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NUCLEOTIDE DIVERSITY IN *16S RDNA* GENE IN BACTERIAL SPECIES SURVIVING IN DIFFERENT AREAS

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Abstract

Microorganisms adapt to harsh conditions easily by performing different important functions. They change their metabolic activities according to the environmental conditions and nutrients availability. What type of changes help microorganisms to survive in automobile polluted area of Rajkot was studied by using molecular analysis. *16s rDNA* gene was amplified from bacterial species isolated from soil samples of polluted and non-polluted area. Phylogenetic and DNA polymorphism analysis was carried out from these gene sequences. Differences in clade and phylogenic relationships of *16s rDNA* gene sequences in same bacteria surviving in different areas was observed. Nucleotide diversity was also observed in bacterial species surviving in different areas. From this, it was concluded that nucleotide variations help bacterial species to survive in harsh environmental conditions.

Key words : *16s rDNA*, pollution, DNA polymorphism.

Introduction

Pollution is the introduction of contaminants into a natural environment that causes instability, disorder, harm or discomfort to the ecosystem. Urbanization in India is increasing more rapidly around the major cities in India. Rajkot is one, of the famous city of Gujarat in India. It is well know for the production of automobiles. Due to increase in industrial activities, population both endemic and floating and vehicular population in this area has led to a number of environmental problems, one of them being soil pollution. Fuel leakages from automobiles get washed away due to rain and seep into the nearby soil. This results in increase in soil pollution through hydrocarbons. Petroleum spillage by these automobile industries reduces microbial diversity in soil through the phenomenon of selectivity (Atlas *et al*., 1991). The microorganisms capable of surviving in such a polluted environment are those that develop specific enzymatic and physiological responses that allow them to use the hydrocarbon compounds as substrates (Mancera-Lopez *et al*., 2007).

Microbes in the soil are the key to carbon and nitrogen recycling. A ton of microscopic bacteria may be active per acre and there may be over one million species of bacteria present. Bacteria are tiny, one-celled organisms. They are similar in size to clay soil particles (<2 μm) to silt soil particles (2–50 μm). They grow and live in thin water films around soil particles. The small bacterial size enables these microbes to grow and adapt to changing environmental conditions more rapidly than larger, more complex microorganisms. In order for bacteria to survive in the soil, they must adapt to many microenvironments. In the soil, oxygen concentrations vary widely from one microsite to another. Large pore spaces filled with air provide high levels of oxygen, which favors aerobic conditions, while a few millimeters away, smaller micropores may be anaerobic or lack oxygen. This diversity in soil microenvironments allows bacteria to thrive under various soil moisture and oxygen levels, because even after a flood (saturated soil, lack of oxygen) or soil tillage (infusion of oxygen) there exist small microenvironments where different types of bacteria and microorganisms can exist to repopulate the soil when environmental conditions improve (Archulate, 2009).

They perform different important functions in soil in order to adapt a particular environmental condition. For example, secretion of various enzymes in soil to decompose organic matter. There are basically four functional soil bacterial groups:

1) Decomposers, which are bacteria that consume simple sugars and simple carbon compounds, such as

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root exudates and fresh plant litter.

2) Bacteria mutualists that form partnerships with plants including the nitrogen fixing bacteria (Rhizobia).

3) Pathogens which are harmful to plants, animals and humans.

4) Lithotrophs or chemoautotrophs bacteria obtain energy from compounds of nitrogen, sulfur, iron, or hydrogen instead of from carbon compounds.

