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Bacterial community analysis of an industrial wastewater treatment plant in Colombia with screening for lipid-degrading microorganisms

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ABSTRACT

The operation of wastewater treatment technologies depends on a combination of physical, chemical and biological factors. Microorganisms present in wastewater treatment plants play essential roles in the degradation and removal of organic waste and xenobiotic pollutants. Several microorganisms have been used in complementary treatments to process effluents rich in fats and oils. Microbial lipases have received significant industrial attention because of their stability, broad substrate specificity, high yields, and regular supply, as well as the fact that the microorganisms producing them grow rapidly on inexpensive media. In Colombia, bacterial community studies have focused on populations of cultivable nitrifying, heterotrophic and nitrogen-fixing bacteria present in constructed wetlands. In this study, culture-dependent methods, culture-independent methods (TTGE, RISA) and enzymatic methods were used to estimate bacterial diversity, to monitor temporal and spatial changes in bacterial communities, and to screen microorganisms that presented lipolytic activity. The dominant microorganisms in the Wastewater Treatment Plant (WWTP) examined in this study belonged to the phyla *Firmicutes, Proteobacteria* and *Bacteroidetes*. The enzymatic studies performed indicated that five bacterial isolates and three fungal isolates possessed the ability to degrade lipids; additionally, the *Serratia, Kosakonia* and *Mucor* genera presented lipase-mediated transesterification activity. The implications of these findings in regard to possible applications are discussed later in this paper. Our results indicate that there is a wide diversity of aerobic Gram-negative bacteria inhabiting the different sections of the WWTP, which could indicate its ecological condition, functioning and general efficiency.

1. Introduction

The biological treatment of industrial and domestic wastewater is an important biotechnological application, constituting a multibillion-dollar global industry. When microbial populations are enhanced in wastewater treatment systems, their beneficial activities result in the efficient removal of organic matter, toxic substances, nutrients and pathogens (Wang et al., 2012). Despite the economic and environmental importance of these processes, the understanding of microbial communities present in wastewater treatment plants (WWTPs) remains limited, especially in developing countries. Bacteria are important contributors to the transformation of complex organic compounds in wastewater treatment systems and are essential for the optimal operation and preservation of biological treatment systems (Moura et al., 2009). Changes in the diversity or metabolism of such communities can affect the whole wastewater treatment process (Guo et al., 2014a); as such, analysis of the population structure and function of these communities has become the basis for solving problems with and optimizing existing and new WWTPs (Cui et al., 2012).

The earliest bacterial diversity studies were performed using culture-dependent methods. These methods only permit the study of the cultivable fraction of bacterial communities, thus underestimating the real diversity present in the environment (Moura et al., 2009). In recent years, several culture-independent molecular techniques have been developed, including Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fernandes et al., 2015), Denaturing Gradient and Temporal Temperature Gel Electrophoresis (DGGE/TTGE) (Lienen et al., 2014), 16S rRNA gene clone libraries (Silva et al., 2013), fluorescent in situ hybridization (FISH)

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(Muszyński et al., 2015), and Next Generation Sequencing techniques like pyrosequencing (Wang et al., 2012). These provide a more realistic understanding of the diversity of bacterial communities present in WWTPs.

Special interest has been placed on WWTPs that treat wastewaters with high levels of organic pollutants in the form of fats, oils and greases (FOG), since the removal of these substances represents an important wastewater treatment challenge at a global level. The use of chemical coagulants, flocculants and aeration usually removes just a fraction of FOG (Facchin et al., 2013), while the residual fraction remains emulsified as droplets in the wastewater. These droplets can continue on to secondary biological treatment systems, which are usually employed to adjust the final effluent before discharging it (Primasari et al., 2011). Biological systems like constructed wetlands are not designed to treat FOG and high levels of these pollutants can hinder their performance, causing negative environmental impacts. Currently, over 95% of these wetlands are subsurface flow wetlands, which are sensitive to inlet FOG, Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) concentrations and must be preceded by an efficient primary treatment system (Wallace and Knight, 2006).

Several techniques involving microorganisms have been implemented in complementary treatment systems to process wastewater with high FOG content. These techniques have used either enzyme extracts of pure cultures obtained by solid state fermentation of agro-industrial residues (Leal et al., 2002) or active consortia grown in the wastewater to be treated (Fadile et al., 2011). Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) can catalyze 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 catalyze other synthetic reactions like transesterification (Escobar-Niño et al., 2014), making them an environmentally relevant class of enzymes that contribute to both wastewater treatment and biofuel synthesis.

