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Amplified Ribosomal DNA Restriction Analysis as a Tool to Characterize Microbial Community Structure of Activated Sludge of Common Effluent Treatment Plant

M. Shah*

Industrial Waste Water Research Laboratory Division of Applied & Environmental Microbiology Enviro Technology Limited Gujarat, India
*Corresponding author: shahmp@uniphos.com

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Abstract ARDRA (Amplified ribosomal DNA restriction analysis) is a simple method based on restriction endonuclease digestion of the amplified bacterial 16S rDNA. In this study we have evaluated the suitability of this method to detect differences in activated sludge bacterial communities fed on domestic or industrial wastewater, and subject to different operational conditions. The ability of ARDRA to detect these differences has been tested in modified Ludzack-Ettinger (MLE) configurations. Samples from three activated sludge wastewater treatment plants (WWTPs) with the MLE configuration were collected for both oxic and anoxic reactors, and ARDRA patterns using double enzyme digestions AluI+MspI were obtained. A matrix of Dice similarity coefficients was calculated and used to compare these restriction patterns. Differences in the community structure due to influent characteristics and temperature could be observed, but not between the oxic and anoxic reactors of each of the three MLE configurations. Other possible applications of ARDRA for detecting and monitoring changes in activated sludge systems are also discussed.

Keywords: *Amplified ribosomal DNA restriction analysis, wastewater treatment plants, 16S ribosomal DNA · Activated sludge*

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1. Introduction

Nowadays, biological wastewater treatment plants (WWTPs) are the most common biotechnological application in the world [1]. From the various alternatives of biological treatment systems that exist, conventional activated sludge (CAS) bioreactors are by far the most commonly used secondary treatment technology [2]. Despite of periodic improvements to the technology since its invention almost a century ago [2] and its ubiquitous global application, little is known about the underlying factors controlling the complex dynamics of the microbial populations interacting in the bioreactors and how those dynamic interactions affect the system's functional stability [3]. Until recently, a major obstacle was that the science behind most of those technology improvements was almost entirely empirical rather than theoretical [2,4]. Major changes to the design of CAS systems were done predominantly from an engineering perspective, greatly underestimating the importance of microbial communities as an integral component of these biological treatment systems [2,5]. Thus, many essential aspects regarding the ecology and dynamics of microbial communities within these systems, necessary for a rational improvement of their design and operation, remain unresolved [5]. Recent efforts have focused on improving the treatment process from a bio-ecological perspective, but so far few studies have been able to establish a clear link between the structure and function of microbial communities and the design and operation of the bioreactors [6]. Most of these efforts have failed due to limiting methodology issues. One of these issues is the modeling of full scale WWTP bioreactors based on studies of lab-scale and pilot scale bioreactors [6,8]. These studies have often been misleading and far from mimicking the real conditions observed in full-scale bioreactors, creating a big gap between their theoretical and their practical contributions [9,10]. Another issue is that many studies had focused on analyzing single bioreactors [11,12], neglecting from their analysis the effect that niche-specific factors may play in the structure and function of microbial communities [4,13,14]. The most notorious, and therefore highly scrutinized, of these issues is culture- and traditional-microscopy-based studies. These studies, aimed to elucidate the diversity of microbes in WWTPs [15,16,17,18], proved to be unreliable, irreproducible and created erroneous perceptions of the dominant populations in the bioreactors [19,20,21,22]. They also failed to consider operational and geographical factors on the

