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The Diversity Analysis of the Microbial Community in Wastewater by Amplified rDNA Restriction Analysis (ARDRA)

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Abstract

Activated sludge, a common biological treatment method for both municipal and industrial waste water, represents a complex microbial community. Due to intricate interactions within the microbial community, process control of waste water treatment plants can be difficult. Population shifts within the microbial community may results from the changes in the plant operating conditions and cause sludge quality problems such as poor sludge settling, compaction and dewatering. Monitoring of the microbial populations may help in the diagnosis and correction of such sludge problems. This study employed a PCR-based 16S rDNA, amplified rDNA restriction analysis (ARDRA) approach to characterize the microbial community structure in wastewater. Samples were collected from two wastewater treatment plants, in Jaipur City, India. Each PCR product was obtained by PCR with eubacteria 16S rDNA. After amplification, the 16S rDNA PCR products were digested with 4-base site specific restriction endonucleases. Restriction pattern was analyzed with four endonucleases (*AluI, MspI, HhaI*, and HaeIII). The result of the bacterial community analysis, by ARDRA revealed that the two wastewater treatment plants carry significantly different microbial population, whereas the diversity among the samples of same plant is not much. These results suggests that Amplified rDNA restriction analysis (ARDRA) is an extremely valuable tool for assessing the diversity from waste water treatment plants.

Key words: ARDRA, Microbial community, Wastewater

1. Introduction

Biological treatment with activated sludge is the most common and appropriate technology for the wastewater treatment process. Activated sludge utilizes micro organisms to break down organic material with aeration and agitation. Although several microorganisms are commonly found in different waste water treatment plants, differences in microbial community have been reported. Therefore, it is important to analyze the microbial community present in specific wastewater treatment plants. Analysis of the structure and function of activated sludge microbial communities could lead to identify the microbial wastewater composition, wastewater treatment plant (WWTP) operation, or manipulations to be done in the activated sludge.

The advent of molecular tools has been proved extremely useful in assessing the changes in microbial community structure in complex environmental samples. Traditionally, the detection of pathogens in water, wastewater, and other environmental samples is restricted by the ability to culture such organisms. The application of molecular techniques to the study of natural and engineered environmental systems has increased our insight into the vast diversity and interaction of microorganisms present in complex environments. Of the various approaches for the understandings of microbial community structures in nature, comparative analysis of 16S rRNA sequence of microorganisms has been universally applied, due to the ubiquity of ribosomal RNA molecules in all microorganisms, to infer relationships among organisms (Pederson *et al.*, 1996; Wise *et al.*, 1999; Lee *et al.*, 2000). The rRNA molecules are comprised of highly conserved sequence domains, interspersed with more variable regions. In general, the essential rRNA domains are conserved across all the phylogenetic domains, thus universal tracts of sequences can be identified (Olsen *et al.*, 1986). Amplified ribosomal DNA restriction analysis (ARDRA) is a simple method based on restriction endonuclease digestion of the amplified bacterial 16S rDNA. Since ARDRA uses universal 16S rRNA gene primers, it is expected to be applicable to the identification of most bacterial species from any kind of environmental sample. ARDRA detects interspecies and interstrain as well as interoperon

variability and enables a relatively fast multiple strain analysis (Heyndrickx et al. 1996). This technique is appropriate to obtain indicative phylogenetic and taxonomic information. Therefore, ARDRA can be designated as a common methodology for a rapid molecular characterization based on the generation of so-called "genetic fingerprints".

ARDRA approach has been successfully tested to detect differences in activated sludge bacterial communities fed on domestic or industrial wastewater, and subject to different operational conditions (Gich et al. 2000). The purpose of this study was to employ similar approach to evaluate the feasibility of the technique in wastewater systems and to detect the differences in microbial communities present in two wastewater treatment plants based in Jaipur, Rajasthan.

2. Materials and Methods

Sample collection

Two wastewater treatment plants (Brahmpuri, and Pratap Nagar, Jaipur) were selected to obtain the samples. Two samples were selected from each site; influent water (water entering the system) and activated sludge samples (combination of raw sewage and microorganisms. Sterile bottles were used to collect the samples and stored in the dark at 4°C until used (1–2 days).

DNA isolation

DNA isolation was carried out by using Potassium Ethyl Xanthogenate: 1 ml volume of homogenous cell culture was pelleted and suspended in freshly made Xs buffer (1% Potassium ethyl Xanthogenate, 100 mM Tris HCl, pH -7.4, 20 mM EDTA, pH -8.0, 1% SDS, 800 mM Ammonium Acetate). Pellet was incubated at 65°C for 2 h, mixed and then incubated on ice for 30 min. The mixture was centrifuged for 10 m at 10,000 rpm. The supernatant was taken to which 1 volume of 100% isopropanol was added. The DNA was precipitated and pelleted, and washed with 70% ethanol. Finally the pellet was resuspended in TE buffer pH-7.4, Tillett & Neilan (2000).

