A (furthest from coast) where lowest abundance values of all groups were observed. This agrees with Montaño (1997), who shows that quantity of heterotrophic bacteria is higher near coasts and decreases as it moves away from continental shelf due to nutrients accumulation and materials that rivers transport until it arrives to sea as described by Escobar (2002).

In this study, gelatin-degrading bacteria group (collagenases) shows higher abundance. That shows that organic compounds input produced by collagen hydrolysis is abundant, this group was represented by *Serratia* sp. genus, according to Win et al. (2006) are enterobacteria, capable to produce three hydrolytic enzymes: lipases, gelatinase, and DNase. Likewise, *Serratialiquefaciences* presented in zones B and C, can produce chemical substances that promote growth in plants, so it was used like additive in many fertilizers that impact in coastal zone (Winn et al., 2006).

The waste disposal by sugar industry produce big quantity of organic waste, composed mostly of cellulose. In these residues, it is possible to find bacteria capable of hydrolyzing this biopolymer, as mentioned by Gaitan and Pérez in 2007, which mentioned that degrading cellulose bacteria are generally aerobic, while under anaerobic conditions, they are unable to completely metabolize this substrate. The genus *Erwinia* sp., which are generally facultative anaerobic organisms, metabolize faster these compounds and transform them into its simplest form and make them available to other organisms (Nissen, 2008). On the other hand, *P. aeruginosa*, Gram-bacteria, which can use carbohydrates, alcohols, aminoacids and a variety of another compounds, also produce exotoxin A, which in plants induces symptoms of rotten roots by their high cellulolytic activity, it is also pathogenic for humans mainly of immunosuppressed patients and responsible for severe nosocomial infections (Pallenori, 2005). When cellulose is degraded, starch is available and its degradation directly influences population of amylolytic bacteria, which in the present study represents 19%; other studies report their presence after sugar industry discharges (Correa-Basurto, 2007).

Amylolytic bacteria abundance registered in Zone A, was represented by *Bacillus subtilis*, which produce hydrolytic enzymes like proteases and amylases. This organism not only help to starch transform, but also nitrogen compounds, improving quality of aquatic environment (Cuervo, 2010) and *Yerseniaruckeri* which is a glucose fermented, negative oxidase and nitrates reducing (Sierralta et al., 2013). These bacteria were predominated in freshwater environments, so that salinity increase has a mitigate affect in gluten fermented process, producing a decrease of metabolic rate of this group, being reflected in their abundance and presence as happened in this study.

With respect to Chitinolytic bacteria, Monroy et al. (2005), mentioned that *Serratia* sp. among other groups suggest a detritus contribution to ecosystem, since they have the capacity to degrade chitin formed principally by filamentous fungi, arthropods exoskeleton. These suspended or dissolved organic matter in water has an important role like nutrition bacteria source, finding disintegrating chitin bacteria added in detritus matter. However, the registered abundance in chitinolytic bacteria in this study was related to biomass where abundant chitin is not available for their use, because chitin have three natural forms (Alpha, Beta and Gamma) that give different properties and allow it to perform different functions: alpha form is rigid and fulfills skeletal functions, meanwhile other two forms, that are able to hydrate, develop mechanical properties like cartilage (Hernández, 2004; Sastoque, 2005).

Most of bacterial groups found in this study correspond to Enterobacteriaceae family, gram negative bacteria that are usually located as saprophytes in digestive tract, although they are ubiquitous germs, being found universally in soil, water and vegetation, as well as being part of normal intestinal microbiota of many animals in addition to man, therefore geographical location of sampled areas is determinant, since there is human

settlements presence that contribute in organic and inorganic compounds drag to body water receptor, coupled with animal excreta and rainfall that produce a considerable source of pollution that alters ecosystem (Ramos et al., 2003).

With exception of *Bacillus subtilis* that belongs to Bacillaceae family, it is a Gram-positive bacterium, this genus is commonly found in fresh and stagnant waters, are particularly active in sediments and is not pathogenic (Cuervo, 2010). It is also necessary to emphasize that *Proteus* sp. and *Citrobacter* sp. gender seem to be related to river influence and wind inputs, which lead to sediments removal and cause that some microorganisms survive longer in water column (Cineros, 2010). Due to above, it is important to recognize that heterotrophic bacteria are highly unstable with respect to changes and fluctuations of the system abiotic factors (Bastardo, 2007).

Regarding to Hydrocarbonoclastic bacteria group, were detected at the three study sample areas and were represented by *Pseudomonas* sp. *Aeromonas* sp. *Vibrio* sp. and *Bacillus* sp. genders present in the ecosystem. Their presence is derived from natural hydrocarbon leaks that exist in Gulf of Mexico, as well as anthropogenic emissions from coastal industrial zones and tourism impact on powered boats use (Ramírez, 2015).

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