UNIVERSIDAD NACIONAL DE COLOMBIA

DIVERSIDAD BACTERIANA ASOCIADA A UNA PLANTA DE TRATAMIENTO DE AGUAS RESIDUALES (PTAR) Y ESTUDIO DE MICROORGANISMOS PRESENTES INVOLUCRADOS EN LA DEGRADACIÓN DE ÁCIDOS GRASOS.

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Universidad Nacional de Colombia
Sede Medellín
Facultad de Ciencias
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Abstract

The operation of wastewater treatment technologies depends on a combination of physical, chemical and biological factors and have to overcome several obstacles to degrade, adsorb and precipitate inorganic nutrients and organic pollutants. Microorganisms present in wastewater play essential roles in the degradation and removal of organic waste and xenobiotic pollutants. Removal of organic pollutants in the form of fats, oils and greases is a demanding procedure in wastewater treatment. Microbial lipases have distinctive industrial attention because of their stability, broad substrate specificity, high yields, regular supply and rapid growth of the enzyme-producing microorganisms. However, under certain conditions, they can catalyze other synthetic reactions like transesterification making of them an environmentally relevant class of enzymes, contributing to the improvement of wastewater treatment and biofuel synthesis. In Colombia, wastewater bacterial community studies have been focused on cultivable populations and to our present knowledge, there is little information regarding the general bacterial communities that inhabit these ecosystems. In this study, culture-dependent, molecular analysis and enzymatic methods were used to estimate bacterial diversity, monitor temporal and spatial bacterial community changes and screen for lipolytic microorganisms with bioremediation potential present in the wastewater. TTGE revealed predominant bands with affiliations to bacterial families *Acetobacteraceae, Bacillaceae, Prevotellaceae, Pseudomonadaceae* and *Veillonellaceae.* Bacterial and fungal isolates had affiliations to the genera *Cronobacter, Leclercia, Enterobacter, Klebsiella, Bacillus, Enterococcus, Escherichia, Kosakonia*, *Serratia, Chromobacterium, Mucor* and *Trichoderma.* Several bacterial and fungal isolates tested positive for lipase enzyme production by qualitative and quantitative methods. To the best of our knowledge, this is the first report for lipase and transesterification activities in the genus *Kosakonia* and the species *K. oryzae*. Our results indicate that there is a wide diversity of aerobic Gram-negative bacteria inhabiting the different sections of the WWTP, which possibly

reflect the ecological condition, functioning and general efficiency of the WWTP in Colombia.

Keywords: Bacterial diversity, Wastewater, Lipase, Transesterification, Fungi.

Resumen

La operación de tecnologías de tratamiento de aguas residuales depende de una combinación de factores físicos, químicos y biológicos. Dichas tecnologías deben superar varios obstáculos para degradar, adsorber y precipitar nutrientes inorgánicos y contaminantes orgánicos. Los microorganismos presentes en el agua residual desempeñan papeles esenciales en la degradación y eliminación de residuos orgánicos y contaminantes xenobióticos. La eliminación de contaminantes orgánicos en forma de grasas y aceites es uno de los procedimientos de mayor reto en el tratamiento de aguas residuales. Las lipasas microbianas atraen un especial interés industrial debido a su estabilidad, amplia especificidad de sustrato, altos rendimientos, suministro regular y rápido crecimiento de microorganismos productores en medios de bajo costo. Sin embargo, bajo ciertas condiciones, las lipasas pueden catalizar otras reacciones sintéticas como transesterificación lo que las convierte en una clase de enzimas relevantes para el medio ambiente, contribuyendo a la mejora del tratamiento de aguas residuales y la síntesis de biocombustibles. En Colombia, los estudios de comunidades bacterianas presentes en aguas residuales se han centrado en poblaciones cultivables y a nuestro conocimiento existe poca información respecto a las comunidades bacterianas generales que habitan estos ecosistemas. En este estudio, se utilizaron métodos dependientes de cultivo, análisis moleculares y métodos enzimáticos para estimar la diversidad bacteriana, monitorear los cambios temporales y espaciales de las comunidades bacterianas y buscar microorganismos lipolíticos con potencial para biorremediación presentes en el agua residual. Las bandas predominantes del TTGE tuvieron afiliación a las familias bacterianas *Acetobacteraceae, Bacillaceae, Prevotellaceae, Pseudomonadaceae* y *Veillonellaceae*. Las bacterias y hongos aislados tienen afiliaciones a los géneros *Cronobacter, Leclercia, Enterobacter, Klebsiella,*

Bacillus, Enterococcus, Escherichia, Kosakonia, Serratia, Chromobacterium, Mucor y *Trichoderma*. Varios aislados bacterianos y fúngicos dieron positivo para producción de enzima lipasa por medios cualitativos y cuantitativos. Hasta la fecha, este es el primer informe de actividades lipasa y de transesterificación en el género y la especie *Kosakonia* y *K. oryzae*. Nuestros resultados indican que existe una amplia diversidad de bacterias aerobias Gram-negativas que habitan en las distintas secciones de la PTAR, que posiblemente reflejan el funcionamiento, condición ecológica, y eficiencia general de la PTAR en Colombia.

Palabras clave: Diversidad Bacteriana, Aguas Residuales, Lipasa, Transesterificación, Hongos.

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Introduction

Biological treatment of industrial and domestic wastewater is a widespread method. Enhancing microorganism populations in wastewater treatment systems allow the efficient removal of organic matter, toxic substances, nutrients and pathogens from the wastewater effluent (Wang *et al.*, 2012). Despite the economic and environmental importance of these processes, the knowledge of microbial communities present in wastewater treatment plants (WWTP) remains incomplete, especially in developing countries. Special interest has been placed on WWTP treating wastewaters with high levels of organic pollutants in the form of fats, oils and greases (FOG) since their removal is a challenging procedure. The process usually implies the use of synthetic coagulants and flocculants plus aeration to remove a fraction of them (Facchin *et al.*, 2013). A residual fraction remains emulsified as droplets in the wastewater and continue to secondary biological treatment systems usually employed to finally adjust the effluent before discharging it to the environment (Primasari *et al.*, 2011). As these biological systems are not designed to treat FOG, high levels can hinder their performance, resulting in environmental and economic repercussions.

Bacteria are among the most important contributors to the transformation of complex organic compounds in wastewater treatment systems. Recently, the analysis of their structure and function has become the basis for problem solving and optimization of existing and new wastewater treatment systems (Cui *et al.*, 2012). Previous studies describe the importance of bacterial assemblies to the proper functioning and preservation of treatment systems (Forster *et al.*, 2003), revealing that changes in the diversity of such communities can compromise the entire process (Moura *et al.*, 2009). Initial diversity studies were carried out using mainly traditional culture-dependent methods that allow only the study of the cultivable fraction of the bacterial communities, leading to an under estimation of the true diversity present in the environment (Moura *et al.*, 2009). The emergence of molecular techniques overcame problems associated with

these methods, allowing a deeper understanding of the true diversity in the bacterial community.

Due to the low efficiency of physicochemical wastewater treatment systems to remove FOGs, several microorganisms have been implemented in complementary treatments to process influents rich in fats and oils. They have been used either as enzyme extracts of pure cultures obtained by solid state fermentation of agro-industrial residues (Leal *et al.*, 2002) or by growing active consortia in the wastewater to be treated. Lipases can catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface (Gupta *et al.*, 2004) and under non-aqueous conditions they can perform synthetic reactions like transesterification (Escobar-Niño *et al.*, 2014), making them an environmentally relevant class of enzymes, contributing to wastewater treatment improvements and biofuel synthesis.

Few studies emphasize on wastewaters from chocolate producing industries and, to our knowledge, only one deals with the study of associated microbial communities associated with them (Patil *et al.*, 2009). In Colombia, there are few studies of bacterial communities associated with wastewater treatment systems (Díaz *et al.*, 2010) (Pérez Peláez *et al.*, 2012) and, to date, there are no studies regarding bacterial diversity by culture independent methods. The aim of this study was to analyse temporal and spatial changes in bacterial diversity associated with the different sections of the WWTP in Compañìa Nacional de Chocolates by culture-dependent and culture-independent (RISA/TTGE) methods and also to find and select lipolytic microorganisms showing transesterification activity, which potentially can contribute to solve environmental problems. This manuscript is presented in two chapters: the first chapter has the bacterial diversity analysis and the second chapter contains the enzymatic analysis for the selected microbial isolates.

1. CHAPTER 1: BACTERIAL DIVERSITY ASSOCIATED TO THE WASTEWATER TREATMENT PLANT (WWTP) IN COMPAÑÍA NACIONAL DE CHOCOLATES, COLOMBIA

ABSTRACT

Operation of wastewater treatment technologies depends on a combination of physical, chemical and biological factors. Microorganisms present in wastewater treatment plants play an essential role in the degradation and removal of organic waste and xenobiotic pollutants. In Colombia, community studies have been focused on populations of cultivable nitrifying, heterotrophic and nitrogen fixing bacteria present in constructed wetlands. However, there is little information regarding the general microbial communities that inhabit these ecosystems. In this study, both culture-dependent and molecular analysis methods were used to estimate bacterial diversity and to monitor temporal and spatial bacterial community changes through the different sections of a wastewater treatment plant. Phylogenetic affiliation of excised TGGE bands and isolates was assessed by the determination of the 16S rDNA sequence. Predominant bands had affiliations to bacterial families *Acetobacteraceae, Bacillaceae, Prevotellaceae, Pseudomonadaceae* and *Veillonellaceae.* Isolates had affiliations to bacteria from the phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes*, including the species *Cronobacter sakazakii, Leclercia adecarboxylata, Enterobacter asburiae, Klebsiella oxytoca, Klebsiella pneumoniae, Bacillus thuringiensis, Enterococcus thailandicus, Bacillus subtilis, Bacillus mycoides, Escherichia coli, Kosakonia oryzae, Serratia* sp., *Bacillus* sp. and *Chromobacterium vaccinii.* Our results indicate a wide diversity of aerobic Gram-negative

bacteria present in the WWTP that changes among the different sections of the WWTP, which possibly reflect the ecological condition, functioning and general efficiency of the WWTP in Colombia.

1.1 INTRODUCTION

Biological treatment of industrial and domestic wastewater (WW) is an important biotechnological application worldwide and constitutes a multibillion industry. By enhancing microbial populations in wastewater treatment systems, their beneficial activities result in efficient removal of organic matter, toxic substances, nutrients and pathogens (Wang et al., 2012). Despite the economic and environmental importance of these processes, understanding microbial communities present in wastewater treatment plants (WWTP) remains a challenge, especially in developing countries. Bacteria are important contributors to the transformation of complex organic compounds in wastewater treatment systems (Moura et al., 2009) and, given the high volume of published works, it is the most studied group of microorganisms present in these systems. Recently, the analysis of their structure and function has become the basis for problem solving and optimization of existing and new wastewater treatment systems (Cui et al., 2012). Previous studies describe the importance of bacterial assemblies to the proper functioning and preservation of biological treatment systems (Forster et al., 2003), revealing that changes in the diversity of such communities can compromise the entire process (Moura et al., 2009).

Initial diversity studies were carried out using mainly traditional microbiological culture-dependent methods that allow only the study of the cultivable fraction of the bacterial communities, leading to an under estimation of the true diversity present in the environment (Moura *et al*., 2009). The emergence of molecular techniques overcame problems associated with these methods, allowing a more realistic understanding of the diversity in bacterial communities. Several molecular techniques like Ribosomal Intergenic Spacer Analysis (RISA) (Yu & Mohn, 2001), Denaturing Gradient and Temporal Temperature Gel Electrophoresis (DGGE/TTGE) (Liu *et al.*, 2007), 16S rDNA clone libraries (Otawa *et al.*, 2006), fluorescent in situ hybridization (FISH) (Björnsson *et al.*, 2002) and pyrosequencing (Wang et al., 2012) have been developed. These technologies have been implemented to investigate changes in wastewater bacterial communities and to broaden the knowledge around the diversity of microbial species and interactions between members of these complex environments (Eisen, 2007).

In terms of peer-reviewed articles, few studies emphasize on bacterial communities present in wastewaters from chocolate producing industries and only one is dedicated to investigate the microbial communities developing in the anode chamber of a microbial fuel cell. In this study, Patil and others (2009) analysed the 16S rRNA genes of this community and reported that it was dominated by clones of *β-Proteobacteria, α-Proteobacteria, Planctomycetes, Firmicutes, Nitrospora, Spirochaetes, Bacteroides* and *γ-Proteobacteria*. In Colombia, wastewater bacterial community studies have been focused on cultivable populations and there is little information regarding the general bacterial communities that inhabit these ecosystems (Díaz et al., 2010) (Pérez Peláez et al., 2012). In these studies, microbial communities were evaluated by culture-dependent methods in search for nitrifying bacteria present in constructed wetlands and peptidolytic bacteria in UASB reactors.

