Research article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Elucidation of the Microbial Community in Activated Sludge Using PCR-DGGE Analysis in Arid and Semi Arid Regions of Rajasthan

Chandra Shivani[1*], Mathur N.[2], Nallapeta S. [3], Nehra S.[1], Bhatnagar P.[4]

[1]Birla Institute of Scientific Research, Statue Circle, Jaipur- 302001
[2]University of Rajasthan, JLN Marg, Jaipur 302004.
[3]Bruker Daltonics pvt Ltd. No.52 2nd Main Road, Vyalikaval, Bangalore
[4]International College for Girls, Gurukul Marg, SFS, Mansarovar, Jaipur 302020

ABSTRACT

Activated sludge is the most commonly used process to treat sewage and industrial waste water by micro organisms. The activated sludge system depends on the activities of microbial communities present in the sludge. However, exact knowledge of the microbial community structure in waste water treatment plants is limited. In this study, the bacterial diversity of activated sludge was investigated in the two waste water treatment plants by using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA fragments. Dominant bands from DGGE profiles were excised and subjected to sequencing to identify the dominant genotypes. Sequence analysis gave insights into the identities of the predominant bacterial populations present. The DNA sequencing results indicated the microbial diversity, revealing that the dominant bacteria present in Bramhapuri waste water treatment plant is *Acinetobacter* sp. whereas the dominant bacteria in Pratapnagar (Delawas) waste water treatment plant is *Alpha-proteobacteria*. Futhermore, cluster analysis of the DGGE profiles indicated significant diversity in the bacterial community by depicting two distinct clusters for each waste water treatment plant. These data endorse the ability of PCR-DGGE method to identify and characterize bacterial community from activated sludge.

KEYWORDS

Acinetobacter, Alpha-proteobacteria, Activated sludge, Bacterial diversity and PCR-DGGE.

Corresponding Author:

Dr. Shivani Chandra,
Associate Professor,
Birla Institute of Scientific Research, Statue Circle, Jaipur- 302001
E Mail: shivani49@gmail.com

INTRODUCTION

The need for clean water is increasing and wastewater treatment can be used as a cost-effective solution for purification of organically polluted industrial waste streams. Biological wastewater treatment especially activated sludge has gained popularity and is now one of the key technologies in environmental biotechnology [1]. Approximately 33% of all waste-water treatment systems within the industry use the activated-sludge process. Activated sludge consists of a mixed community of microorganisms that metabolize and transform organic and inorganic substances into environmentally acceptable forms. The typical microbiology of activated sludge consists of approximately 95% bacteria and 5% higher organisms. However, exact knowledge of the microbial community structure in waste water treatment plants is limited. The activated sludge process is generally complex to fully comprehend and thus difficult to be effectively operated and controlled. This is partly due to the complex way the sludge communities behave as well as the methodological limitations related to the knowledge on the microbiology of this process.

Application of molecular biology techniques allows us to detect and enumerate microorganisms in their natural habitat and so to determine the structure, function and dynamics of bacterial communities. 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 [2,3,4] . 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 [5] . Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used among the culture-independent fingerprinting techniques [6] It is based on the separation of polymerase chain reaction (PCR) amplicons of the same size but different sequences. Descriptions and comparisons of activated sludge bacterial communities have been carried out since early 90s. Currently the basic tools used in a comparative analysis of bacterial communities without previous cultivation are DGGE and FISH [7]. It is also used to verify the dominance of microorganisms in wastewater biological treatment, as a supplementary support method for screening of the dominant microorganisms from activated sludge [8].

In this work, an attempt has been made to open the “black box” of the activated sludge community to evaluate the bacterial diversity in activated sludge of two wastewater treatment plants located in arid and semi-arid region of Rajasthan by using PCR-DGGE technique.

MATERIAL AND METHODS

Sampling Sites

The two waste water treatment plants used in the study were Bramhapuri and Pratapnagar (Delawas), located in Jaipur, Rajasthan. Two samples, influent water and activated sludge were collected from each of the waste water treatment plants over the period of two years.