composition of the communities [23,24,25,26]. With the development and application of modern culture independent molecular techniques in ecological studies of wastewater treatment systems [20,27,28,29], the capacity of researchers to understand the true dynamics of microbial communities in these ecosystems has greatly been improved [10]. However, de los Reyes [30] explains that advanced molecular studies of microbial communities in WWTPs have led to the emergence of a microbial community ‘‘structure-function’’ paradigm that has not yet been fully clarified. Linking changes in system design and operation with the ecological factors controlling community assembly in the bioreactors will be critical in fully clarifying this ‘‘structure function’’ paradigm and resolving important operational issues, such as: sludge bulking (e.g. [31]), poor biochemical removal (e.g. [32]), and system instability (e.g. [33,34]); ultimately resulting in more stable and predictable systems [4]. Moreover, the study of CAS systems could provide, in turn, a research platform for developing and validating ecological principles that could be used to predict the behavior of microbial communities in other engineered and natural ecosystems [35,36,37]. In this study, another molecular biology technique, the amplified ribosomal DNA restriction analysis (ARDRA), is applied to activated sludge samples. Even faster than hybridization and probing, ARDRA has been used in the analysis of mixed bacterial populations from different environments [38,39,40]. Although ARDRA gives little or no information about the type of microorganisms present in the sample, it can be used for a quick assessment of genotypic changes in the community over time, or to compare communities subject to different environmental conditions.

2. Materials and Methods

2.1. Description of the WWTP Configurations

The activated sludge samples were collected from the oxic and the anoxic reactors of three WWTPs. D1 has an anoxic tank (D1-an) of 350 m^3, and an oxic tank (D1-ox) of 1500 m^3, arranged in a modified Ludzack-Ettinger (MLE) configuration. It treats about 1500 m3/day of domestic wastewater. The WWTP (D2) was built to operate as an Orbal but, due to the low influent load, only the two inner channels are in use. Of these two, the outer one (4500 m^3, designated as D2-an) operates under anoxic conditions, while the other (3500 m^3, designated as D2-ox) is under oxic conditions. Internal recycling from the inner to the outer channel allows the system to work in MLE configuration. The WWTP treats approximately 9000 m3/day of domestic wastewater. Finally, the third WWTP studied is a pilot plant that treats industrial wastewater from a food-processing factory. It has an oxic reactor of 3.5 l (designated as IN-ox), and an anoxic reactor of 2.2 l (designated as IN-an) in an MLE configuration. The influent flow to the pilot plant is 0.83 l/day.

2.2. Sampling and Analytical Determinations

Samples of the inlet and the outlet of each reactor were collected in order to determine influent characteristics and reactor efficiencies. The conservation of the samples, their processing and all analytical measurements were carried out according to APHA [41]. Activated sludge samples of 50 ml for DNA extraction were collected in sterile Falcon tubes and frozen at –20°C until processing.

2.3. 16S rDNA Amplification

DNA extraction was carried out in Nalgene sterile tubes by the phenol-chloroform method described by Moore [42]. DNA was purified with Bio-Spin chromatography columns (Bio-Rad, Hercules, CA, USA) to eliminate proteins and nucleotides. PCR amplification of 16S rDNA, using the primer set of fD1 and rP1, was performed as described previously [43]. PCR products were purified using the QIA quick PCR Purification Kit (Quiagen, Valencia, CA, USA).

2.4. rDNA Restriction Fragments Separation by Electrophoresis

Double restriction endonuclease digestions were performed for every sample with *Alu*I+*Msp*I (Promega, Madison, WI, USA) as described previously [38]. Restriction enzymes were chosen on the basis of their high average number of restriction sites per taxon [40]. Separation of digested products in polyacrilamide gels were performed as described elsewhere [38]. The gel was digitallized using a scanner AGFA Arcus II and the images were contrasted using the NIH Image Program 1.59

2.5 Data Analysis

The patterns of each sample were compared by identifying, from different samples, fragments of identical size in the same digestion. Pairwise comparations of the band patterns were manually performed, and a presence/absence matrix was constructed. In this way, the Dice similarity coefficient [44] was obtained for every pair of samples, enabling us to generate a similarity dendrogram. The data were computed by using the SPSS program version for Macintosh 4.0.