Amongst the consequences resulting from a change in the diversity of bacterial communities, is the bulking or clogging of biological wastewater treatment systems (Guo et al., 2014). A shift in bacterial diversity from cocci- and bacillus-like bacteria to filamentous-like bacteria can clog the narrow compartments in constructed wetlands impeding the wastewater flow (Nivala et al., 2012). A constructed wetland is a basin filled with filter material, usually gravel, planted with vegetation tolerant to saturated conditions (Wallace & Knight, 2006). As wastewater flows through the substrate, several physical, chemical and biological processes like volatilization, filtration, sedimentation, precipitation, adsorption and plant/microbial metabolism remove pollutants (Adrados *et al.*, 2014). Vegetation plays a vital role as it provides surfaces and a suitable environment for microbial growth and filtration. Since the 1950s, CWs have been used effectively to treat wastewaters with different configurations, scales and designs and, currently, over 95% of these wetlands are subsurface flow wetlands (Wallace & Knight, 2006).

The horizontal sub-surface flow constructed wetland (HSFCW) in Compañía Nacional de Chocolates (CNC) has been experiencing a progressive clogging process. In order to maintain the designed operational parameters, part of the wetland (the first 15 meters within the inlet) has to be removed and washed, causing operational and economic costs. This study aims to use both culture-dependent and molecular analysis as complementary methods to estimate bacterial diversity and to monitor temporal and

spatial bacterial community changes throughout the different sections of this WWTP. This study is the first report of the general structure of microbial communities present in a full scale industrial WWTP in Colombia.

1.2 MATERIALS AND METHODS

1.2.1 Sampling and chemical analysis

Water samples were collected in a WWTP, located in Rionegro (Antioquia, Colombia) that treats domestic and industrial wastewater released from the chocolate factory property of Compañía Nacional de Chocolates SAS (1200 employees). The WWTP deals with a volume flow rate of 2 L/s (172.8 m^3/day) and an average daily BOD and COD load of 985 kg/d and1560 kg/d respectively (Appendix G). It consists of four consecutive different sections (Figure 1-1): Two main channels with metallic screening grids that conduct the industrial WW and the domestic wastewater to the homogenization tank and to the Domestic WW pre-treatment respectively. These two effluents mix at the Imhoff tank entrance and are treated at the Imhoff tank, to then pass through the Horizontal Subsurface Flow Constructed Wetland (HSFCW) for secondary treatment before it is discharged. The HSFCW consists of four basins with an area of 1250 m^2 each and a total area of 5000 m^2 (0.5 ha).

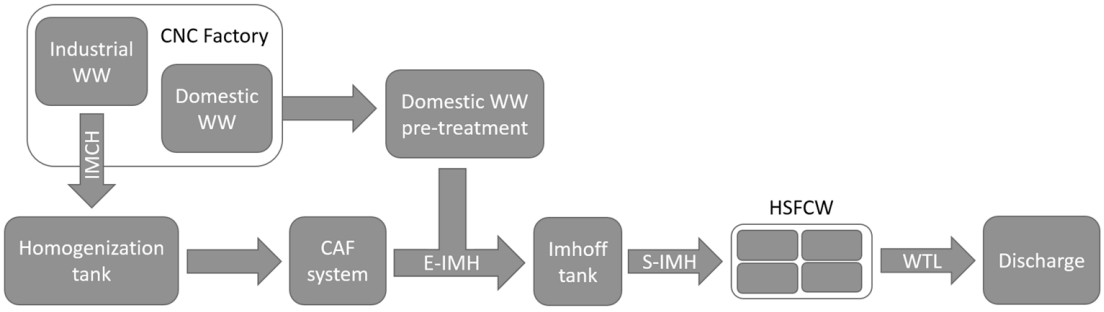

Figure 1-1. WWTP arrangement and sampling sites

Four WW samples (300 mL) were collected through a year and a half period (June (S1), September (S2), December 2013 (S3) and December 2014 (S4)) from four different sites of the WWTP: Industrial Main Channel (IMCH), Imhoff influent (E-IMH) and effluent (S-IMH), and at the HSFC Wetland effluent (WTL). All water samples were transported on

ice to the Molecular and Cell Biology Laboratory from the Universidad Nacional for processing and analysis.

Water temperature, pH, conductivity and dissolved oxygen (DO) were determined on site with a Hach® HQ40d Model portable meter (United States). Influent characteristics, namely chemical oxygen demand (COD), biochemical oxygen demand (BOD), Fat, oil and grease (FOG) content, Total Nitrogen (N), Total Phosphorous (P), Total Organic Carbon (TOC), Total solids (TS) and Total Suspended Solids (TSS) were determined following Standard Methods (Eaton *et al.*, 2005) by a certified laboratory.

1.2.2 DNA extraction

Total DNA from WW samples for the RISA and TTGE analysis was extracted using the Ultra CleanTM Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA); according to the manufacturer's protocol. Final DNA aliquots were quantified using ND-100 Nanodrop Thermo Scientific spectrophotometer (Thermo Fisher Scientific Inc, MA). DNA of each bacterial isolate was obtained by lysing a loop-full of the culture in 50uL of TE 0.1X at 100ºC for 10 min and centrifuging at 5000 rpm for 5 min. The crude lysates supernatant was diluted to a 1:10 dilution with Milli Q water and used immediately for PCR amplifications. Genomic DNA from the cultivable fraction for each culture media was extracted using the Ultra CleanTM Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA), according to the manufacturer's protocol.

1.2.3 PCR amplifications

To amplify the 16S rDNA, different sets of primers were used according to the technique employed. To amplify 16S rDNA for isolate identification, the 27F (50-AGAGTT TGATCCTGGCTCAG-30) and 1492R (50-GGTTACCTTGTTACGACTT-30) (Espejo *et al.*, 1998) were used. The ribosomal intergenic spacer regions (RISA) between the 16S and 23S rDNA were amplified using the primers G1 (50-GAAGTC GTAACAAGG-30) and L1 (50-CAAGGCATCCACCGT-30) (Jensen *et al.*, 1993). Primers 341F-GC and 907R described by Moreno and collaborators (2006) were used for the PCR-TTGE analysis of the V3 and V6 regions from the 16S rRNA gene.

1.2.4 Temporal Temperature Gel Electrophoresis

TTGE was performed on a Dcode system (Bio-Rad Lab, Hercules, CA, USA) as described by Gómez *et al*., (2011). The products were separated on a 7% (w/v) acrylamide–bisacrylamide gel containing 7 M of urea as denaturant, 1.25x TAE, 0.1% TEMED, 1 gL–1 ammonium persulfate and acrylamide–bisacrylamide solution (from 40% stock) following the manufacturer's recommendation (BioRad). The temperature ranged between 66–68 °C with a ramp rate of 0.1ºC for 15h at 55 V. The gel was stained for 30 min with SybrGreen (Invitrogen, Paisley, UK) in 1.25× TAE and visualized by VWR® Blue LED Transilluminator.

1.2.5 DNA sequence and phylogenetic analysis

Selected bands excised from the TTGE gel were eluted in Milli Q water overnight and re-amplified using the same primers but without the GC complement. Isolates displaying unique RISA profiles were identified by 16S rRNA gene sequencing analysis. Both amplification products were purified and sent to Macrogen (Seoul, Korea) to be sequenced on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA). The sequences obtained were edited using BioEdit® (Hall, 1999), and the presence of chimeric sequences was evaluated with DECIPHER (Wright *et al.*, 2012). To determine the phylogenetic affiliation of the isolates and the excised bands, similarity searches were performed using the BLAST program. A phylogenetic analysis via the Neighbor-Joining method (Saitou & Nei, 1987) with 1000 bootstrap replicates using MEGA v6.1 (Tamura *et al.*, 2007) with reference sequences obtained from the NCBI (Altschul *et al.*, 1997) and RDP databases (Cole *et al.*, 2005).

1.2.6 Sequence accession numbers

Twenty-two isolates and nine TTGE band 16S bacterial rDNA gene sequences were deposited in the GenBank database and received the accession numbers KT275815 to KT275836 and KT275837 to KT275845 respectively.

1.2.7 Bacteriological analysis

One millilitre of each wastewater sample was serially diluted up to 10^{-3} and 200uL were plated on commercial LB agar (Difco labs) with (LBw) and without (LB) wastewater supplement and as duplicates. The LBw culture medium was elaborated as instructed by the manufacturer but replacing the distilled water by autoclaved and filtered wastewater obtained from the Imhoff tank exit.

Plates were incubated under aerobic conditions at 25 ± 2 °C and growth was observed after 24 h and 72 h of incubation. Colonies with different morphological characters (colony, size, elevation and pigmentation) were isolated from different media plates and sub-cultured to obtain pure bacterial cultures. For further maintenance of cultures, only LB was used. On the third day, the entire cultivable fraction was retrieved and stored in LB broth at -20ºC until DNA extraction. The remaining wastewater sample was centrifuged and stored at -20ºC for total DNA extraction.

Pure colonies were re-isolated in LB agar at least three times and pure isolates were characterized by RISA. Isolates with different RISA patterns were identified through 16S rRNA gene sequencing. RISA patterns were resolved by polyacrylamide gel electrophoresis (PAGE) using the GelCompar Software (Applied Biosystems, Belgium). For long-term preservation, all isolates were stored in LB medium in 20% glycerol and at -20°C and -80 °C.

A t test was performed to the colony forming units data for both culture media to analyse if the differences in CFU/mL between both culture media were significantly different based on the statistical hypothesis that no significant differences exist between them.

1.2.8 Bacterial community analysis

GelCompar Software (Applied Biosystems, Belgium) was used to analyse RISA and TTGE profile patterns from the DNA samples. The quantitative data matrix for the number and intensity of the fragments was used for statistical and diversity analysis in PAST software version 2.17c (Hammer *et al.*, 2001). Diversity indices (Appendix A) and significant differences in bacterial diversity were assessed by the “Diversity indices” and

“Diversity t test” options of the PAST program. For these analyses, band number was considered to represent species number or Taxa (S) and band intensity was considered to represent the relative abundance or number of individuals of each bacterial species (Fromin *et al.*, 2002) (Ribeiro *et al.*, 2013). The “Principal Components” analysis option was implemented to view overall similarities and dissimilarities between sampling sites and dates (Boon *et al.*, 2002).

1.3 RESULTS

1.3.1 Wastewater characterization

The global average influent (IMCH) and effluent (WTL) parameters give the WWTP a general efficiency of 99% for COD, BOD, FOG and total solids removal. On site measurements indicated a general average pH of 5.1 and temperature of 25ºC through all the sampling sites with variable measurements in dissolved oxygen between the different sections of the WWTP (Table 1-1).

Table 1-1. Mean of different physicochemical parameters at different sampling sites. pH, Temperature, Dissolved Oxygen and Conductivity were measured in situ

PARAMETER	SAMPLING SITE			
	IMCH	E-IMH	S-IMH	WTL
BOD (mg O2/L)	5699	2209.8	1354.2	26.9
COD (mg O2/L)	9027.3	2908.7	1889.6	98.5

FOG (mg/L)	392	134.8	38	24.6
TS (mg/L)	3326	2238.2	1070.6	513.1
TSS (mg/L)	1076	393	224.2	58.8
N (mg N/L)	30.2	28.4	43.4	20.7
P (mg P/L)	6.2	8.9	5.1	-
TOC (mg C/L)	2686.1	1951	713.8	18
DO (mg/L)	1.7	4.2	0.2	1.6
T (°C)	28.1	24.8	24.5	21.3
pH	4.5	6.3	4.6	6.8
Conductivity (mS)	156.6	36.1	135.4	11.4

1.3.2 Bacterial diversity through TTGE

Changes in the total bacterial community structure of the four different sampling sites were evaluated through TTGE profiles of partial 16S rRNA genes (Figure 1-2). The results of the banding profiles ranged from one to six bands per lane with several migration patterns. The dendrogram generated using Dice coefficient and the UPGMA algorithm with the GelCompar II program indicated the presence of a core community in the WWTP that is constant throughout time and space (Figure 1-2). The Diversity indices (Appendix A) and the Diversity t test analysis unveiled significant differences between the sampling sites and some differences between the sampling dates as displayed by the Principal Components Analysis (Figure 1-3).