Some of these species are important for nitrogen cycling and degradation of pollutants. They convert energy in soil organic matter into forms useful to the rest of the organisms in the soil food web. A number of bacteria decomposers can break down pesticides and pollutants in soil (Magdoff *et al*., 2001). Many bacteria produce a layer of polysaccharides or glycoproteins that coats the surface of the cell. Some form a slime layer while others form a thick gelatinous capsule which reduces water loss from the bacterial cell and is used by the bacteria to form biofilms so that they can attach to structures (Nester *et al*., 2007). Nitrogen-fixing bacteria (Rhizobia) form symbiotic associations with the roots of legumes like alfalfa and clovers. Rhizobia are gram negative rod-shaped bacteria. Visible nodules are created where bacteria infect a growing root hair. The plant supplies simple sugars to the bacteria and the bacteria convert nitrogen (N2) from air into a nitrogen form (NO^{3-} or NH^{4+}) that plant can use (Magdoff *et al*., 2001). Sulfur-reducing bacteria under anaerobic conditions make sulfur less available to plants by converting sulfur to H_2S in water-saturated soils, precipitating out into the soil as an insoluble metal sulfide. Under well-aerated conditions, sulfur oxidizing bacteria generates SO_4^- by oxidizing metal sulfides to elemental sulfur or thiosulfate ($S_2O_3^{2-}$) (Tugel *et al*., 2000). A large group of bacteria are actinomycetes that grow hyphae like fungi and are similar to fungus in their function. Various kinds of antibiotics produced by actinomycetes help in degradation of hard compounds, such as chitin, lignin, keratin, cellulose fungal and animal polymers (Sylvia *et al*., 2005).

It has been reported that adapted communities previously exposed to hydrocarbons exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination. Organic compounds of low molecular weight and simple molecular structure are preferred by many microorganisms. Compounds with more complex structures, such as polycyclic aromatic hydrocarbons (PAHs), with more than five benzene rings, are more resistant to microbial breakdown. According to Atlas (1995), the most prevalent bacterial hydrocarbon degraders, in decreasing order, belong to the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Nocardia*, *Arthrobacter* and other *coryneforms*, *Vibrio*, *Bacillus*, *Micrococus* and *Acinetobacter*. Other genera of bacteria able to degrade hydrocarbons include *Actinomyces*, *Aeromonas* and *Alcaligenes* (Atlas and Cerniglia, 1995). To understand, change in bacterial diversity due to automobiles pollution and type of organisms surviving in industrial area of Rajkot; soil sample was collected from the industrial area of Rajkot near field marshal. Bacterial diversity of this industrial area was compared with the bacterial diversity of Saurashtra university campus. This was done to know the type of organisms capable of surviving in polluted and non-polluted soil. Biodiversity of bacterial species surviving in both this area were studied by isolating and identifying them using biochemical tests and sequencing of *16s rDNA* gene. Population genetics is a branch of the evolutionary biology that tries to determine the level and distribution of genetic polymorphism in natural populations and also to detect the evolutionary forces (mutation, migration, selection and drift) that could determine the pattern of genetic variation observed in natural populations. Ideally, the best way to quantify genetic variation in natural populations is by comparison of DNA sequences (Kreitman, 1983). Hence, DnaSP software was used to determine genetic variation in *16s rDNA* sequences of bacterial species due to hydrocarbon pollution. This was done to study genetic variation among the bacterial population in polluted and non-polluted environment. It had helped to study the change in conserved *16s rDNA* sequences due to adaptive capacity of microorganisms and variation in diversity of bacterial species at genetic level.

Materials and Methods

Sample collection, bacterial isolation and identification

Soil samples were collected from two sites *viz*. Saurashtra University campus and an industrial site near Aaji dam, Rajkot which were referred as non-polluted and polluted sites, respectively.

Appropriate dilutions of each of the samples were prepared, which were further inoculated in N-broth for 24 h at 37 °C. The cultures were further streaked on the agar plates and incubated for 24 h at 37ºC. The well isolated colonies observed on the next day, were subcultured and finally preserved at 4°C. Further, biochemical tests were performed from these colonies to identify bacterial species on the basis of Bergy's manual classification.