DNA isolation

For the isolation of DNA from sludge samples, 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.[9] .

PCR Amplification

PCR were standardized to precisely amplify the 16S conserved region (1.5 kb) for each sample. The universal primer sequences were used for 16S rDNA amplification, Fwd: 5'-GAGTTGGATCCTGGCTCAG -3' and Rev: 5'-AAGGAGGGGATCCAGCC-3'. The variable V3 region of 16S rDNA was PCR amplified to obtain a PCR product of 220 bp with primers to conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers were primer 1: 5'-CCTACGGGAGGCAGCAG-3', primer 2: 5'-ATTACCGCGGCTGCTGG-3', and primer 3: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG-3', that contained the same sequence as primer 1 but has at its 5' end an additional 40-nucleotide GC-rich sequence (GC clamp). A combination of primers 1 and 2 or primers 3 and 2 was used to amplify the 16S rDNA.

DGGE Analysis

PCR products were resolved on 8% (w/v) polyacrlamide gels in 0.5X TAE using denaturing gradients ranging from 40% to 80% (where 100% denaturant contains 7M urea and 40% formamide). For each sample 10 µl of PCR product was loaded after mixing with equal volume of loading dye to the bottom of the well. Electrophoresis was carried out at low voltage (20V) for 20 min and then at 200 volts for 3 hrs at a constant temperature of 60^0C. The gels were stained for 20 min with ethidium

bromide and washed twice for 5 min with Milli-Q water prior to UV transillumination in UVI gel documentation system (UVItec, Cambridge, United Kingdom). The DGGE bands were excised and subsequently sequenced.

RESULTS AND DISCUSSION

Bramhapuri Wastewater treatment plant

PCR-DGGE analysis was done for the influent water and activated sludge samples for two consecutive years. In the first year, the influent water sample produced a total of six bands whereas nine bands were observed in the activated sludge sample. The next year, a total of six bands were produced. All of these six bands were common to both the influent water and activated sludge samples (Figure 1).

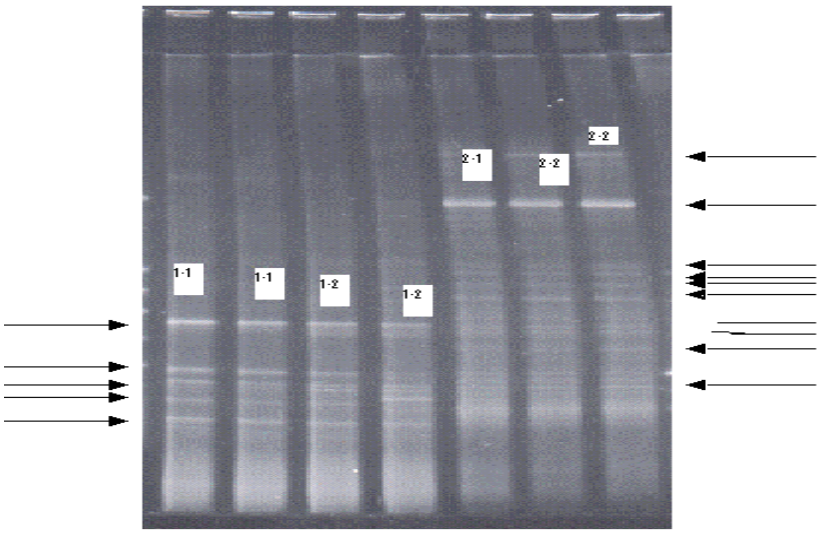

Figure 1: DGGE analysis for year 2009 where lanes 1 and 2 are samples from Bramhapuri location and sample 3 and 4 from Pratap nagar location

For the determination of the more specific community structure traits, a sequencing analysis of the bands was performed. The results of the alignment of the obtained sequence, using the BLAST suggested up to ~ 90% similarity with *Acinetobacter* sp. These results suggest that the dominant bacterial population in this WWTP is *Acinetobacter* sp.