The bands common to all or most lanes, as well as unique banding patterns were successfully re-amplified and sequenced. Nine representative bands were selected, obtaining sequences with 200-500 bp approximately. These bands were linked to specific bacterial groups according to similarity in 16S rDNA sequences held in public databases (Table 1-2). Affiliation by Maximum-Likelihood tree (Appendix B) shows the phyla *Firmicutes, Proteobacteria* and *Bacteroidetes* including families like *Acetobacteraceae, Bacillaceae, Pseudomonadaceae, Prevotellaceae* and *Veillonellaceae*.

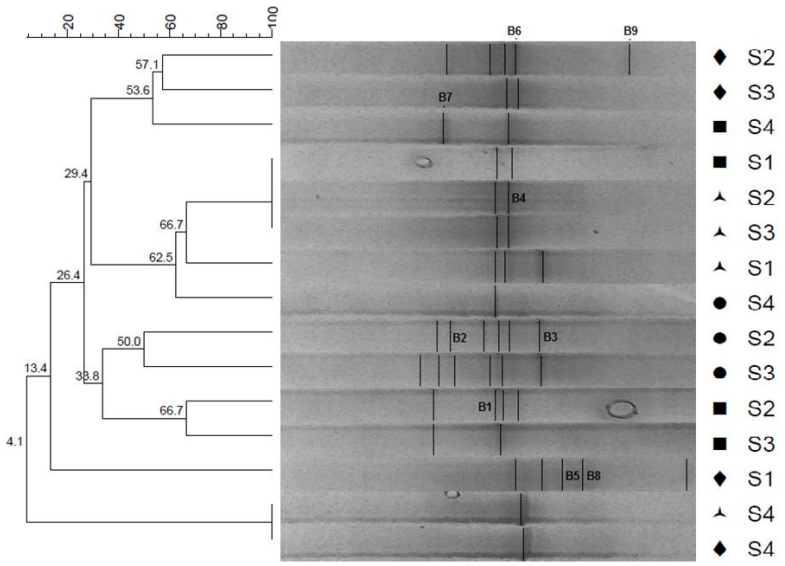

Figure 1-2. Dice-UPGMA clustering analysis of bacterial diversity TTGE patterns at different sections of the WWTP. Dendogram represents the clustering of WWTP taken from different sampling sites ((■) IMCH, (●) EIMH, (▲) SIMH, (◆) WTL) and times (S1, S2, S3, S4).

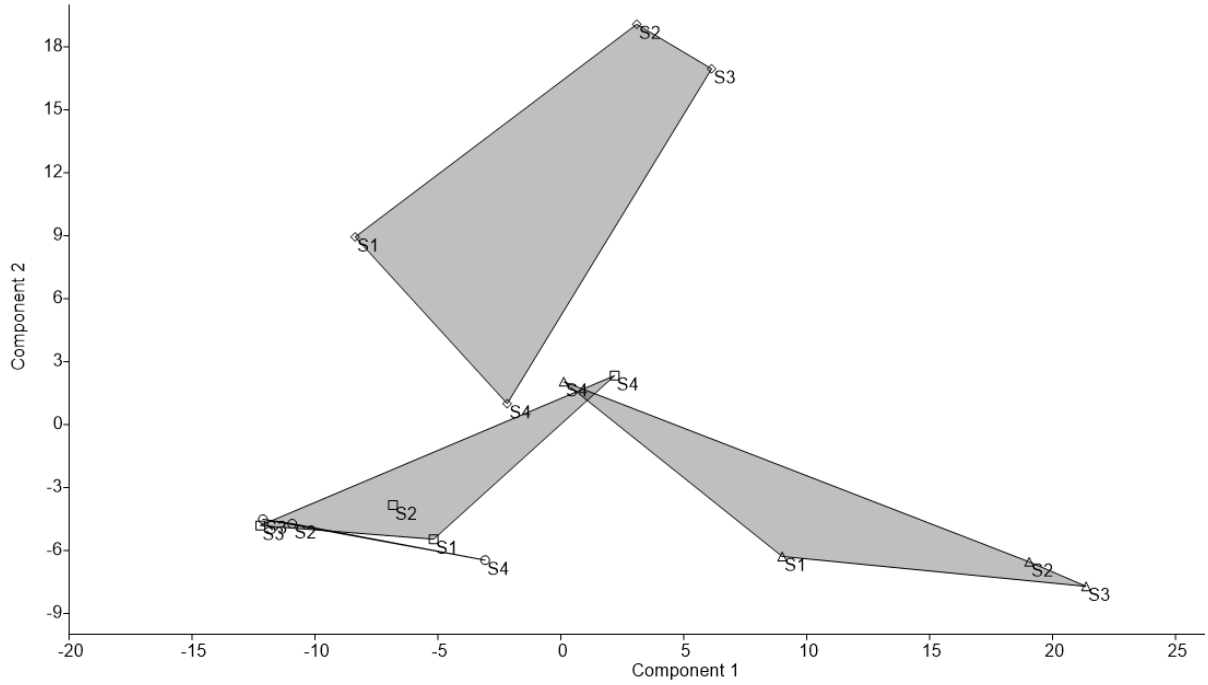

Figure 1-3. Principal components analysis for the TTGE quantitative matrix of the total DNA from the different sampling sites ((□) IMCH (○) EIMH (△) SIMH (◇) WTL) and times (S1, S2, S3, S4).

Table 1-2. Phylogenetic affiliations of major TTGE bands

Origin	Sample	Accession #	Closest relative accession #	Phylogenetic affiliation

			[Bases compared] (Similarity %)		
IMCH	S2-Band1	KT275843	NR104863.1 [300/337] (89%)	*Firmicutes*	*Veillonellaceae*
	S4-Band7	KT275841	NR104749.1 [446/457] (98%)		*Bacillaceae*
EIMH	S2-Band2	KT275840	NR113411.1 [287/313] (92%)	*Bacteroidetes*	*Prevotellaceae*
	S2-Band3	KT275837	NR125626.1 [442/457] (97%)	*α-proteobacteria*	*Acetobacteraceae*
SIMH	S2-Band4	KT275844	NR044111.1 [489/527] (93%)	*Firmicutes*	*Veillonellaceae*
WTL	S1-Band5	KT275845	NR117597.1 [240/287] (84%)	*Firmicutes*	*Veillonellaceae*
	S2-Band6	KT275838	NR113122.1 [308/323] (95%)	*Bacteroidetes*	*Prevotellaceae*
	S2-Band8	KT275842	NR043656.1 [317/359] (88%)	*Firmicutes*	*Veillonellaceae*
	S2-Band9	KT275839	NR103934.1 [176/207] (85%)	*γ-proteobacteria*	*Pseudomonadaceae*

1.3.3 Bacterial community analysis

The objective of the RISA analysis was to compare the similarities and differences of the cultivable and total bacterial communities. Diversity indices (Appendix A) and the Diversity t test analysis for the band profiles and intensities among different sampling sites and times were very different and revealed significant changes in diversity between both culture media and the total DNA as seen by the Principal components analysis in Figure 1-4.

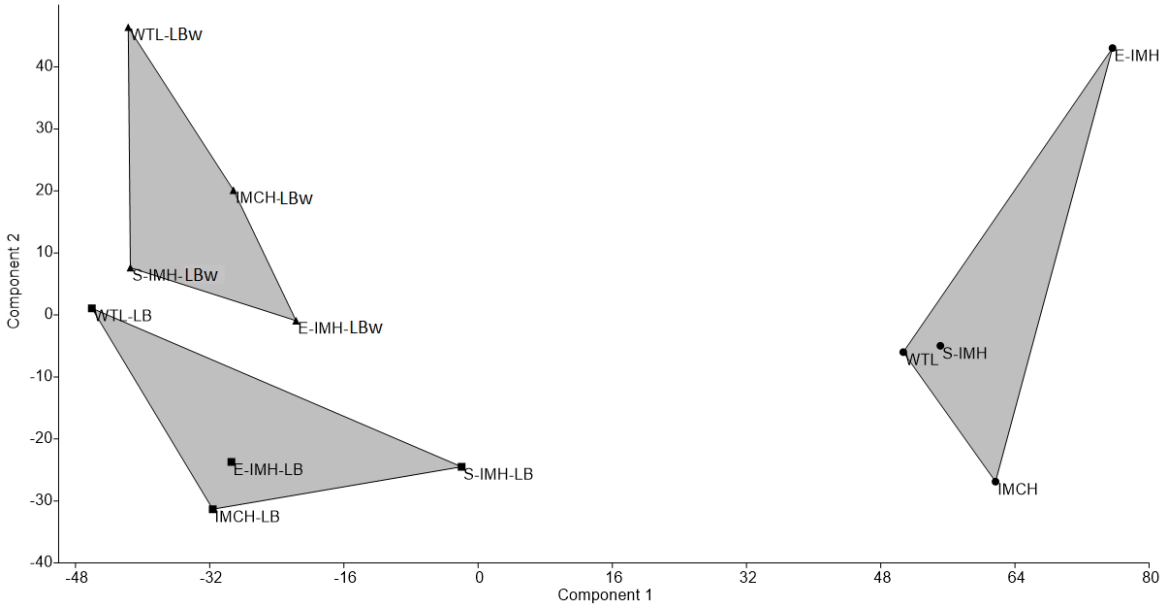

Figure 1-4. Principal components analysis for the RISA quantitative matrix of the total DNA and cultivable fractions from the different sampling sites. (▲) LBw culture medium, (■) LB culture medium, (●) total DNA

1.3.4 Molecular identification of bacterial isolates

Eighty strains recovered from LB and LBw medium were analysed by RISA with the GelCompare II software to differentiate the ITS-patterns and group similar bacterial isolates (Appendix C). Twenty-one groups were stablished with 70% or more similarity values between the isolates and one isolate from each group was selected for 16S rDNA sequence analysis. An additional isolate was selected for its colony morphology (Appendix E) but was unsuccessful in amplifying the ITS region (WTLIsolate001, *Chromobacterium vacinii*).

The twenty-two bacterial sequences had high similarity percentages (98–100%) with reported sequences in the GenBank database. Based on the phylogeny (Appendix D) of the related sequences from the BLAST search and the reference sequences obtained from the Ribosomal Database Proyect (RDP), eleven bacterial species were identified (Table 1-3) as follows: *Cronobacter sakazakii, Leclercia adecarboxylata, Enterobacter asburiae, Klebsiella oxytoca, Klebsiella pneumoniae, Bacillus thuringiensis, Enterococcus thailandicus, Bacillus subtilis, Bacillus mycoides, Escherichia coli, Kosakonia oryzae, Serratia sp, Bacillus* sp. and *Chromobacterium vaccinii.*

Table 1-3. Taxonomic identification of bacterial strains isolated from the WWTP.

Origin	Sample	Accession #	Closest relative accession # [Bases compared] (Similarity %)	Phylogenetic affiliation	
IMCH	IsolateB029-LBw	KT275832	KJ806502.1 [1271/1277] (99%)	*γ-Proteobacteria*	*K. pneumoniae*
	IsolateB052-LBw	KT275833	NR116033.1 [1403/1411] (99%)		*Ko. oryzae*
	IMCHisolate054-LBw	KT275815	NR114077.1 [912/918] (99%)		*Cr. sakazakii*
	IMCHisolate055-LBw	KT275816	NR104933.1 [1364/1376] (99%)		*L. adecarboxylata*
EIMH	EIMHisolate001-LB	KT275817	NR024640.1 [900/902] (99%)	*γ-Proteobacteria*	*E. asburiae*
	EIMHisolate002-LB	KT275818	NR041749.1 [1345/1354] (99%)		*K. oxytoca*
	EIMHisolate005-LBw	KT275819	HM161855.1 [1331/1338] (99%)		*E. asburiae*
	IsolateB008-LBw	KT275835	KJ806502.1 [1364/1367] (99%)		*K. pneumoniae*
SIMH	SIMHisolate001-LBw	KT275820	KJ806502.1 [1096/1103] (99%)	*γ-Proteobacteria*	*K. pneumoniae*
	SIMHisolate002-LBw	KT275821	NR112780.1 [1388/1392] (99%)	*Firmicutes*	*B. thuringiensis*
	SIMHisolate004-LBw	KT275822	NR114015.1 [1423/1424] (99%)		*E. thailandicus*
	SIMHisolate005-LBw	KT275823	NR024640.1 [963/967] (99%)		*E. asburiae*
	IsolateB006-LBw	KT275834	NR114043.1 [595/604] (99%)	*γ-Proteobacteria*	*Serratia sp.*
	IsolateB013-LBw	KT275836	KJ806502.1 [1400/1406] (99%)		*K. pneumoniae*
	SIMHisolate014-LBw	KT275824	NR115001.1 [1403/1406] (99%)	*Firmicutes*	*B. subtilis*
	SIMHisolate017-LBw	KT275825	CP009692.1 [1414/1420] (99%)		*B. mycoides*
	SIMHisolate027-LB	KT275826	KJ806502.1 [1015/1020] (99%)		*K. pneumoniae*
	SIMHisolate032-LB	KT275827	CP007394.1 [1344/1344] (100%)	*γ-Proteobacteria*	*E. coli*
	SIMHisolate036-LBw	KT275828	NR112558.1 [1109/1110] (99%)		*E. coli*
WTL	WTLisolate001-LB	KT275830	NR109451.1 [986/989] (99%)	*β Proteobacteria*	*C. vaccinii*
	WTLisolate014-LB	KT275829	NR112780.1 [1416/1422] (99%)	*Firmicutes*	*B. subtilis*

WTLisolate021-LBw	KT275831	JX680066.1 [613/649] (94%)	*Bacillus sp.*

1.3.5 Bacterial count

Colony counts varied between both culture media for all the sampling sites, but remained constant through the different sampling dates (data not shown). No statistical significances were observed for CFU/mL between the culture media (Table 1-4).