DNA isolation, *16S rDNA* amplification and sequencing

Bacterial DNA was extracted according to the method given by [8] followed by quality assessment using 1% agarose gel electrophoresis and measurement of concentration and purity using microplate reader (μ Quant, Bio-Tek instruments, USA). The amplification of 16S rDNA was performed in a 25-μL final volume containing 1 μL total DNA, 20 μM 8F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.2 μM 1517R primer (5'-ACGGCTACCTTGTTACGACTT -3'), 10 mM of dNTPs mix, 25mM $MgCl_2$ and 1 U Taq DNA polymerase (Genei-Merck). The reaction conditions were as follows: 95°C for 5 min followed by 30 cycles of denaturation at 95 °C for 1.5 min., annealing at 54 °C for 1 min and primer extension at 72°C for 3.5 min, followed by a final extension at 72 °C for 7 min. The reaction products were separated by running 5 μL of the PCR mixture on a 2% (w/v) agarose gel and staining the bands with ethidium bromide. Subsequently, the PCR products were sequenced using the amplifying primers and BigDye Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems, Foster City, California, USA) as per manufacturer's protocol. After purification, the reaction products were analyzed on an ABI PRISM Genetic Analyzer 3130 (Applied Biosystems). Sequence editing was performed using Bioedit. All sequences were submitted to the NCBI Genbank database using the standalone submission tool Sequin.

Bioinformatics' analyses

Sequences obtained were analyzed by bioinformatics tools for accurate identification and mutation analysis of *16s rDNA* gene of bacteria surviving in polluted and non-polluted area.

Sequence analysis of *16s rDNA* gene

16s rDNA gene sequence was obtained by using cycle sequencing kit commercially available from Applied Biosystems. Cycle sequencing reaction was carried out in PCR and then sequencing was done in genetic analyzer by loading cycle sequencing product in it. Further, sequences obtained were analyzed by bioinformatics tools for accurate identification and mutation analysis of *16s rDNA* gene of bacteria surviving in polluted and non-polluted area.

[1] NCBI Blast analysis

16s rDNA gene sequences obtained from genetic analyzer were paste in NCBI basic alignment tool for comparison with the sequence present in the database. Accuracy of results was determined by recording the query coverage and percent identity of sequences with sequences available in NCBI database. The basic local alignment search tool (BLAST) finds regions of local similarity between sequences. This program compares nucleotide or protein sequences with the sequences available in the databases and calculates the statistical significance of matches. BLAST can also be used to infer functional and evolutionary relationships between sequences as well as to identify members of gene families.

[2] Multiple sequence alignment

Sequences of all identified bacterial species were downloaded from the NCBI database. Then, multiple sequence alignment of *16s rDNA* gene sequences obtained from genetic analyzer was done with the sequences downloaded from NCBI database for phylogenetic analysis. Multiple sequence alignment was done by using bioinformatics softwares, clustalX and tree view.

Clustal X is a general purpose multiple sequence alignment program for DNA or proteins. It attempts to calculate the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. The basic alignment method used in ClustalX can be divided into three steps: 1) all pairs of sequences are aligned separately in order to calculate a distance matrix giving the divergence of each pair of sequences; 2) a guide tree is calculated from the distance matrix; 3) the sequences are progressively aligned according to the branching order in the guide tree. Two problems with this approach are that there is no guarantee to find the global optimal solution and that misaligned regions in the beginning cannot be corrected later on. The advantage, however, is that the algorithms is fast even for alignments of hundreds of sequences. A well chosen set of heuristics, including the use of a guide tree, also means that alignments are generally of high quality (Thompson *et al*., 1997).

[3] Sequin

Sequences of *16s rDNA* gene was submitted in the NCBI database using sequin software. Sequin is a stand-alone software tool developed by the NCBI for submitting and updating entries to the GenBank sequence database. Sequin 11.90 is currently available from the NCBI. Sequin runs on Macintosh, PC/Windows and UNIX computers. A number of powerful sequence annotation tools have been integrated into Sequin.

[4] Bioedit

Bioedit is a biological sequence alignment editor written for Windows 95/98/NT/2000/XP/7. An intuitive

multiple document interface with convenient features makes alignment and manipulation of sequences relatively easy on your desktop computer. Several sequence manipulation and analysis options and links to external analysis programs facilitate a working environment, which allows you to view and manipulate sequences with simple point and click operations.