16S rDNA amplification

PCR amplification was carried out to obtain a 1.5 kb fragment of 16S rRNA gene. Reaction volume was 25 µl with 50 ng of extracted DNA, 200 µM of each dNTPs, 1 U Taq polymerase (Bangalore Genei), 10X Taq buffer and 1.5 mM $MgCl_2$, both supplied with the enzyme, and 20 pmol of each primer: forward 5'-GAGTTGGATCCTGGCTCAG -3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The PCR parameters were 5 min initial denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C, 45 s annealing at 65°C, and 1 min extension at 72°C, finishing with 7 min extension at 72°C. PCR products were electrophoresed in 1.5 % agarose gel for 1 h at 60 V.

Amplified ribosomal DNA restriction analysis (ARDRA)

Four restriction enzymes were used for the restriction digestion of the amplified DNA samples. *AluI, HaeIII, HhaI* and *MspI* were used. *HaeIII, HhaI* and *MspI* were used in single digestion where as *AluI* + *MspI* were used for double digestion. The protocol was standardized for restriction digestion to obtain the best possible results, for all the enzymes for their peak efficacy. The incubation of the reaction mixture was carried out in the PCR Thermal Cycler at 37°C for 4 h. Table 1. details the optimized conditions for each enzyme reaction. After the incubation the samples were electrophoresed on 2% agarose gel at 50V for 1 h.

Data analysis

The patterns of each sample were compared by identifying, from different samples, fragments of identical size in the same digestion. Pairwise comparison of the band pattern was manually performed, and a presence/absence matrix was constructed. NTSYS software was used to prepare summaries of relationships using cluster analysis to obtain the phylogenetic tree.

3. Results and Discussion

Activated sludge systems are widely used as a method of biological wastewater treatment. Microbial population present in the activated sludge can markedly affect the treatment of the waste. Therefore, it is extremely important to understand the structure of the microbial community. In the last decade, a set of molecular tools have been developed and applied for the investigation of the microbial community composition and dynamics in activated sludge systems, in both cultivation dependent and independent manners (Pike and Carrington, 1972; Wagner et al., 1993; Juretschko et al., 2002). ARDRA is a rapid, accurate and reliable technique to assess the microbial diversity. The band pattern obtained indicates the structure of the community present in the environmental system. Our results clearly indicated differences in the microbial community composition amongst the activated sludge systems studied. Significant differences have been observed between the restriction patterns of the two waste water treatment plants. All the enzymes used for the digestions showed the difference in the banding pattern of the two plants. However, not much difference was observed among the samples from the two sites except for the restriction pattern obtained from *HhaI* (Fig 2). This enzyme showed