Table 1-4. Mean, Standard deviation and p-Value from the t test of bacterial plate counts (CFU/mL) in LB and LBw culture media for each sampling site.

Sampling	LB (CFU/mL)		LBw (CFU/mL)		p-value
Site	Mean	SD	Mean	SD	
IMCH	6.6×10^5	5.1×10^4	6.6×10^5	8.5×10^4	0.993
E-IMH	8.2×10^5	7.0×10^4	1.0×10^6	7.0×10^4	0.766
S-IMH	3.3×10^5	5.0×10^4	5.6×10^5	7.1×10^4	0.545
WTL	7.1×10^4	1.2×10^4	5.2×10^4	1.6×10^4	0.451

1.4 DISCUSSION

Little is known regarding bacterial community structure of WWTP in Colombia. Díaz and collaborators (2010) and Pérez-Pelaez and collaborators (2012) studied cultivable bacterial community of several WWTP. These studies focused on changes between the nitrifying bacterial communities present in different constructed wetlands and peptidolytic bacterial strains isolated from UASB reactors. Peptidolytic bacterial isolates were associated to the *Proteobacteria, Actinobacteria* and *Firmicutes* phyla. In this research, the general bacterial diversity associated with an industrial wastewater treatment plant in Colombia was examined using both culture dependent (agar plating) and culture independent techniques (RISA, TGGE and sequence analysis of the 16S rRNA gene). Variations among the bacterial populations were identified based on the culture media used for plating, the sampling sites and times and between cultivable and molecular methods.

TTGE culture-independent analysis showed significant variations in diversity among sampling sites as expected since each wastewater treatment system exerts different types of physical and chemical variables that probably select for different types of bacteria (Jordaan & Bezuidenhout, 2013) (Table 1-1). Diversity variations were also detected among sampling dates (Figures 1-2 and 1-3) which suggests that, in general, the different sections of the WWTP have not reached a stable bacterial community (Appendix A). Variations between sampling dates were expected for the IMCH and E-IMH sampling sites as these sampling sites may suffer variations associated to the production in the factory that alters the influent composition. More stable bacterial communities were expected for the Imhoff exit (S-IMH) and the wetland exit (WTL) in concordance with the structure, physicochemical characteristics and purpose of these treatment systems and the constant effluent they are expected to give (Samsó & García, 2013) (Tilley *et al.*, 2014).

Variations in bacterial diversity at the WTL and S-IMH sampling sites perhaps are a consequence of the constant washing processes for the WTL and variations associated to the production line in the factory that may alter the influent composition for the first three sampling sites (IMCH, E-IMH and S-IMH). An exception to the diversity variation trend among sampling dates was the fourth sampling date (S4) in which all the different

sampling sites were very similar (Figure 1-3) in terms of bacterial composition. At this sampling date (S4), the E-IMH sampling site was the most different (Figure 1-3). This sampling site (E-IMH) is characterized by an inlet of domestic wastewater coming from the restrooms of the factory (Figure 1-1) that is diminished through the Imhoff tank and subsequent HSFCW treatment systems.

Sequence and phylogenetic analysis of the excised bands from the TTGE (Table 1-2 and Appendix B) revealed several different phylogenetic groups for each sampling site/time belonging to *Firmicutes, Bacteroidetes, α-proteobacteria* and *γ-proteobacteria* with families like *Acetobacteraceae, Bacillaceae, Pseudomonadaceae, Prevotellaceae* and *Veillonellaceae*. Most of these families are Gram-negative anaerobes or facultative anaerobes with the exception of *Acetobacteraceae* and *Pseudomonadaceae* which are predominantly aerobic (Komagata *et al.*, 2014) (Mandic-Mulec *et al.*, 2015) (Rosenberg, 2014) (Palleroni, 2012) (Marchandin & Jumas-Bilak, 2014). (Marchandin & Jumas-Bilak, 2014). These results are consistent with the reports of Wagner and Loy (2002) stating that the most frequently retrieved sequences in WWTPs are affiliated with the *α-,β-* and *γ-proteobacteria*, *Bacteroidetes* and *Actinobacteria*. The families present at the HSFCW are also consistent with the results of similar investigations regarding this type of treatment system, affirming that predominant bacterial populations associated with them belong to *γ -Proteobacteria, Bacteroidetes* and *Firmicutes* families (Adrados et al., 2014).

The families found in this study belong to phylogenetic groups harboring genera related to the most frequently encountered in filamentous build-up (*Sphaerotilus, Micothrix, Haliscomenobacter, Nostocoida, Thiothrix, Chloroflexi* and *Meganema*) as reported by Wagner and Loy (2002) and Martins and collaborators (2004). By the methods employed, no specific filamentous bacteria were identified in the microbial diversity of the WWTP, which probably indicates that the clogging phenomenon presented is not associated with this event or that the methods employed were insufficient to detect them. It is known that filamentous bacteria are reluctant to cultivation methods or are difficult to maintain in pure cultures and that molecular methods often fail to detect them and yield better results when coupled with *in situ* hybridization techniques employing specific probes (Wagner & Loy, 2002). There are also problems associated with inherent biases present in PCR-based molecular methods, including differential primer preference; efficiency of cell lysis; DNA extraction and purification (Jin *et al.*, 2011).

Possibly, the build-up phenomena presented in the WWTP may not be caused by a shift in diversity towards filamentous bacteria. Some authors suggest that changes in the bacterial metabolism might be associated with clogging events in WWTPs. This type of non-filamentous clogging involves the presence of Gram-negative bacteria that under certain nutritional conditions (nitrogen/phosphorus imbalance) have an increased production of extracellular polymeric substances (Glymph, 2011) (Guo et al., 2014). In addition, it is more likely that these Gram-negative bacteria become attached to the gravel and root surfaces and aid the build-up process in the HSFCW wastewater treatment system. Glymph (2011) suggests that the optimal balance of C:N:P to prevent a change in the metabolism of Gram-negative bacteria must be 100:10:1. For Imhoff tank outlet (S-IMH) leading the wastewater into the wetland, the ideal C:N:P ratio should be around 713.8:71.4:7.1 based on the total organic carbon (TOC) content for this sample (Table 1-1). The actual nitrogen and phosphorus values for this sample (43.4 mg N/L and 5.1 mg P/L) indicate that there is an imbalance in the ratio of nutrients specially marked by a lack of nitrogen in the wastewater (Table 1-1). This could be the main reason for the clogging considering that no filamentous bacterial species or families were found and that the majority of the isolated species and families by culture-dependent (Appendix D and Appendix E) and culture-independent (TTGE) methods are Gram-negative (Tables 1-2 and 1-3).

Other unbalanced values with respect to the optimal ones required for ideal functioning of the HSFCW are the BOD and COD measurements at the S-IMH sampling site (Table 1-1). Caselles-Osorio and collaborators (2007) concluded that there is a direct relationship between inlet effluent parameters like BOD, COD and TSS with the accumulation of solids in the bed and a subsequent clogging of the HSFCW near the inlet zone. Wallace and Knight (2006) stablished that ideal Surface Loading Rate (SLR) values for BOD and COD for the inlet effluent of HSFCWs should be between 4-6 g/m^2 d. The actual SLR values for the inlet effluent (S-IMH) of the studied HSFCW are 46.8 and 65.3 g/m^2 d (Appendix G), implying that the HSFCW is overloaded and that solid accumulation in the bed is a probable cause for the frequent clogging of the bed. A plausible solution for this issue is an upgrade of the primary treatment in the WWTP, in this case the Imhoff tank, to prevent the income of large amounts of solids and organic matter to the HSFCW (Caselles-Osorio et al., 2007) (See Chapter 2). Besides a saturation of the filter media, this excess of solids

can cause growth inhibition of beneficial microorganisms that dwell on HSFCWs since these solids are usually hard or non-biodegradable by the microorganisms commonly present at these treatment systems (Caselles-Osorio et al., 2007).

Bacterial community analysis through RISA for the cultivable and total bacterial communities inhabiting the WWTP (Figure 1-4) revealed significant differences between the culture media implemented and between the cultivable and the total bacterial communities. The statistical analysis for every sampling site showed that the diversity present in the LBw medium and the LB medium was completely different, leading to a direct influence of the supplement in the development of certain types of bacteria (Stewart, 2012). As expected, the cultivable diversity profile at all the sampling sites is dissimilar from the diversity obtained by culture-independent methods since the cultivable portion is usually very low (Handelsman, 2004). These results highlight the importance of both techniques (dependent and culture-independent) for a fuller understanding of the status of the WWTP and the possible paths that can be taken to repair the problems it faces (Dashti *et al.*, 2015) (See Chapter 2).

Abundance of cultivable bacteria varied greatly, in terms of colony forming units, among the sampling sites but remained constant through time. Bacterial counts fluctuated between 4.9×10^3 and 1×10^6 colony forming units (CFU/ml) on average, with no significant difference between the culture media (Table 1-4). The high counts of heterotrophic bacteria in both culture media for the first three sections of the plant can be related to the presumed high nutrient content of the wastewater treated as indicated by the COD, BOD and DO measurements (Table 1-1) for these sites (Heiskary & Markus, 2001). This is also in agreement with the very low counts for the wetland exit (WTL) and the physicochemical parameters of this sampling site that indicate a possible low nutrient content effluent that selects for oligotrophic bacteria incompatible with nutritive culture media such as Luria-Bertani.

The sequence analysis for the isolates obtained revealed a very different diversity of species from the ones retrieved from the TTGE bands. The isolates were predominantly aerobic or facultative anaerobes and the family *Bacillaceae* was common between the culture dependent and culture independent methods (Tables 1-2 and 1-3). The reported genera are frequently encountered in wastewater samples by culture dependent methods

(Ferreira da Silva *et al.*, 2007). The families and species found have also been reported as part of the microbiota present in oil spills (Al-Awadhi *et al.*, 2012), hydrocarbon contaminated sites (Sarma *et al.*, 2010); (Zafra *et al.*, 2014) and as part of the natural microbiota present in cocoa beans during the late stages of fermentation (Kavroulakis & Ntougias, 2011) (Papalexandratou *et al.*, 2011). The results obtained indicate that the methods employed were an adequate approach to elucidate the bacterial diversity and dynamics present in the WWTP and a possible cause for the clogging problem. Culture dependent and culture independent methods are complementary techniques that should be used hand in hand for a better understanding of bacterial communities and for potential studies regarding functional properties of the isolates.