[5] MEGA 5.05

MEGA 5.05 is specifically designed to reduce the time needed for mundane tasks in data analysis and to provide statistical methods of molecular evolutionary genetic analysis in an easy to use computing workbench (Tamura *et al*., 2011).

[6] DnaSP 5.10

DnaSP is a software package for a comprehensive analysis of DNA polymorphism data. Version 5 implements a number of new features and analytical methods allowing extensive DNA polymorphism analyses on large data sets. The program addressed to molecular population geneticists, calculates several measures of DNA sequence variation within and between populations, linkage disequilibrium parameters and Tajima's D statistic (Librado and Rozas, 2009).

Results

Isolation and identification of bacteria from soil samples using biochemical tests

Soil samples were collected from two different areas of Rajkot. Among them Aaji dam area was considered as polluted area because of presence of various automobile industries, whereas Saurashtra university campus was consider as non-polluted area. From this soil samples, 42 morphologically distinct colonies were separated. Among these colonies 23 bacterial colonies were isolated from polluted area and 20 bacterial colonies were isolated from non polluted area. From these bacterial samples, 19 bacteria were identified up to genus and species level using biochemical characteristics.

Bacterial species identified from the polluted soil samples were *Bacillus azotoformans*, *Bacillus larvae*, *Bacillus alvei*, *Bacillus coagulans*, *Lactobacillus casei*, *Corynebacterium xerosis*, *Corynebacterium kutceri*, *Enterobacter munditii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Bacillus marinus*, *Enterococcus hirae*, *Enterococcus faecium*. There was one species of *Bacillus azotoformans*, one species of *Bacillus larvae*, one species of *Bacillus alvei*, one species of *Bacillus coagulans*, two species of *Lactobacillus casei*, one species of *Corynebacterium xerosis*, one species of *Corynebacterium kutsceri*, two species of *Enterococcus mundtii*, three species of *Staphylococcus aureus*, two species of *Staphylococcus epidermidis*, one species of *Bacillus marinus*, one species of *Enterococcus hirae*, and two species of *Enterococcus faecium*.

Bacterial species identified from non-polluted soil samples were *Bacillus azotoformans*, *Lactobacillus fermantii*, *Enterobacter aerogen*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Bacillus megaterium*, *Bacillus sphericus*, *Streptococcus mitis.* There was one species of *Bacillus azotoformans,* one species of *Lactobacillus fermentii,* one species of *Enterobacter aerogen,* ten species of *Staphylococcus aureus,* two species of *Staphylococcus epidermidis,* one species of *Staphylococcus saprophyticus,* one species of *Bacillus megaterium,* two species of *Bacillus sphaericans* and one species of *Staphylococcus mitis.*

Identification of bacteria using *16s rDNA* gene

For molecular identification of these bacterial species, *16s rDNA* gene sequence amplification was carried out using colony PCR method. However, only seven bacterial species (*Bacillus azotoformans*, *Bacillus coagulans*, *Corynebacterium kutceri*, *Lactobacillus fermantii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*) gave positive response in colony PCR. Colony PCR product run on 2% agarose gel gave 500 bp *16s rDNA* gene intact band in all the seven organisms (fig. 20). Since, *16s rDNA* gene was not amplified from the rest of the twelve bacterial species using colony PCR, so DNA was isolated from all the 19 bacterial species for accurate amplification of *16s rDNA* gene. Intact bands of genomic DNA were obtained on agarose gel (fig. 19). Purity of DNA samples ranged from 1.2 to 1.7 and concentration of DNA samples ranged from 15 to 245 ng/µl. Further, *16S rDNA* gene was amplified from the DNA samples (fig. 21) and sequencing analysis of gene was done using ABI 3130 genetic analyzer. *16s rDNA* gene sequence of all bacterial species were obtained.

These sequences were further analysed by bioinformatics tools for accurate identification of *16s rDNA* gene. NCBI BLAST tool was used to confirm the percent identity of *16s rDNA* gene sequence with relative bacterial sequence available in the database. All the sequences obtained showed query coverage in range from 80-95% and percent identity in range from 77 to 93%. Identified sequences were submitted in the NCBI database using sequin software. Among 31 sequences submitted accession number of nine sequences were

obtained and other sequences are under communication process.