The class of bacteria identified in present study is in agreement with the previous studies. The *Acinetobacter* species have been identified from waste water treatment plants since early 1990s [10] . *Acinetobacter* organisms which are heterotrophic works on enhanced biological phosphorus removal. These organisms release phosphorus, thereby obtaining the energy to uptake readily biodegradable

organics. This ability enables *Acinetobacter* to become dominant. *Acinetobacter* species is also known to be predominant micro-organism involved in enhanced phosphorus uptake. Other researchers reported that *Acinetobacter* spp. were predominant when enumerated using the analytical profile index method. For example, Hart and Melmed (1982) estimated *Acinetobacter* spp. at 56% to 66% of the total population, Buchan (1983) reported 48% to 66%, Lötter (1985) 56% to 66%, Lötter and Murphy (1985) ca. 60% to 70% and Kerdachi and Healey (1987) 73% [11,12,13] . Bramhapuri waste water treatment plant basically deals with textile wastes and bacteria of this genus are known to be involved in biodegradation, leaching, and removal of several organic and inorganic man-made hazardous wastes that are known to be produced by textile dyes. Also, among microbial communities involved in different ecosystems such as soil, fresh water, wastewater, and solid waste, several strains belonging to the genus *Acinetobacter* have been identified. Thus, the presence of *Acinetobacter* sp as dominant bacteria seems justified.

Pratapnagar (Delawas) Wastewater treatment plant

For the Delawas location five bands were observed for the influent water, and for the activated sludge, three dominant and three faint bands were observed in the first year. For the next year, a total of ten bands were observed.

A similar band pattern was produced by both, the influent water and activated sludge samples (Figure 2). Sequence analysis of the excised bands revealed up to 100% similarity with *alpha proteobacteria.*

This location basically deals with mixed wastes, domestic and industrial. *Alphaproteobacteria* is known to be associated with bulking in industrial waste water treatment plants [14]. Large population of *Alphaproteobacteria* has been observed in waste water treatment plants. Wagner *et al.* (1993) studied bacterial community structure in activated sludge samples using group specific oligonucleotide probes for in situ analysis [15].

Probing activated sludge with fluorescently labeled oligonucleotide probes specific for the alpha, beta and gamma subclasses of the *proteobacteria* had revealed that the microbial consortia are dominated by the *Proteobacteria* (approximately 80%), a phylum containing a majority of the traditional gram negative bacteria. Arroyo *et al.* 2010, provided information about bacterial community structure in natural wastewater treatment systems treating different types of wastewater using the direct sequencing of the 16S ribosomal RNA codifying genes [16].

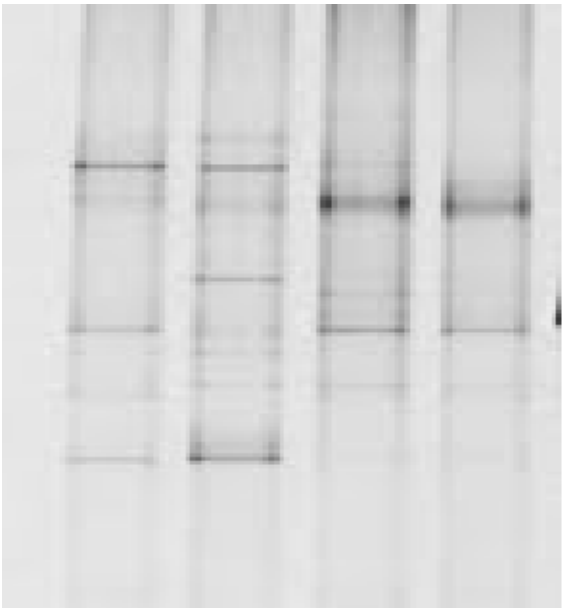

Figure 2: DGGE analysis for year 2009 where lanes 1 and 2 are samples from Bramhapuri location and sample 3 and 4 from Pratapnagar location.