3. Results and Discussion

3.1. Influent Characteristics, Operational Parameters and Removal Efficiencies

Table 1 summarizes the principal influent characteristics and operational parameters for each of the three WWTPs. The nitrite and nitrate concentrations in the influents were always under 1 ppm N. The chemical oxygen demand (COD) removal efficiencies of all WWTPs were over 85%. The nitrification efficiencies were 83% and 94% for reactors D1 and IN, respectively, while reactor D2 showed complete nitrification. Reactors D1 and D2 had denitrification efficiencies of 16% and 64%, respectively. Reactor IN also achieved complete denitrification.

3.2. Differences between Industrial and Domestic WWTP Communities

The restriction patterns obtained by electrophoresis are shown in Figure 1. The differences between restriction

patterns in all the communities subject to domestic wastewater are smaller than those between domestic wastewater WWTPs and those treating the industrial wastewater, as reflected in the dendrogram and in the Dice similarity coefficient matrix shown in Figure 2. Two main factors can explain these results. First, industrial WWTP communities are subject to much higher COD, ammonia and organic nitrogen inputs than the domestic WWTP communities, as shown in Table 1. Besides, the temperature was higher in the industrial wastewater reactors (38°C) than in all the domestic wastewater reactors, where it remained between 10 and 12°C (Table 1).

Table 1. Principal characteristics and operational parameters of the treatment plants studied

Operational parameter	Reactor		
	D1	D2	D3
sCOD(mg/l)	144	157	8539
pCOD(mg/l)	96	149	1138
$N\text{-}NH_4^+$(mg/l)	11	18	655
sNorg(mg/1N)	2	3	327
pNorg(mg/1N)	<1	8	-
$MLSS_{anoxic\ reactor}$(mg/l)	1600	3048	7080
$MLSS_{oxic\ reactor}$(mg/l)	1600	4082	4080
$\%VSS_{anoxic\ reactor}$	50%	47%	49%
$\%VSS_{oxic\ reactor}$	49%	48%	71%
$\theta_{anoxic\ reactor}$(h)	20.3	5.6	63.4
$\theta_{oxic\ reactor}$	15.7	24	100.8
θ_c(day)	>30	22	>30
External recycle(%)	75	290	150
Internal recycle(%)	200	290	1000
$T_{mixed\ liquor}$(°C)	10	12	38

s: soluble, p: particulate, θ: sludge residence time
COD, chemical oxygen demand
MLSS, mixed liquor suspended solids
VSS, volatile suspended solids

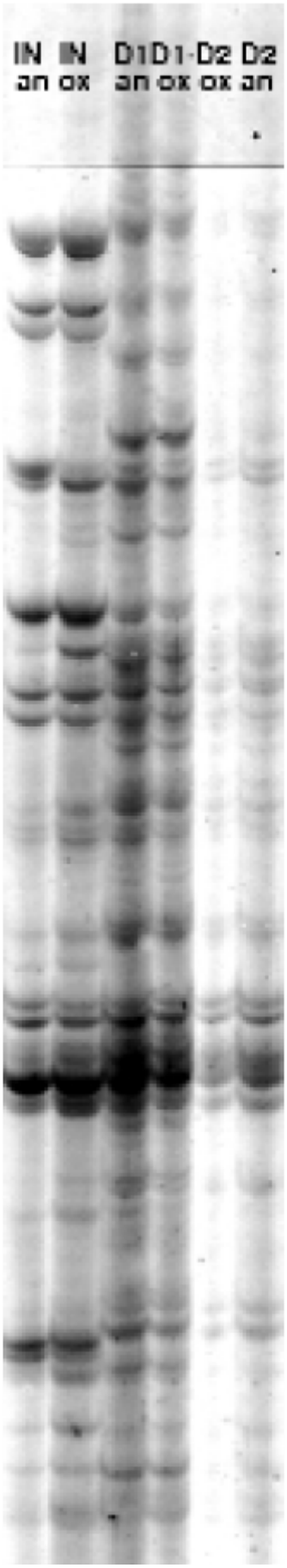
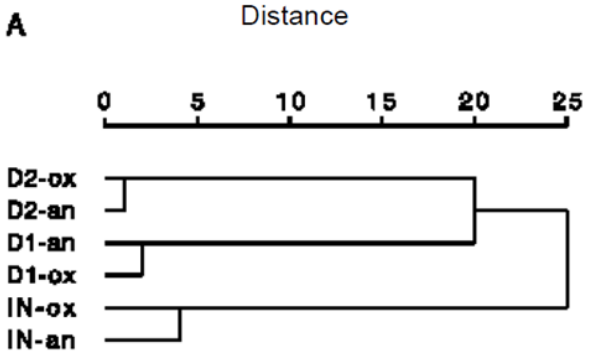