Mutiple sequence analysis and phylogenetic relationship between sequences

Multiple sequence alignment of *16s rDNA* gene sequences obtained from polluted and non-polluted bacterial species was done with the sequences available in the NCBI database to study the genetic variation among the bacterial species isolated from polluted and non-polluted area. Phylogenetic trees were constructed from the multiple sequence analysis data to gain information about organism's evolutionary relationships. *Bacillus azotoformans* (polluted site) showed an evolutionary relatedness with sequence having accession no. AB014099.1 whereas *Bacillus azotoformans* (non polluted site) showed a relatedness with sequence having accession no. NR_041641.1, AB363732.1, BAC16SRRA, X60609.1 (fig. 22). *Enterococcus faecium* of polluted and non-polluted site were in separate clade but in one cluster. Polluted site *Enterococcus faecium* showed evolutionary closeness with sequences having accession no. HQ259240.1, HQ012007.1, JN104690.1, JN128743.1, JN104688.1, JN128744.1, JN128745.1, and JN104694.1 (fig. 23). Similarly, *S. mitis* of polluted site and non-polluted site was in one cluster but in separate clade. *S.mitis* of non-polluted site was closely related with sequence having accession no. HQ634538.1 (fig. 24). *S. epidermidis* of polluted and nonpolluted site were closely related with each other. They were in same clade and one cluster. Sequence having accession no. AB617539 was evolutionary closely related with *S. epidermidis* of polluted site (fig. 25).

DNA Polymorphism analyses using DnaSP 5.10

This command analysed sequentially several data files. It computed a number of measures of the extent of DNA polymorphism and also performed some common neutrality tests. Haplotype/Nucleotide Diversity was determined by the change in following parameters of DNA polymorphism.

1) The number of Segregating Sites (S)
2) The total number of mutations (Eta)
3) The number of haplotypes (NHap) (Nei, 1987).
4) Haplotype (gene) diversity and its sampling variance (Nei, 1987).
5) Nucleotide diversity (Pi) (Nei, 1987) and its sampling variance (Nei, 1987).
6) The average number of nucleotide differences (k) (Tajima, 1983).
7) Theta (mutation per gene or per site) from (Eta) or from (S) (Watterson, 1975 and Nei, 1987).

Four identical bacterial species (*Bacillus azotoformans*, *Enterobacter faecium*, *Staphylococcus epidermidis* and *Staphylococcus mitis*) were obtained from polluted and non-polluted area. Pairwise alignment of *16s rDNA* sequences obtained from these bacterial species was done. These aligned sequences data was loaded in DnaSP 5.10 software and various parameters mentioned above were calculated. Mutational analysis showed that there were 151, 138, 103 and 195 mutations in *B. azotoformans*, *E. faecium*, *S. epidermidis* and *S. mitis* respectively, which were isolated from polluted area. Nucleotide diversity in *B. azotoformans* was 0.51186, *E. faecium* was 0.41566, *S. epidermidis* was 0.29942, and *S. mitis* was 0.52139. Hence, it was observed that there was remarkable change in the *16s rDNA* sequences at nucleotide level.

Further, multiple sequence alignment of all bacterial species identified was done with the sequences available in the NCBI database and file was loaded in the DnaSP 5.10 software and DNA polymorphism was studied. Bacterial species from non-polluted and polluted area showed a remarkable change in segregating sites (S), total number of mutations (Eta), number of haplotypes, haplotype diversity, nucleotide diversity (Pi), average number of nucleotide differences (k), mutation per gene, and mutation per site was observed.