1.5 ACKNOWLEDGEMENTS

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2. CHAPTER 2: DETECTION AND QUANTIFICATION OF LIPASE POSITIVE FUNGAL AND BACTERIAL ISOLATES ASSOCIATED WITH THE WWTP FROM COMPAÑÍA NACIONAL DE CHOCOLATES, COLOMBIA

ABSTRACT

Wastewater treatment plants (WWTP) have to overcome several obstacles to degrade, adsorb and precipitate inorganic nutrients and organic pollutants present in wastewater prior to its release into the environment. Special interest has been placed on WWTP treating wastewaters with high levels of organic pollutants in the form of fats, oils and greases (FOG) since their removal represents an important challenge in wastewater treatment worldwide. Several microorganisms have been implemented in complementary treatments to process effluents rich in fats and oils. They have been used either as enzyme extracts of pure cultures obtained by solid state fermentation of agro-industrial or by growing active consortia in the wastewater to be treated. Microbial lipases have distinctive industrial attention because of their stability, broad substrate specificity, high yields, regular supply and rapid growth of microorganisms producing them on inexpensive media. However, under certain conditions, they can catalyse other synthetic reactions like transesterification making them an environmentally relevant class of enzymes, contributing to wastewater treatment improvements and biofuel synthesis. The aim of this study was to select and identify lipase positive isolates *in vitro* suitable for further studies

and applications in wastewater treatment systems. Five bacterial isolates and three fungal isolates tested positive for lipase enzyme production and were related to *Klebsiella*, *Kosakonia*, *Serratia*, *Mucor* and *Trichoderma* genera. Selected isolates belonging to *Serratia* and *Kosakonia* genera had lipase mediated transesterification activity. To date, this is the first report for lipase and transesterification activities in the genus *Kosakonia* and the species *K. oryzae*.

2.1 INTRODUCTION

Wastewater treatment plants (WWTP) have to overcome several obstacles to degrade, adsorb and precipitate inorganic nutrients and organic pollutants present in wastewater prior to its release into the environment. Special interest has been placed on WWTP treating wastewaters with high levels of organic pollutants in the form of fats, oils and greases (FOG) since their removal represents an important challenge in wastewater treatment worldwide. FOG removal usually implies the use of chemical coagulants, flocculants and aeration to remove only a fraction of them (Facchin et al., 2013). A residual fraction remains emulsified as droplets in the wastewater and secondary biological treatment systems are usually employed to finally adjust the effluent before discharging it (Primasari et al., 2011). As these biological systems are not designed to treat FOG, high levels can hinder their performance causing negative environmental impacts. Popular biological treatment systems like constructed wetlands are sensitive to inlet FOG, BOD and COD concentrations and require an efficient primary treatment system preceding them. A constructed wetland is a basin filled with filter material, usually gravel, planted with vegetation tolerant to saturated conditions(Wallace & Knight, 2006)(Wallace & Knight, 2006) (Wallace & Knight, 2006). As wastewater flows through the substrate, several physical, chemical and biological processes like volatilization, filtration, sedimentation, precipitation, adsorption and plant/microbial metabolism remove pollutants (Adrados et al., 2014). Vegetation plays a vital role as it provides surfaces and a suitable environment for microbial growth and filtration (Wallace & Knight, 2006).

Some of the consequences of high levels of FOG in biological wastewater treatment systems involve a decline of the cell-aqueous phase transfer rates, filamentous microorganism blooms, development of unpleasant odours and are frequently associated with a decrease in the general efficiency of WWTPs (Cammarota & Freire, 2006). Due to the low efficiency of physicochemical wastewater treatment systems to remove FOG and the grave consequences they entail to biological treatment systems or natural ecosystems, several techniques involving microorganisms have been implemented in complementary treatments to process wastewater rich in fats and oils. They have been used either as enzyme extracts of pure cultures obtained by solid state fermentation of agro-industrial residues (Leal et al., 2002) or by growing active consortia in the wastewater to be treated. An successful application example is the study of Fadile and

collaborators (2011) who selected and optimized a bacterial consortium treating synthetic and restaurant wastewater with high content of fats and oils. The implementation of this consortium was able to reduce the COD and the lipid content by 83 and 81% respectively.

Fungal consortia have also been stablished for high oil content wastewater. Djelal and Amrane (2013) tested a fungal consortium consisting of *Aspergillus niger, Mucor hiemalis* and *Galactomyces geotrichum* on dairy wastewater achieving a successful reduction on the COD values for the effluent at the industrial biological tank. Mixed bacterial and fungal consortia have also been proposed to treat complex oily wastewaters like Palm Oil Mill Effluents (POME). Safitri and collaborators (2014) implemented a mixed consortium consisting of *Trichoderma viride, Phanerochaete chrysosporium* and *Bacillus* sp. to treat POME with a successful reduction in Chemical oxygen demand (COD), Biological oxygen demand (BOD) and Total Suspended Solids (TSS).

Microbial lipases have distinctive industrial attention because of their stability, broad substrate specificity, high yields, regular supply and rapid growth of microorganisms producing them on inexpensive media (Hasan *et al.*, 2006). Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) can catalyse the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface (Gupta et al., 2004). However, under certain conditions, they can catalyse other synthetic reactions like transesterification (Escobar-Niño et al., 2014), making them an environmentally relevant class of enzymes, contributing to wastewater treatment improvements and biofuel synthesis. About 2 % of the vast microbial biodiversity has been explored for enzyme production, hence justifying the quest for new lipases with improved properties (Kanmani *et al.*, 2015).

The horizontal sub-surface flow constructed wetland (HSFCW) in Compañía Nacional de Chocolates (CNC) has been experiencing a progressive clogging process. In order to maintain the designed operational parameters, part of the wetland (the first 15 meters within the inlet) has to be removed and washed, causing operational and economic costs. This study aims to select and identify lipase positive isolates *in vitro* suitable for further studies and potential applications in this wastewater treatment plant.

2.2 MATERIALS AND METHODS

2.2.1 Microbial isolates origin

The Twenty-two bacterial isolates characterized in Chapter 1 (Table 1-2) and five additional fungal isolates obtained from the wastewater treatment plant were used for this study. Fungal isolates were isolated from serial dilutions of raw wastewater from the Imhoff tank, plated on PDAw culture media respectively. PDAw culture medium was elaborated from PDA culture medium (Difco Labs) as instructed by the manufacturer but replacing the distilled water by autoclaved and filtered wastewater obtained from the Imhoff tank exit (S-IMH). For long-term preservation, all bacterial isolates were stored in LB medium in 20% glycerol at -20°C and -80°C. Fungal isolates were preserved in sterile distilled water at 4ºC.

2.2.2 Detection of lipase positive isolates

Pure isolates were cultured at 30ºC on Tween 20 agar plates described by El-Betsawy and collaborators (2005), to detect the presence of lipase through the formation of a white precipitate halo around the colony. The medium contained Peptone 1%, NaCl 0.5%, CaCl2 0.01%, Agar 2% and Tween 20 1%, isolates were incubated at 30ºC for up to 5 days.

2.2.3 Lipase quantification

Lipase positive isolates were cultured in a lipase induction liquid culture media containing 1% peptone, 1% yeast extract, 0.5% glucose and 1% olive oil that was autoclaved separately and mixed when cooled (Prasad, 2013). Isolates were incubated for 48h at 30ºC and 150rpm and 1.5 mL of the culture were centrifuged at 6000g for 30 minutes at 4ºC. Two hundred microliters of the supernatant was used for the reaction, adding 250µL of phosphate buffer 50mM (pH 7) and 50µL of p-nitrophenyl palmitate 2.5mM. The reaction was incubated at 30ºC for 30 minutes and stopped by placing the tubes in ice and adding 100µL of KOH 0.5M. The inoculum for each isolate was standardized to OD_{600}=1 and each isolate had its own negative control reaction (without substrate) and a general culture medium negative control without inoculum. Each microbial isolate had two

repetitions for the reaction and absorbance measurements were made in triplicate on a Multiskan Plus Model 355 Microplate Reader (Thermo Labsystems, Finland). Calculations for enzyme units per millilitre of supernatant were made according to Formula 1 (Sigma Aldrich).

$$U/mL = \frac{(Am - Ab)(Vf)(Fd)}{(0.018)(T)(Ve)}$$

Formula 1. Calculation for enzyme units per milliliter of supernatant for absorbance of the product p-nitrophenol at 405nm. Am sample absorbance; (Ab) blank absorbance; (Vf) volume for the final reaction in milliliters; (Fd) dilution factor; (0.018) micromolar extinction coefficient of p-nitrophenol at 405nm; (T) total time for the reaction (Ve) volume in milliliters of supernatant used for the reaction (Sigma Aldrich).

2.2.4 Detection of transesterification activity

Transesterification activity test was done according to the method described by Escobar-Niño and collaborators (2014) with some modifications. One millilitre of the supernatant of the culture used for the lipase quantification assay was stored at -80ºC for at least 24h and lyophilized in a FreeZone 2.5 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO, USA). The lyophilized supernantant was mixed with 1mL of p-nitrophenyl palmitate dissolved in n-hexane and 60µL of absolute ethanol. The reaction mix was incubated at 200rpm and 30ºC for 16h and was left still for the enzyme to decant for 10 minutes. 25µL of the reaction was mixed with 1mL of NaOH 0.05M. Each microbial isolate had two repetitions for the reaction and the absorbance was measured in triplicate on a Multiskan Plus Model 355 Microplate Reader (Thermo Labsystems, Finland). Each isolate had its own negative control reaction (without absolute ethanol), a general culture medium negative control reaction without inoculum and a general negative control reaction of p-nitrophenyl palmitate in n-hexane and absolute ethanol. Isolates with absorbance measurements above the ones obtained by the negative controls were considered positive for transesterification activity mediated by lipases.

2.2.5 Fungal DNA extraction and PCR amplifications

DNA from fungal isolates was obtained through CTAB fenol-chloroform method as described by Perea and collaborators (2003). To amplify the ITS region for fungal

identification the primers ITS1 (5´TCCGTAGG TGAACCTTGCGG 3´) and ITS4 (5´TCCTCCGCTTATTGATATGC 3´) were used as described by White and collaborators (1990)

2.2.6 DNA sequence and phylogenetic analysis

The fungal sequences obtained were edited using ChromasPro 1.34 software (http://www.technelysium.com.au/ChromasPro.html). To determine the phylogenetic affiliation of the fungal isolates, similarity searches were executed using the BLAST program and a phylogenetic analysis via the Neighbor-Joining method (Saitou & Nei, 1987) with 1000 bootstrap replicates using MEGA (6.1) (Tamura et al., 2007) with reference sequences obtained from the NCBI (Altschul et al., 1997).

2.2.7 Sequence accession numbers

Three fungal ITS rRNA gene sequences were deposited in the GenBank database and received the accession numbers from KT275846 to KT275848.

2.3 RESULTS

2.3.1 Detection of lipase positive isolates

Five of the Twenty-two bacterial isolates tested showed fatty acids precipitate halos in the Tween 20 medium with calcium chloride (Figure 2-1). Three of the five fungal isolates tested were positive for lipase presence in Tween 20 culture medium.

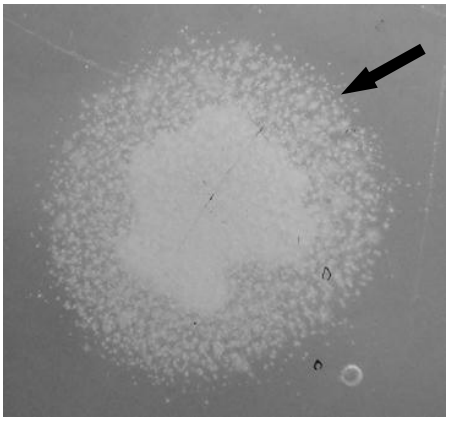


Figure 2-1. Example of lipase positive bacterial isolate (IsolateB006) displaying precipitation halo (Arrow) on Tween 20 culture media with $CaCl_2$. Picture taken from the back of the Petri dish through a stereoscope.

2.3.2 Lipase quantification

Eight positive microbial isolates for lipase activity were quantified. Figure 2-2 shows the values observed in enzyme units per millilitre of enzyme extract and their standard deviations for each sample.