very different patterns for sample 1-1 and sample 1-2 where as a very similar patterns are observed for sample 2-1 and sample 2-2 except the absence of some bands in sample 2-1. The absence of differences between the patterns does not ensure that the composition of the communities is exactly the same. However, significant composition changes in the community should be detected with the restriction enzymes used (Moyer et al. 1996). The phylogenetic tree (Fig 4) depict the analogous results that are indicative of the fact that there is difference between the microbial community composition of sample 1-1 and sample 1-2 that is observed in the distribution of the nodes for both the samples at a point whereas the microbial community composition of sample 2-1 and sample 2-2 are similar. However, the two plants carry different microbial populations as both the samples from the two plants branched out. Restriction analysis from *HaeIII* show almost akin patterns for sample 1-1 and sample 1-2 except one band (Fig 2.). This shows that most of the bacterial population is similar in the two samples, except for one community that is represented by that single band in 1-1. The restriction patterns were very similar in sample 2-1 and sample 2-2. The phylogenetic tree (Fig 5) suggests that similar microbial biota exist in sample 2-1 and sample 2-2. The phylogenetic tree depicts a complete dissimilarity among the sample 1-1 and sample 1-2 because a completely different node depicts sample 1-1, this suggests that there is a variation in the microbial population between these two samples. There is a little difference observed between sample 1-2, sample 2-1 and sample 2-2 but the distance from the node of sample 1-1 and the rest of the samples show that the microbial community composition of sample 1-1 is very different from rest of the samples. Also, the intensity of the band present in sample 1-1 suggests the dominancy of that microbial population present in the sample. Very interesting results were observed in the restriction patterns that were achieved after the restriction digestion of the samples by enzyme *MspI* (Fig 2). The result exemplify that the sample 1-1 and sample 1-2 share comparable microbial biota as shown by the similar restriction patterns for both the samples. Likewise the identical restriction patterns portray the correspondence of the microbial community of sample 2-1 and sample 2-2. The phylogenetic tree obtained equivalent to the restriction bands authenticate the elucidation that sample 1-1 and sample 1-2 have similar bacterial community and also that the sample 2-1 and sample 2-2 have comparable community composition. Also the similarity amongst sample 1-1, sample 1-2 sample 2-1 and sample 2-2 can be observed. The origination and the no difference in the distance between the samples from two The restriction patterns obtained after restriction digestion of the samples by restriction enzyme *Msp I* + *Alu I* clearly depicts the difference between the populations in the two plants. However, in concord to the band patterns the results of the phylogenetic tree (Fig 6) obtained by the comparison amid the samples show that sample 1-1 and sample 1-2 have alike microbial biota as well the sample 2-1 and sample 2-2 have identical bacterial community. The cluster analysis also represent that there is similarity amongst the bacterial community composition of sample 1-1, sample 1-2, sample 2-1 and sample 2-2. Previous works demonstrated that double restriction endonuclease digestions are sensitive enough to detect important composition changes in the community (Acinas et al. 1997; Martínez-Murcia et al. 1995; Moyer et al. 1996). In their study, the absence of differences between the patterns of the among the samples from the same site lead to the conclusion that there were probably no significant changes between the microbial communities , however, in this study clear differences were obtained between the samples from two different sites. This suggests that the two wastewater treatment plants differ in their microbial population. ARDRA has been used previously to assess the microbial diversity. Gich et al. showed the difference between the industrial and domestic wastewater treatment plant communities by using ARDRA. They 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. In their study the differences in the community structure due to influent characteristics and temperature were observed, however, no differences were observed between the oxic and anoxic reactors of each of the three MLE configurations. Similar conclusions were drawn by Ehlers and Cloete (1999). They used protein fingerprints to evaluate the differences between the microbial community structures among P-removing, non-P-removing and N-removing systems. 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. Their study indicated no difference in the community, which they explained as 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. This implies that analysis of the microbial community structure is important for understanding the role of microorganisms in

relation to the treatment processes that occur within wastewater treatment plants.

Figure 1. PCR products of extracted DNA by a 16S rDNA primer

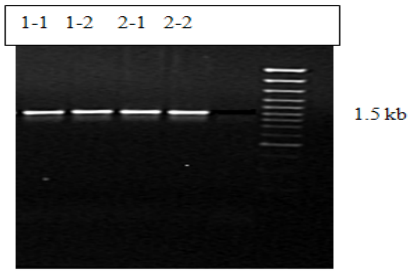


Figure 2. Restriction pattern of PCR-amplified fragment of 16S rDNA genes digested with *Msp*I and *Hae*III, and *Hha*I. 1-1 for influent water, 1-2 for activated sludge site 1; 2-1 for influent water, and 2-2 for activated sludge site 2

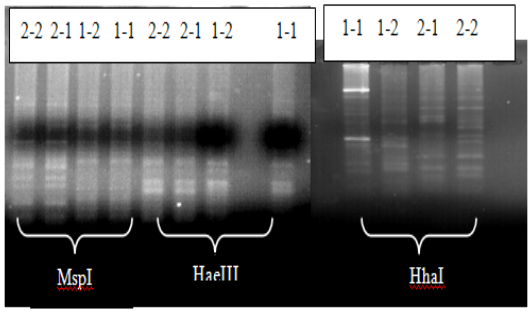


Figure 3. Restriction pattern of PCR-amplified fragment of 16S rDNA genes digested with *Alu*I and *Msp*I. 1-1 for influent water, 1-2 for activated sludge site 1; 2-1 for influent water, and 2-2 for activated sludge site 2

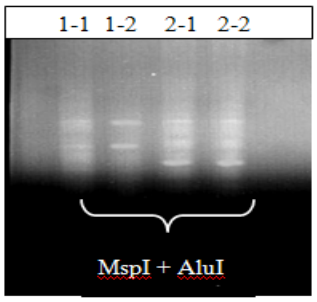


Figure 4. Dendrogram of genetic similarity matrix value of 16S rDNA genotypes analyzed by PCR-ARDRA using enzyme *Hha*I. 1-1 for influent water site 1, 1-2 for activated sludge site 1; 2-1 for influent water, and 2-2 for activated sludge site 2