Figure 1. Polyacrilamide gel showing *Alu*I+*Msp*I digestions of the whole eubacterial communities from the six reactors studied: D1-anoxic reactor; D1-oxic reactor; D2-anoxic reactor; D2-oxic reactor; IN-an, industrial wastewater anoxic reactor; IN-ox, industrial wastewater oxic reactor

3.3. Differences between the Oxic and Anoxic Reactors in MLE Configurations



B

Variable	D1-an	D1-ox	D2-ox	D2-an	IN-ox
D1-ox	.9778				
D2-ox	.7458	.7586			
D2-an	.7458	.7586	.9971		
IN-ox	.8766	.8909	.7246	.7246	
IN-an	.7170	.7908	.7273	.7273	.9524

Figure 2. Differences in restriction patterns. (A) Dendrogram of eubacterial 16S rDNA-ARDRA similarities obtained by digestion with AluI+MspI. (B) Dice similarity coefficient matrix. Reactors D1-an, D1-ox, D2-an, D2-ox, IN-an and IN-ox, as in Figure 1

No significant differences have been observed between the restriction patterns of oxic and anoxic reactors of all the systems studied, as shown in the dendrogram (Figure 2A). The absence of differences between the patterns does not ensure that the composition of the communities is exactly the same. However, significative composition changes in the community should be detected with the restriction enzymes used [11]. Previous works demonstrated that double restriction endonuclease digestions are sensitive enough to detect important composition changes in the community [38,40]. The absence of differences between the patterns of the oxic and the anoxic reactors leads to the conclusion that there were probably no significant changes between the microbial communities of the two reactors. Similar conclusions were drawn by Ehlers and Cloete [45] by using protein fingerprints to evaluate the differences between the microbial community structures among P-removing, non-P-removing and N-removing systems. Thus, the similarity of endonuclease restriction patterns among the samples agrees with the high similarity of protein fingerprints in bacterial communities of different activated sludge systems. Given the residence times and the internal recycle values of the systems studied, the generation times of the microorganisms are probably too long to observe significant differences in community composition among the anoxic and oxic reactors. Therefore, the aerobic and anaerobic populations apparently do not have enough time to change while inside the oxic or anoxic reactors, and therefore they merely coexist. Despite the absence of changes in community composition, there are probably differences in the microbial activity developed in the oxic and anoxic reactors. In each of these, only the part of the community able to grow under the conditions found in the reactor is active, whereas the rest is not able to develop activity until it reaches the other reactor. Facultative anaerobic bacteria could be active in both oxic and anoxic reactors. Further work, using specific rRNA targeted probes, will be necessary to determine the metabolic activity of a given group of microorganisms in each of the reactors.

4. Concluding Remarks

ARDRA is able to detect differences between activated sludge communities from industrial and domestic wastewater treatment plants, and these differences could be due to influent composition and temperature. However, differences in the community compositions of the anoxic and oxic reactors of each of the three MLE configurations studied have not been observed. Before this study, ARDRA had only been applied in raw sewage samples to detect the presence of rotaviruses by comparing the pattern of the samples with known viral patterns. ARDRA is a promising first approach in the evaluation of the changes in activated sludge communities of WWTPs caused by modifications in influent composition, temperature and other operational conditions. However, the effects that changes or perturbations have on a system can only be detected as long as they cause changes in community composition. Further studies will be required to evaluate the effect of other parameters on the activated sludge microbial communities, as well as to see how sensitive is ARDRA to them.

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