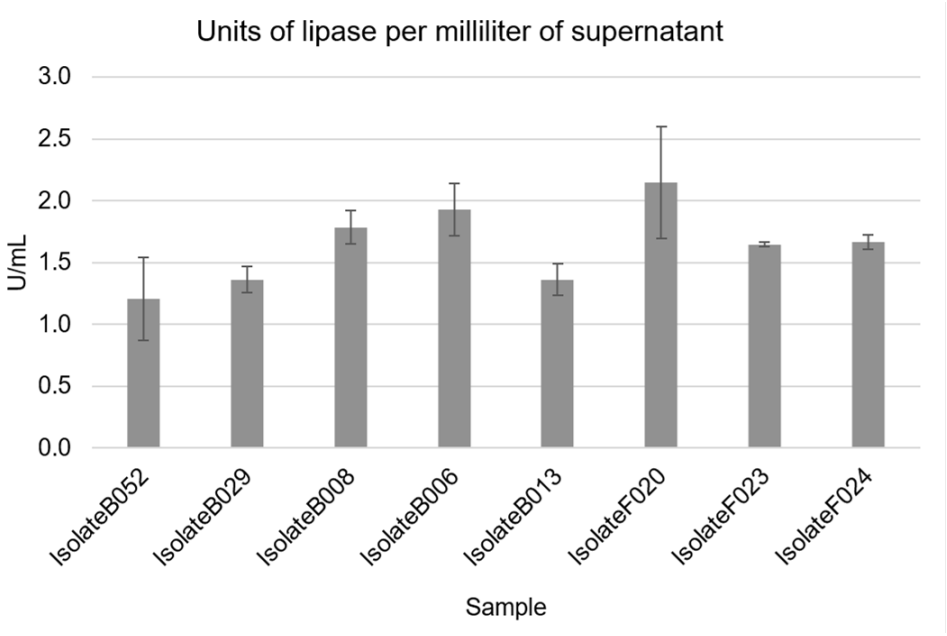


Figure 2-2. Average lipase activity values observed for eight microbial isolates in enzyme units per milliliter of enzyme extract and their standard deviations quantified by release of p-nitrophenol and absorbance at 405nm (Formula 1).

2.3.3 Detection of transesterification activity

IsolateB006 and IsolateB052 were chosen to be tested for transesterification activity because of the high lipase activity of IsolateB006 (Figure 2-2) and for the low pathogenicity of IsolateB052 (Peng *et al.*, 2009). These isolates tested positive with higher absorbance values (Figure 2-3) than the negative controls, determining the presence of transesterification activity mediated by lipases in non-aqueous conditions.

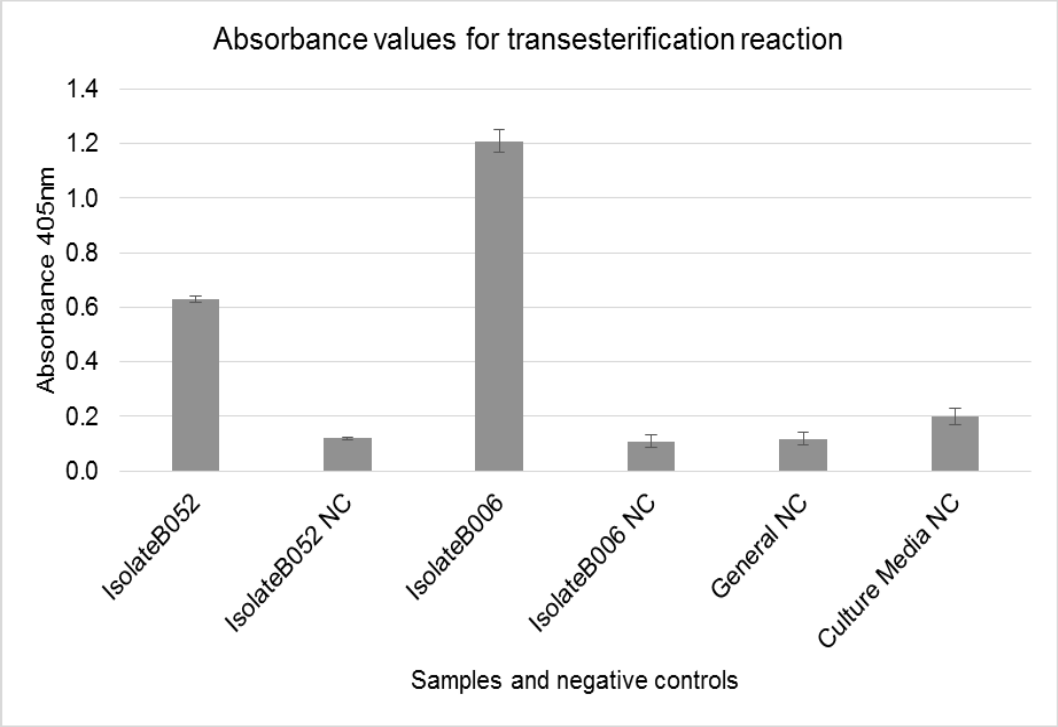


Figure 2-3. Transesterification activity absorbance measurements of the isolates tested and the negative controls (NC) of the reactions quantified by release of *p*-nitrophenol and absorbance at 405nm.

2.3.4 Molecular identification of fungal isolates

Three fungal isolates were sequenced for identification. All of the sequences had high similarity percentages (98–100%) with reported reference sequences in the GenBank database (Table 2-1). Based on the phylogeny of the related sequences from the BLAST search the fungal isolates were identified at the phylum level belonging to *Zygomycota* and *Ascomycota* with two different genera *Mucor* and *Trichoderma* (Figure 2-4 and Appendix F).

Table 2-1. Taxonomic identification of lipase positive fungal isolates

Origin	Sample	Accession #	Closest relative accession # [Bases compared] (Similarity %)	Phylogenetic affiliation	
IMH	IsolateF023-PDAw	KT275847	NR103628.1 [222/233] (95%)	*Mucormycotina*	*Mucor sp.*
	IsolateF024-PDAw	KT275848	NR077179.1 [400/435] (92%)	*Sordariomycetes*	*Trichoderma sp.*
	IsolateF020-PDAw	KT275846	NR077179.1 [468/562] (83%)		*Trichoderma sp.*

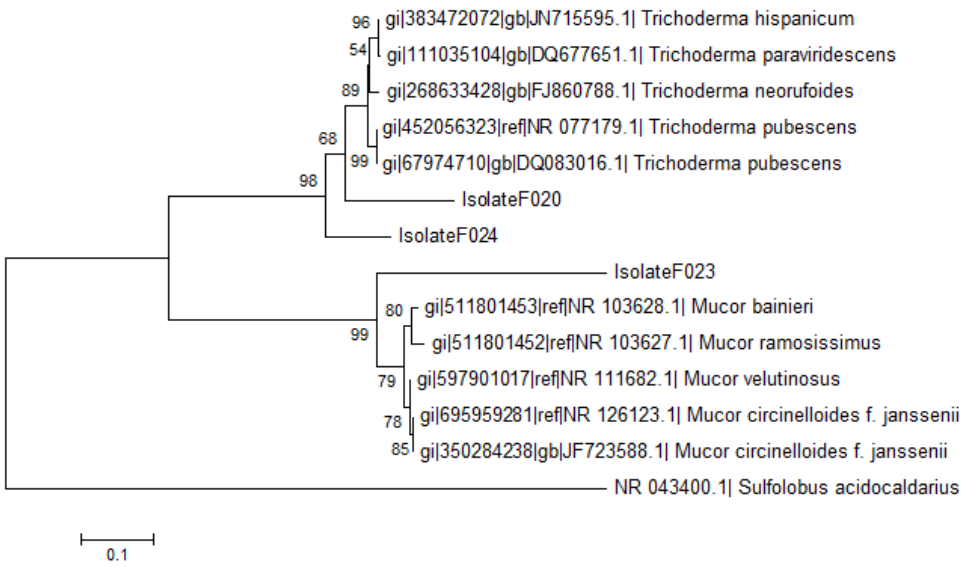


Figure 2-4. ITS rDNA-based dendrogram showing phylogenetic relationships of fungal isolates using the Neighbour-Joining method.

2.4 DISCUSSION

Microbial lipases have distinctive industrial attention because of their stability, broad substrate specificity, high yields and regular supply making them an environmentally relevant class of enzymes (Kapoor & Gupta, 2012). A small percentage of the vast microbial biodiversity has been explored for enzyme production, validating the search for new lipases with improved properties. The aim of this study was to select and identify lipase positive isolates *in vitro* (Figures 2-1 and 2-2) and five bacterial isolates and three fungal isolates were observed to have lipase positive reactions. Bacterial and fungal isolates retrieved from the wastewater treatment system were observed to degrade lipids (Figure 2-2) and are related to the genera *Klebsiella*, *Kosakonia*, *Serratia, Mucor* and *Trichoderma* (Table 1-2 and Table 2-1), with all of them having previous reports for lipase positive activity and implementation in wastewater treatments except the genus *Kosakonia*. To the best of our knowledge, this is the first report for lipase and transesterification activities in the recently created *Kosakonia* genus (Brady *et al.*, 2013).

Kosakonia oryzae (IsolateB052, Table 1-2, Appendix D and E), a diazotrophic bacteria previously known as *Enterobacter oryzae* (Peng et al., 2009) is a freshly described species isolated from rice roots in China and Italy (Meng *et al.*, 2015). This species displayed less lipase activity than the *Klebsiella*, *Serratia* and fungal isolates (Figure 2-2); transesterification activity was also detected implying that besides that function, the enzyme is also tolerant to organic solvents like n-hexane (Figure 2-3). Dashti and collaborators (2015) have recently reported this species in oily Olive-pomace by culture-independent methods (DGGE). Olive-pomace is a waste by-product of olive oil and the presence of this species in that environment may validate its lipase enzyme production, although it has not been detected in WWTPs nor tested in complementary wastewater treatments. This species is reported as a facultative anaerobe (Peng *et al*., 2009) and was recovered from culture media supplemented with wastewater form the Imhoff tank exit (S-IMH). This implies that the isolate can possibly tolerate the chemical conditions of the S-IMH including the small amount of dissolved oxygen (DO) present at this treatment system (Table 1-1). It could also be compatible with the HSFCW due to its reported close relationship with plant roots of rice and beans (Peng *et al*., 2009) (Meng *et al*., 2015) (Kandjimi *et al.*, 2015).

Serratia sp. (Isolate006) showed the highest lipase and transesterification activity from the bacterial isolates (Figures 2-2 and 2-3). This species has been previously characterized for its extracellular lipase enzyme production with very good results by Abdou (2003) and Prasad (2013). Zhao and collaborators (2008) proved that the lipase enzyme secreted by this species was very stable in organic solvents providing it with unique properties for organic synthesis including enantiomeric resolution. Recently, Li and collaborators (2012) made the first report for the transesterification activity of the *S. marcescens* lipase for biofuel production and demonstrated how it successfully catalysed the transformation of waste grease into biodiesel when cloned and expressed in recombinant *E. coli* cells. This species has also been used to effectively treat wastewater with high levels of fats, oils and grease (FOG) by Prasad and Manjunath (2011) who arranged a consortium consisting of *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens, S. marcescens* and *Staphylococcus aureus*.

Klebsiella genus is a well-recognized lipase producer. This genus has been detected in several FOG and hydrocarbon contaminated sites and has been previously implemented in bioremediation applications (Chamkha *et al.*, 2011) (Eman *et al.*, 2012) (Rocha *et al.*, 2013) (Alkhatib *et al.*, 2013) (Zafra et al., 2014) (Allamin *et al.*, 2014). The isolates of this genus (IsolateB029, B008 and B013) had variable lipase production with the p-nitrophenyl palmitate reaction (Figure 2-2). This is consistent with the results obtained by Rodrigues and collaborators (2009) where different isolates of the same species (*Klebsiella pneumoniae*) had variable degradation efficiencies for the same compound. A factor that may enhance their hydrocarbon or FOG degradation is that this genus usually secretes biosurfactants that help increase the surface area of hydrophobic substrates making them more bioavailable (Ron & Rosenberg, 2002) (Lee *et al.*, 2008) (Jain *et al.*, 2013) (Jamal *et al.*, 2014). *Serratia* sp. also produces several types of biosurfactants known as Serrawettins (Matsuyama *et al.*, 2011).

Mucor sp. (IsolateF023) and *Trichoderma* sp. (IsolateF020 and F024) isolates (Table 2-1 and Figure 2-4) displayed greater lipase activities than most bacterial isolates (Figure 2-2) with IsolateF020 being the highest producer. These genera are frequently documented for their lipase production and their ability to degrade hydrocarbons (Suseela *et al.*, 2014). *Mucor* sp. is able to degrade motor oil (Szewczyk & Długoński, 2009) and its lipase has confirmed transesterification activity (Carvalho *et al.*, 2015). *Trichoderma* sp. has the

ability to degrade wildfire-generated toxic hydrocarbons (Andreolli *et al.*, 2015) and heavy crude oil (Hamzah *et al.*, 2012). Fungal isolates can provide additional beneficial properties for wastewater treatment besides lipase production since they can excrete other enzymes that help reduce the chemical and biological oxygen demand (COD and BOD) in the effluent (See Chapter 1) like laccases and peroxidases (Wang *et al.*, 2015) (Murugesan *et al.*, 2014) (Marchut-Mikolajczyk *et al.*, 2015).