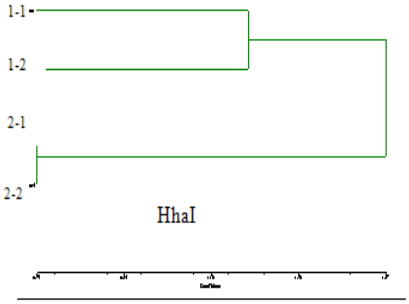


Figure 5. Dendrogram of genetic similarity matrix value of 16S rDNA genotypes analyzed by PCR-ARDRA using enzyme *HaeIII*. 1-1 for influent water, 1-2 for activated sludge site 1; 2-1 for influent water, and 2-2 for activated sludge site 2

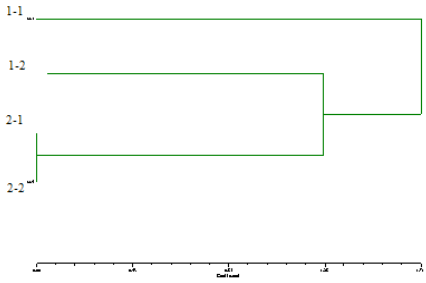
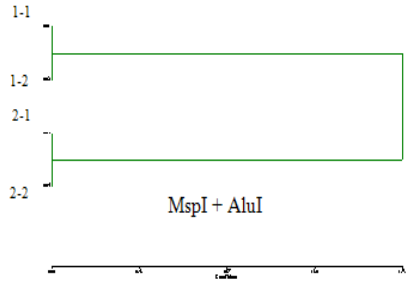


Figure 6. Dendrogram of genetic similarity matrix value of 16S rDNA genotypes analyzed by PCR-ARDRA using double digestion with enzymes *AluI* and *MspI*. 1-1 for influent water, 1-2 for activated sludge site 1; 2-1 for influent water, and 2-2 for activated sludge site 2



4. Conclusion

Our results indicate that the two wastewater treatment plants based in Jaipur, share some common microbial population however, the total microbial community is fairly different. The samples from the same treatment plant were similar in community structure. Despite the limited sampling, our study clearly revealed the broad diversity of bacteria involved in two plants. In order to better classify the microbial populations of the two plants, further investigations are needed. However, in conclusion, we can interpret that ARDRA is a powerful molecular biology tool to detect differences between activated sludge communities and to analyze the microbial diversity in wastewater treatment plants.

References

Acinas, S.G., Rodríguez-Valera, F., Pedrós-Alió C.1997. Spatial and temporal variation in marine bacterioplancton diversity as shown by RFLP fingerprinting of PCR amplified 16S rDNA. *FEMS Microbiol Ecol.* 24, 27–40.

Ehlers, M.M., Cloete, T.E. (1999) Comparing the protein profiles of 21 different activated sludge systems after SDS-PAGE. *Water Res* 33, 1181–1186

Gich, F.B., Amer, E., Figueras, J.B., Abella,C.A., Balaguer, M.D.,M, Poch. 2000. Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA) *Internatl Microbiol* 3,103–106.

Heyndrickx M., Vauterin L., Vandamne P., Kersters K., De Vos P. 1996. Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J. Microbiol.* 26, 247-259.

Juretschko, S., A. Loy, A. Lehner, M, Wagner. 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Syst. Appl. Microbiol.* 25, 84-99.

Lee, D.H., S.A. Noh, C.K. Kim. 2000. Development of molecular biological methods to analyze bacterial species diversity in freshwater and soil ecosystems. *J. Microbiol.* 38, 11-17.

Martínez-Murcia, A.J., Acinas, S.G., Rodríguez-Valera, F. 1995. Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hipersaline environments. *FEMS Microbiol Ecol.*17, 247–256

Moyer, C.L., Tiedje, J.M., Dobbs, F.C., D.M. Karl. 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small- subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl Environ Microbiol.* 62, 2501–2507.

Olsen G.J., D.J. Lane, S.J. Giovannoni, N.R. Pace, D.A. Stahl. 1986. Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* 40, 337-365

Pederson, K., J. Arlinger, L. Hallbeck, Pettersson. 1996. Diversity and distribution of subterranean bacteria in groundwater at Oklo in Gabon, Africa, as determined by 16S rRNA gene sequencing. *Mol. Ecol.* 5, 427-436.

Pike, E.B., E.G. Carrington.1972. Recent developments in the study of bacteria in the activated-sludge process. *Water Pollut. Control.* 71, 583-605.

Tillett, D., B.A, Neilan,. 2000. Xanthogenate nucleic acid isolation from cultured and environmental Cyanobacteria. *Journal of Phycology.* 36, 251-258.

Wagner M., Amann R., Lemmer H., K.H., Schleifer. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl Environ Microbiol.* 59, 1520–1525.

Wise, M.G., J.V. McArthur, L.J. Shimkets. 1999. Methanotroph diversity in landfill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Appl. Environ. Microbiol.* 65, 4887-4897.

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