They concluded that the municipal wastewater treatment system presented a high diverse community in both macrophytes with *gammaproteobacteria* and *Alphaproteobacteria*, respectively, as the most abundant groups. This is in agreement with our findings. Reid *et al.* 2008, studied the bacterial composition of a waste water treatment system reliant nitrogen fixation, they confirmed that despite changes in wastewater composition and dissolved oxygen levels, the bacterial community composition appeared stable and was dominated by *Alphaproteobacteria* and *Betaproteobacteria* [17]. Thus, it can be inferred that alpha proteobacteria is one of the dominant bacterial species found in waste water systems. The Proteobacteria kingdom is the largest and most diverse in the domain bacteria. As a group, these organisms show extreme metabolic diversity and represent the majority of known gram-negative bacteria of medical, industrial, and agricultural significance. This is an evolutionarily, geologically, and environmentally important group. This is in agreement with our findings for Pratapnagar (Delaws) location, since this waste water treatment plant basically deal with non specific wastes.

The study also revealed some uncultured bacteria which is in concerence with Ziembińska et al. (2009). [18] In this study they used denaturing gradient gel electrophoresis (DGGE), combined with cloning and sequencing of 16S rRNA to estimate biodiversity and temporal community changes in activated sludge and revealed a high diversity of uncultured bacteria.

Cluster analysis

Cluster analysis of the DGGE profiles depicted that the two waste water treatment plants carry different microbial populations (Figure 3 and Figure 4).

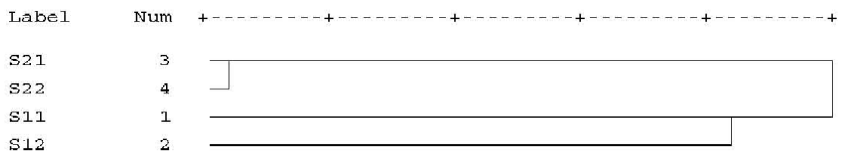

Figure 3: Cluster analysis using SPSS-2008

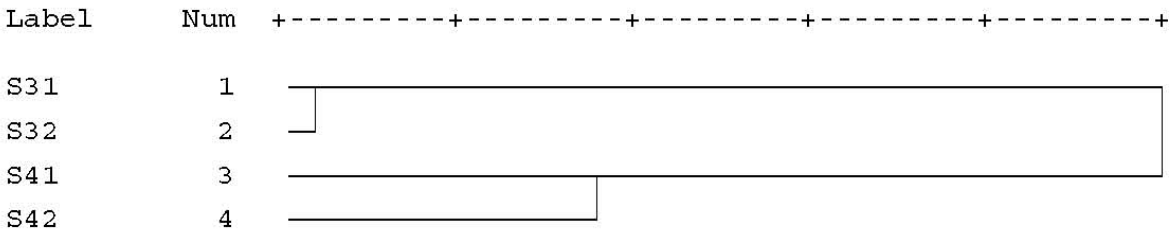

Figure 4: Cluster analysis using SPSS-2009

The dendrogram depicts two distinct clusters for each of the waste water treatment plants suggesting that the two waste water treatment plants carries different bacterial population (Figure 5).