The bacterial and fungal isolates tested in this study displayed lipase activity by qualitative and quantitative methods degrading Tween 20 on agar plates and p-nitrophenyl palmitate potentially making them useful for future bioremediation strategies in this WWTP. These isolates can provide a different alternative to contribute to solve the clogging phenomena presented at the HSFCW. Most of them have been previously reported as lipase producing microorganisms and have been tested alone or in mixed cultures in wastewater treatment systems to treat wastewater high in FOG content successfully. To date, this is the first report for lipase and transesterification activities in the genus *Kosakonia* and the species *K. oryzae*.

2.5 ACKNOWLEDGEMENTS

This research was funded by the project 201010013022 of the Universidad Nacional de Colombia and the 201010012909 agreement with Compañía Nacional de chocolates S.A.S. According to the Colombian legislative framework of the Environmental Ministry of Colombia, this project is subscribed to “Permiso Marco No. 0255” established by the Autoridad Nacional de Licencias Ambientales (ANLA) to the Universidad Nacional de Colombia in March 14, 2014. Special thanks to Juliana Morales Rodriguez, Dr. Mauricio Alejandro Marín Montoya, Dr. Blanca Fabiola Espejo Benavidez and M.SC. Mario Andrés Rodríguez Pineda for all the support provided.

3. Chapter 3

3.1 Conclusions

The general bacterial diversity associated with an industrial wastewater treatment plant in Colombia was examined using complementary culture dependent (agar plating) and culture independent techniques (RISA, TGGE and sequence analysis of the 16S rRNA gene). Spatial and temporal changes were described among the different sections and sampling dates in the WWTP. By the methods employed, no filamentous bacteria were detected suggesting that the clogging phenomena at the HSFCW might involve a nutrient and chemical imbalance supported by the general physico-chemical parameters obtained for the WWTP. The bacterial and fungal isolates tested in this study displayed lipase activity by qualitative and quantitative methods potentially making them useful for immediate and future bioremediation strategies. To date, this is the first report for lipase and transesterification activities in the genus *Kosakonia* and the species *K. oryzae* and the first description of the general structure of microbial communities present in a full scale industrial WWTP in Colombia.

3.2 Recommendations

We recommend the Multi-Locus Sequence Analysis approach to confirm the species of the unresolved isolates.

The TGGE or pyrosequencing may be done with a single copy gene like *rpoB* to obtain a better understanding of the diversity of the WWTP.

The bacterial and fungal isolates more adequate for *in situ* applications are IsolateB052 (*Kosakonia oryzae*) and IsolateF023 (*Mucor* sp.).

Lipase positive isolates should be comprehensively studied to determine the best implementation method for the WWTP like enzyme extracts, bio-stimulation or bio-augmentation with pure or mixed cultures.

A. Appendix A: Diversity indices for the TTGE and RISA analysis.

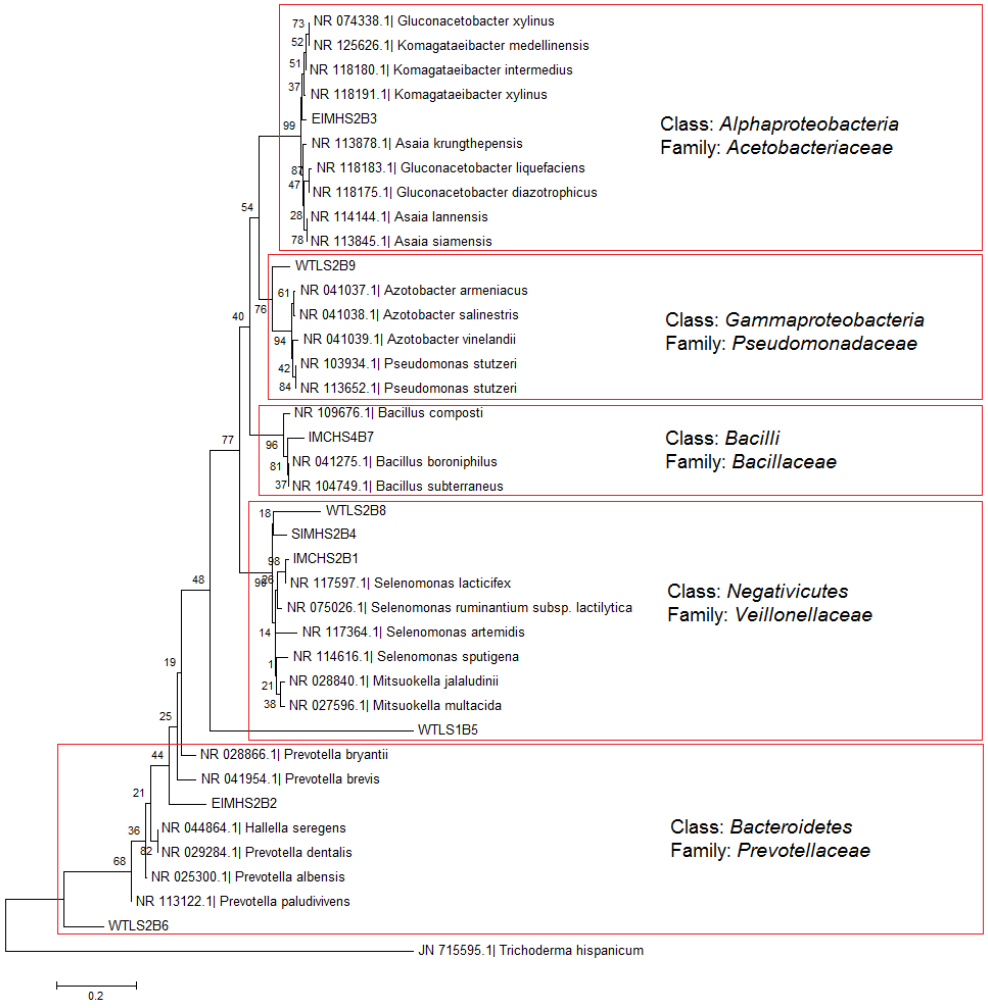
Appendix A: Diversity indices for TTGE and RISA analysis.

TTGE	IMCH				E-IMH			S-IMH				WTL			
	S1	S2	S3	S4	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Taxa (S)	2	4	2	2	6	6	1	3	2	2	1	5	5	2	1
Individuals	12	23	15	27	26	36	10	44	44	48	26	42	64	40	19
Dominance (D)	0.5	0.3	0.8	0.5	0.2	0.2	1.0	0.3	0.5	0.5	1.0	0.2	0.2	0.5	1.0
Simpson (1-D)	0.5	0.7	0.2	0.5	0.8	0.8	0.0	0.7	0.5	0.5	0.0	0.8	0.8	0.5	0.0
Shannon (H)	0.7	1.2	0.4	0.7	1.6	1.5	0.0	1.1	0.7	0.7	0.0	1.6	1.5	0.7	0.0

RISA	IMCH			E-IMH			S-IMH			WTL		
	Total	LB	LBr	Total	LB	LBr	Total	LB	LBr	Total	LB	LBr
Taxa (S)	7	6	7	9	5	8	6	5	8	6	4	8
Individuals	271	184	218	377	134	190	207	171	215	227	127	215
Dominance (D)	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.2	0.3	0.2
Simpson (1-D)	0.8	0.8	0.8	0.9	0.8	0.8	0.7	0.8	0.8	0.8	0.8	0.8
Shannon (H)	1.8	1.7	1.9	2.2	1.6	2.0	1.6	1.6	2.0	1.8	1.8	1.9

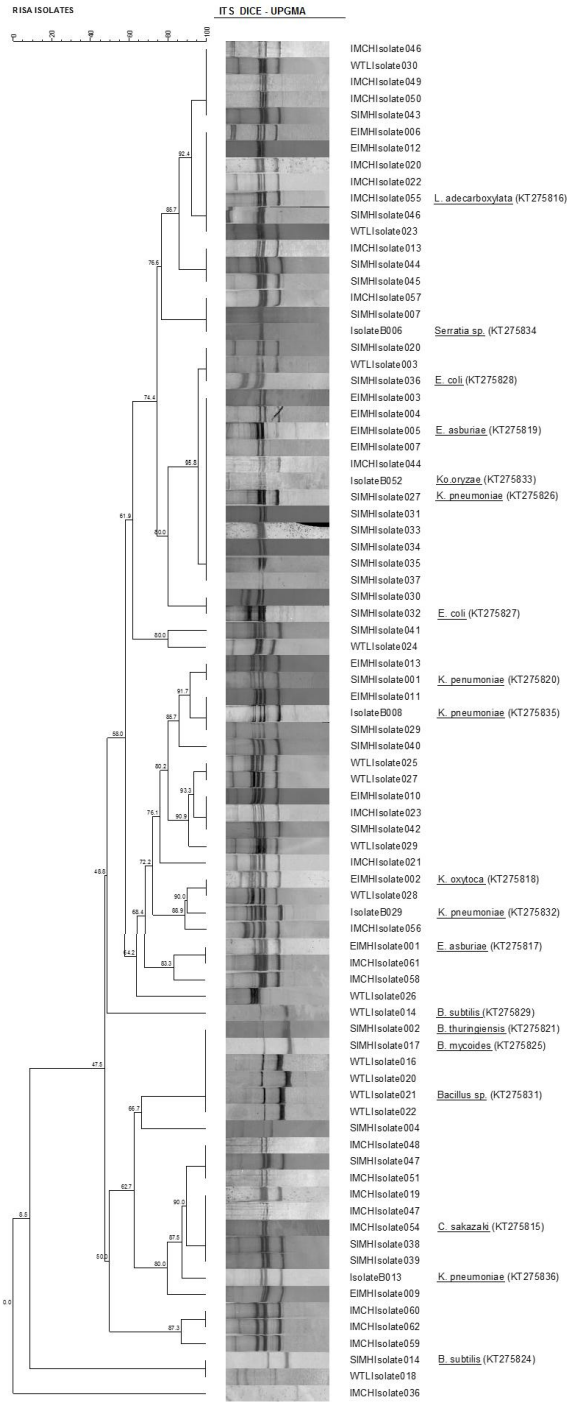
B. Appendix B: Phylogenetic analysis of the TTGE excised bands.

Appendix B: Phylogenetic analysis based on 16S rRNA gene sequences of 9 bands excised from the TGGE of total DNA samples from the WWTPS. The tree was produced by the Neighbor-Joining method using MEGA 6 and rooted with a *Trichoderma hispanicum* ITS sequence.



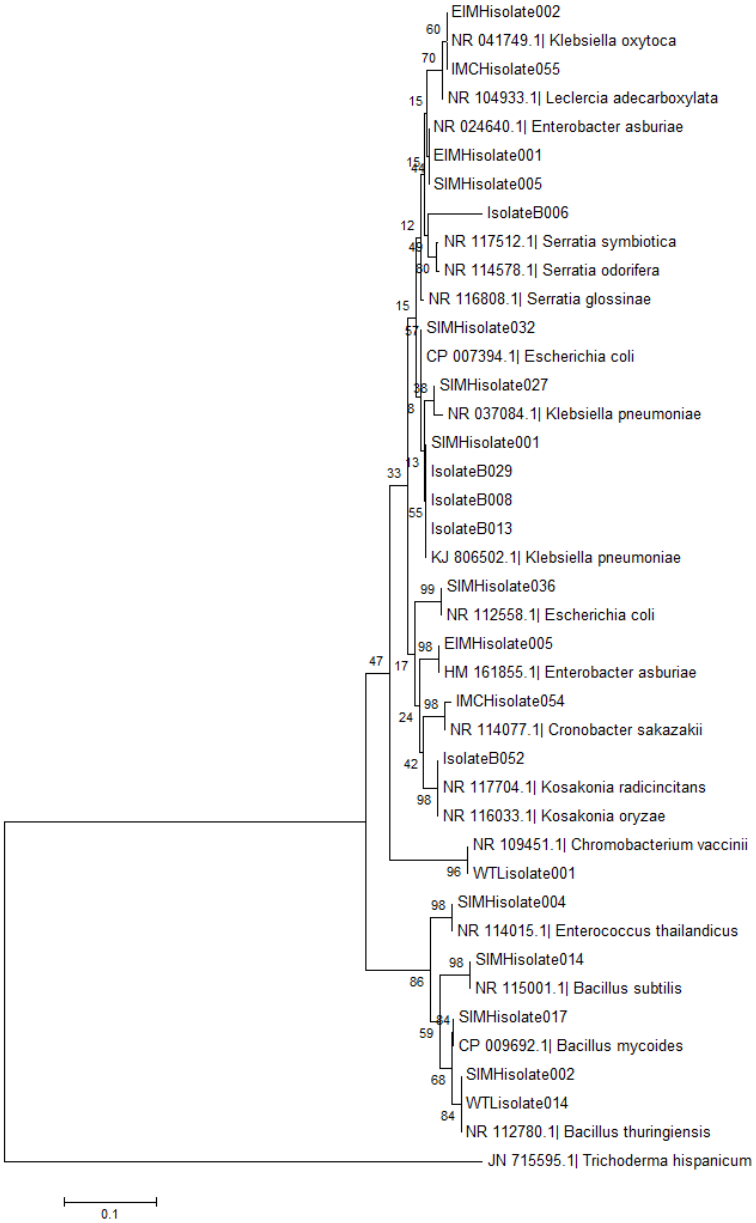
C. Appendix C: RISA analysis of the isolates.