To date, few peer-reviewed articles have focused on bacterial communities present in wastewaters from chocolate-producing industries, and only one has investigated the development of microbial communities in the anode chamber of a microbial fuel cell. In that study, Patil and collaborators (Patil et al., 2009) analyzed the 16S rRNA genes of one such community and reported that it was dominated by clones of α-, β- and γ- *Proteobacteria, Planctomycetes, Firmicutes, Nitrospora, Spirochaetes,* and *Bacteroides*. In Colombia, studies of wastewater bacterial communities have focused on cultivable populations (Díaz et al., 2010; Pérez-Peláez et al., 2011) and there is little information available about the bacterial communities that inhabit these ecosystems.

The horizontal sub-surface flow constructed wetland (HSFCW) from the Compañía Nacional de Chocolates SAS chocolate factory has been experiencing a progressive clogging process. In order to maintain the designed operational parameters, part of the wetland (the first 15 m within the inlet) has to be removed and washed, which entails operational and economic costs. This study used both culture-dependent and culture-independent analysis as complementary methods to estimate bacterial diversity, monitor temporal and spatial changes in bacterial communities throughout the different sections of this WWTP, and select and identify lipase-positive isolates in vitro that merit further study and have potential applications in this WWTP.

2. Materials and methods

2.1. Sampling and WWTP data

Water samples were collected from a WWTP located in Rionegro (Antioquia, Colombia) that treats domestic and industrial wastewater released from the chocolate factory of the Compañía Nacional de Chocolates SAS (1200 employees). The WWTP handles 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 and 1560 kg/d, respectively. It consists of four different consecutive sections. Two main channels with metallic screening grids conduct the industrial wastewater and the domestic wastewater to the homogenization tank and the domestic wastewater pre-treatment system, respectively. These two effluents mix at the Imhoff tank entrance and are treated in the Imhoff tank (70m^3). They then pass through the HSFCW for secondary treatment before being discharged. The HSFCW consists of four basins with a total area of 6000 m^2 (0.5 ha).

Four wastewater samples (300 mL) were collected from four different sites of the WWTP over a one-and-a-half-year period June (S1), September (S2), December (S3) 2013 and December 2014 (S4). These sites were: the Industrial Main Channel (IMCH), the Imhoff influent (E-IMH), the Imhoff effluent (S-IMH), and the HSFC wetland effluent (WTL). All water samples were transported on ice to the Molecular and Cellular Biology Laboratory of the Universidad Nacional de Colombia 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). The following influent characteristics were determined by a certified laboratory, in accordance with Standard Methods (Rand et al., 1976): COD, BOD, FOG content, total nitrogen (N), total phosphorus (P), total organic carbon (TOC), total solids (TS) and total suspended solids (TSS).

2.2. Microbiological analysis

One milliliter of each wastewater sample was serially diluted up to 10^{-3} and 200 μL of sample were plated on commercial LB agar (Difco labs) in duplicate, with (LBw) and without (LB) a wastewater supplement. The LBw culture medium was prepared according to the manufacturer's instructions, but with the distilled water replaced by filtered wastewater obtained from the Imhoff tank effluent (S-IMH). The plates were incubated under aerobic conditions at 25 ± 2 °C and growth was observed after 24 h and 72 h of incubation. Bacterial colonies with different morphological characteristics (colony, size, elevation and pigmentation) were isolated from different media plates and sub-cultured to obtain pure bacterial cultures. For further culture maintenance, only LB was used. On the third day, the entire cultivable fraction was retrieved and stored in LB broth at −20 °C until DNA extraction. The remaining wastewater sample was centrifuged and stored at −20 °C 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 GelCompar software (Applied Biosystems, Belgium) (García et al., 2016). RISA-PAGE was performed in a Mini-PROTEAN Tetra cell electrophoresis unit with 7% polyacrylamide gels (acrylamide/bis-acrylamide 29:1) for 100 min at 130 v. For long-term preservation, all isolates were stored in LB medium with 20% glycerol, at −20 °C and −80 °C. A "*t*-test" was performed on the colony forming unit data for both culture media, in order to analyze whether there were significant differences in the CFU/mL of the two media, based on the statistical hypothesis that no significant differences existed between them.

Fungal isolates were obtained from serial dilutions of raw wastewater from the Imhoff tank. These were plated on PDAw culture medium, which was elaborated from PDA culture medium (Difco Labs) according to the manufacturer's instructions, but with the distilled water replaced by autoclaved and filtered wastewater obtained from the Imhoff tank exit (S-IMH). For long-term preser-