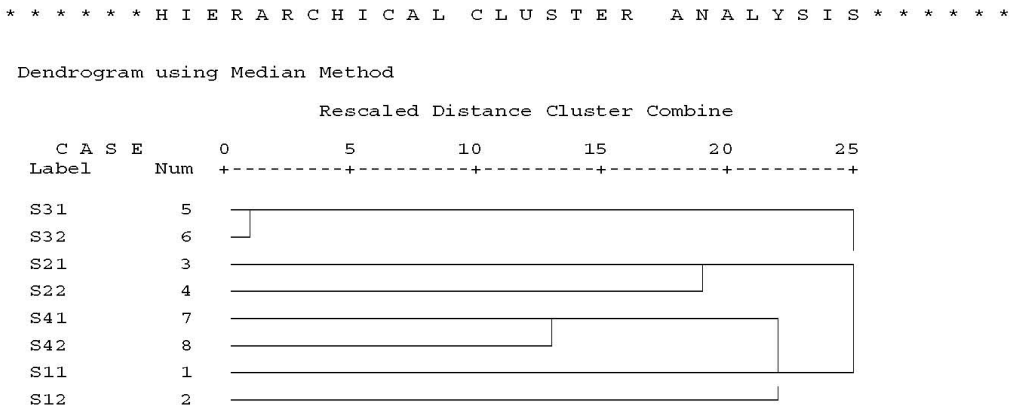

Figure 5: Hierarchical Cluster Analysis using SPSS

However, no significant difference was observed over the period of two years. This suggests that diversity of bacterial community did not change much over a period of two years. One striking observation was that influent water and activated sludge bacterial population was similar in both the locations. This suggests that no specific bacteria are being used by these waste water treatment plants (Table I, II and III).

Table I: Case processing summary

Cases					
Valid		**Missing**		**total**	
N	**Percent**	**N**	**Percent**	**N**	**Percent**
11	78.6%	3	21.4%	14	100.0%

a. Binary squared Education Distance used.

Table 2: CLUSTER Proximity Matrix

Case	Matrix File Input							
	S11	**S12**	**S21**	**S22**	**S31**	**S32**	**S41**	**S42**
S11		4.000	6.000	5.000	7.000	7.000	5.000	3.000
S12	4.000		6.000	7.000	5.000	5.000	5.000	3.000
S21	6.000	6.000		3.000	7.000	7.000	9.000	7.000
S22	5.000	7.000	3.000		6.000	6.000	8.000	8.000
S31	7.000	5.000	7.000	6.000		.000	6.000	8.000
S32	7.000	5.000	7.000	6.000	.000		6.000	8.000
S41	5.000	5.000	9.000	8.000	6.000	6.000		2.000
S42	3.000	3.000	7.000	8.000	8.000	8.000	2.000	

Table 3: Median Linkage Agglomeration Schedule

Stage	Cluster Combined		Coefficient	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	5	6	.000	0	0	7
2	7	8	2.000	0	0	4
3	3	4	3.000	0	0	6
4	1	7	3.500	0	2	5
5	1	2	2.875	4	0	6
6	1	3	4.594	5	3	7
7	1	5	4.086	6	1	0

In conclusion, it can be said that since the two waste water treatment plants deal with different kinds of wastes and thus the dominant bacteria present in each plant are different. To the best of our knowledge, this is the first study assessing the bacterial population in these two waste water treatment plants. The results from present study indicates that even though the bacterial community structure is different in the Bramhapuri and Pratapnagar (Delawas) waste water treatment plants, the influent water and the activated sludge of the individual plant does not carry much bacterial diversity. This implies that the two working waste water treatment plants are not using specific bacteria to ensure the maximum efficiency of the plant. The results from this study would be beneficial for the operators and engineers of the waste water treatment plants to further improve on the process and increase the efficiency of the working plants.

The results from this study also indicated that PCR-DGGE is a powerful technique to identify bacterial population in environmental samples. This is in agreement with the findings from de Araújo and Schneider (2008). [19] They conducted a systematic study was conducted with artificial consortia to test whether denaturing gradient gel electrophoresis (DGGE) is a reliable technique to obtain such community data under conditions where results would not be affected by differences in DNA extraction efficiency from cells. Their results demonstrated that DGGE was suitable for identification of all important community members in the three-membered artificial consortium, but not for identification of the dominant organisms in this small community.