Appendix C: Ribosomal Intergenic Spacer Analysis (RISA) of the 80 isolates obtained from the WWTP.



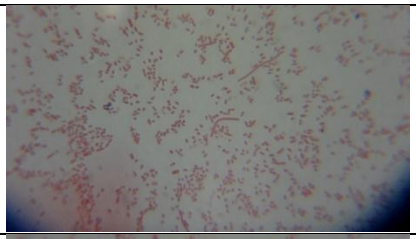
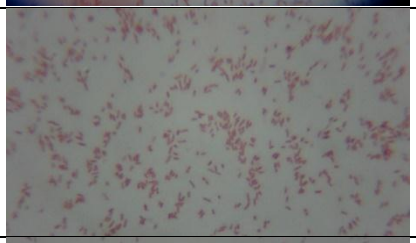
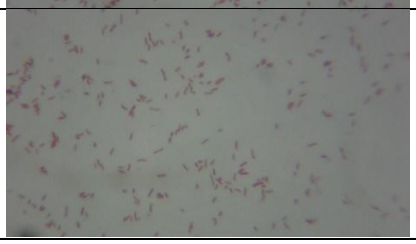
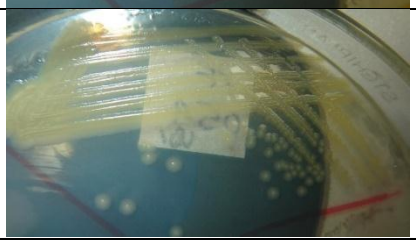
D. Appendix D: Phylogenetic analysis of the isolates

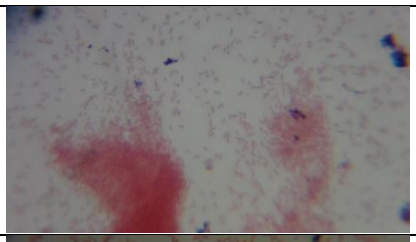
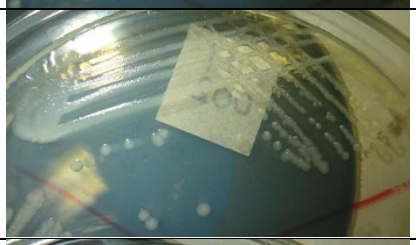
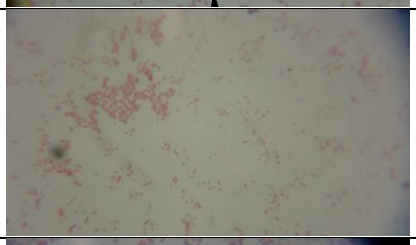
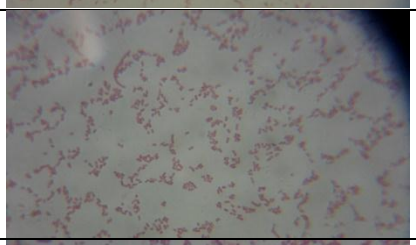
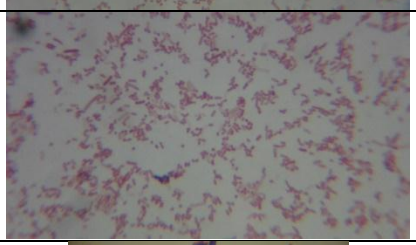
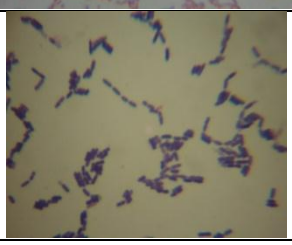
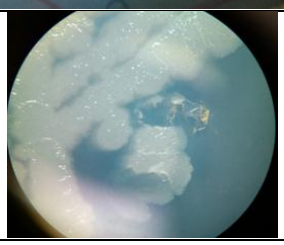
Appendix D: Phylogenetic analysis based on 16S rRNA gene sequences, of 22 isolates from the WWTP. The tree was produced by the Neighbor-Joining method using MEGA 6.

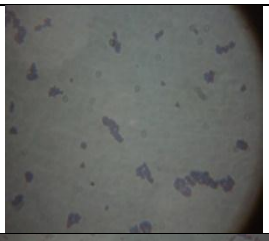
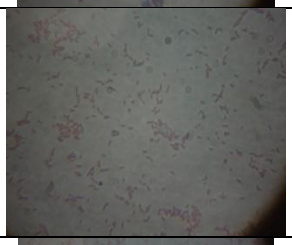
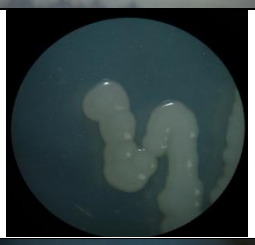
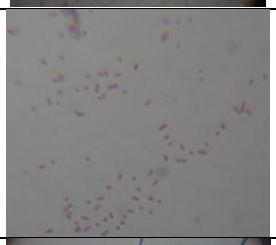
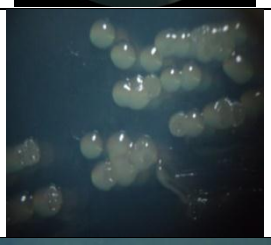
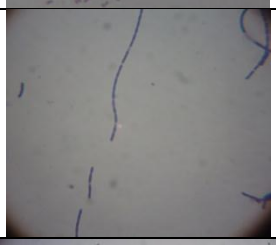
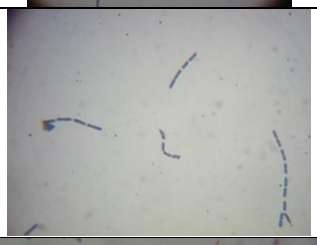
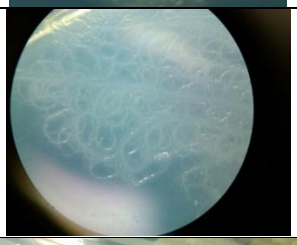
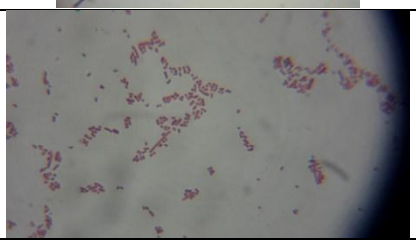


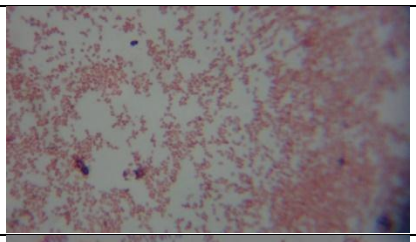
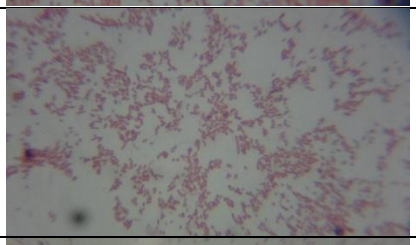
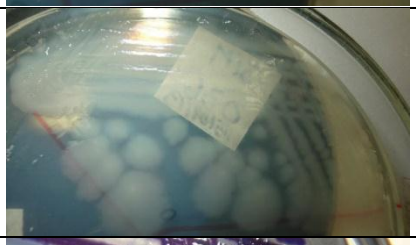
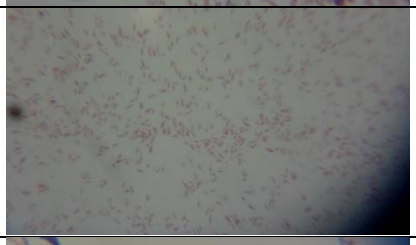
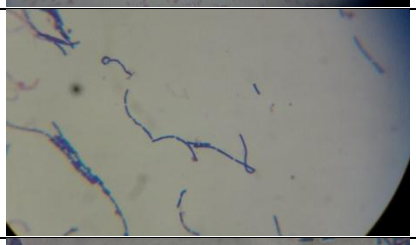
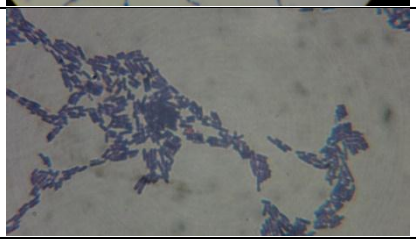
E. Appendix E: Gram stain and colony morphology of the isolates

Appendix E: Gram stain and colony morphology of the bacterial isolates sequenced

Origin	Sample	Gram stain	Colony Morphology	Phylogenetic affiliation
IMCH	IsolateB029-LBw			*K. pneumoniae*
	IsolateB052-LBw			*Ko. oryzae*
	IMCHisolate054-LBw			*Cr. sakazakii*

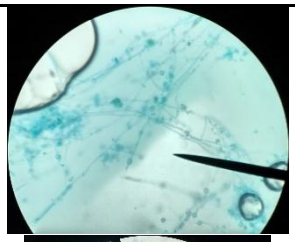
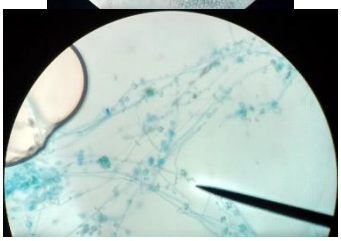
	IMCHisolate055-LBw			*L. adecarboxylata*
EIMH	EIMHisolate001-LB			*E. asburiae*
	EIMHisolate002-LB			*K. oxytoca*
	EIMHisolate005-LBw			*E. asburiae*
	IsolateB008-LBw			*K. pneumoniae*
SIMH	SIMHisolate001-LBw			*K. pneumoniae*
	SIMHisolate002-LBw			*B. thuringiensis*

	SIMHisolate004-LBw			*E. thailandicus*
	SIMHisolate005-LBw			*E. asburiae*
	IsolateB006-LBw			*Serratia sp.*
	IsolateB013-LBw			*K. pneumoniae*
	SIMHisolate014-LBw			*B. subtilis*
	SIMHisolate017-LBw			*B. mycoides*
	SIMHisolate027-LB			*K. pneumoniae*

	SIMHisolate032-LB			*E. coli*
	SIMHisolate036-LBw			*E. coli*
WTL	WTLisolate001-LB			*C. vaccinii*
	WTLisolate014-LB			*B. subtilis*
	WTLisolate021-LBw			*Bacillus sp.*

F. Appendix F: Colony morphology of the fungal isolates

Appendix F: Colony morphology of the fungal isolates sequenced

Origin	Sample		Colony	Phylogenetic affiliation
	IsolateF020-PDAw			*Trichoderma sp.*
IMH	IsolateF023-PDAw			*Mucor sp.*
	IsolateF024-PDAw			*Trichoderma sp.*

G. Appendix G: Average Loading and average Surface Loading Rate calculations for the IMCH and S-IMH sampling sites of the WWTP.

Appendix G: Average Loading and average Surface Loading Rate calculations for the IMCH and S-IMH sampling sites of the WWTP (Table 1-1)

BOD and COD daily average loading for the IMCH sampling site (1mg/L=1g/m^3) when Q=2 L/s (172.8 m^3/day)

BOD= 5699 mg/L = 5699 g/m^3=984787.2 g/d=985 kg/d

COD= 9027.3 mg/L = 9027.3 g/m^3=1559917.4 g/d=1560 kg/d

BOD and COD daily average Surface Loading Rate (SLR=mass per day/surface area) for the S-IMH sampling site(1mg/L=1g/m^3) when Q=2 L/s (172.8 m^3/day) and the surface area of the HSFCW is 5000m^2

BOD=1354.2 mg/L=1354.2 g/m^3=234005.8 g/m^3 d, SLR=46.8 g/m^2 d.

COD=1889.6 mg/L=1889.6 g/m^3=326523 g/m^3 d, SLR=65.3 g/m^2 d.

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