REFERENCES

1. Watanabe K. and Baker PW. Environmentally Relevant Microorganisms. Jour. Of Biosci. Bioeng. 2000; 89(1):1-11.
2. Pederson K., J. Arlinger L., Hallbeck and Pettersson. Diversity and distribution of subterranean bacteria in groundwater at Oklo in Gabon, Africa, as determined by 16S rRNA gene sequencing. Mol. Ecol. 1996; (5): 427-436.
3. Wise MG, McArthur JV and Shimkets LJ. 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. 1999; (65): 4887-4897.
4. Lee DH, Noh SA and Kim CK. Development of molecular biological methods to analyze bacterial species diversity in freshwater and soil ecosystems. J. Microbiol. 2000 (38): 11-17.
5. Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR and Stahl DA. Microbial ecology and evolution: a ribosomal rRNA approach. Annl. Rev. of Microbiol .1986; (40)**:**337–365.
6. Muyzer G., Ellen C. De Waal and Uitierlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl.Environ. Microbiol. 1993; 59 (3): 695-700.
7. Ziembińska A., Raszka A., Truu J., Surmacz-Górska J. and Miksch K. Molecular analysis of temporal changes of a bacterial community structure in activated sludge using denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH). Polish journal of microbiology .The Polish Society of Microbiologists 2007; 56 (2) 119-127.
8. Zhou S, Wei C, Ke L and Wu H. PCR-DGGE as a supplemental method verifying dominance of culturable microorganisms from activated sludge. J Microbiol Biotechnol. 2010; 20(11):1592-6.
9. Tillett D. and Neilan BA. Xanthogenate Nucleic Acid Isolation from Cultured and Environmental Cyanobacteria. Jour. of Phycol 2000; 36(1): 251-258.
10. Blackall L L, Parlett JH, Hayward AC, Minnikin DE, Greenfield PF and Harbers AE. *Nocardia pinensis* sp. nov., an actinomycete found in activated sludge foams in Australia. Jour. of Gen. Microbiol. 1989; (135): 1547–1558.
11. Hart MA and Melmed LN. (1982). Microbiology of nutrient removing activated sludge. Water Sci Tech. 1982; (14): 1501-1502.
12. Buchan L. Possible biological mechanism of phosphorus removal. Water Sci Tech. 1983; (15): 87-103.

13. Kerdachi DA and Healey JK. The reliability of the cold perchloric acid extraction to assess metal-bound phosphate. In: Ramadori R (ed.) Phosphate Removal from Wastewaters. Permagon Press, Oxford.1987.
14. Levantesi C, Beimfohr C, Geurkink B, Rossetti S, Thelen K, Krooneman J, Snaidr J, van der Waarde J and Tandoi V. Filamentous Alpha proteobacteria associated with bulking in industrial wastewater treatment plants. Syst Appl Microbiol.2004; 27(6):716-27.
15. Wagner M., Amann R and Lemmer H., Karl-Heinz Schleifer. Probing activated Sludge with Oligonucleotides Specific for Proteobacteria: Inadequacy of Culture-Dependent Methods for Describing Microbial Community Structure. Appl. Environ. Microbiol. 1993; 59 (5): 1520-1525.
16. Arroyo P, Ansola G Blanco I, Molleda P, de Luis Calabuig E and Sáenz de Miera LE. Comparative analysis of the composition of bacterial communities from two constructed wetlands for municipal and swine wastewater treatment. J Water Health. 2010; 8(1):147-57.
17. Reid NM, Bowers TH and Gareth LJ. Bacterial community composition of a wastewater treatment system reliant on N2 fixation. Appl. Microbiol Biotech. 2008; 79(2): 285-292.
18. Ziembińska A., Ciesielski S. and Miksch K. Ammonia oxidizing bacteria community in activated sludge monitored by denaturing gradient gel electrophoresis (DGGE). Jour. of Gen. Appl. Microbiol. 2009; 55 (5): 373-380.
19. De Araújo JC and Schneider RP. DGGE with genomic DNA: suitable for detection of numerically important organisms but not for identification of the most abundant organisms. Water Res. 2008; 42(20):5002-10.