Tajima's test using DnaSP software

This command calculated the D test statistic proposed by Tajima (1989), for testing the hypothesis that all mutations are selectively neutral (Kimura, 1983). The D test is based on the differences between the number of segregating sites and the average number of nucleotide differences. Tajima's test is based on the neutral model prediction that estimates of S / a1, and of k, are unbiased estimates of q,

Where,

1) S is the total number of segregating sites. a1 = S (1 / i) from i =1 to n-1
2) n= the number of nucleotide sequences
3) k is the average number of nucleotide differences between pairs of sequences (Tajima 1983).
4) q=4Nu (for diploid-autosomal; N and u are the effective population size, and the mutation rate per DNA sequence per generation, respectively).

A positive value of Tajima's D indicated that there has been 'balancing selection' and the data showed a few divergent haplotypes, whereas a negative value

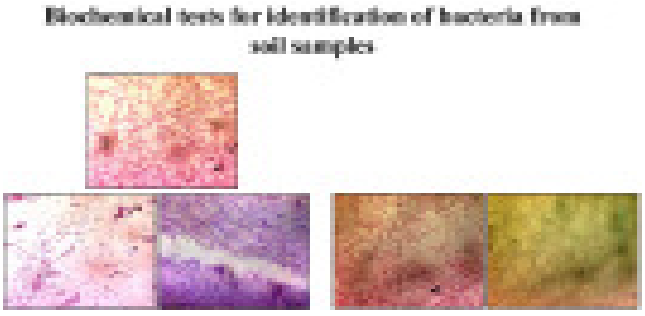

Fig. 1 : Gram staining.

Fig. 2 : Spore staining.

Fig. 3 : Starch hydrolysis test.

Fig. 4 : Glucose phosphate broth test.

Fig. 5 : Optochin test.

Fig. 6 : Casein agar test.

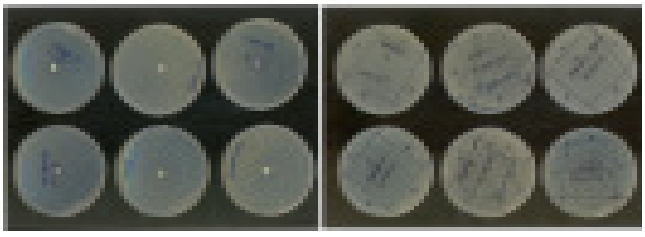

Fig. 7 : Novobiocin test.

Fig. 8 : Gelatin agar test.

suggested that 'purifying selection' have occurred and the data reveal an excess of singletons.

Discussion

Different bacterial genera have been characterized from hydrocarbon polluted soils in different geographical and ecological contexts (Van Hamme *et al*., 2003; Maila *et al*., 2004; Maila and Cloete, 2005; Maila *et al*., 2006;

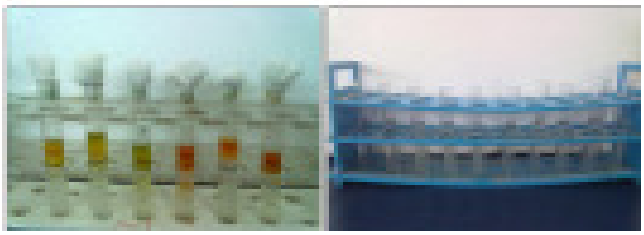

Fig. 9 : Mannitol fermentation test.

Fig. 10 : Nitrate reduction test.

Fig. 11 : Glucose Phosphate broth and 2% peptone test.

Fig. 12 : Citrate test.

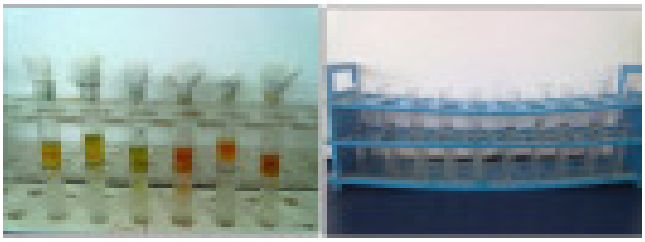

Fig. 13 : Mannitol fermentation test.

Fig. 14 : Nitrate reduction test.

Hamamura *et al*., 2006). Although, experimental and climatic conditions differed considerably in each study, some organisms tend to be common habitants of the hydrocarbon polluted soils. In the present studies, bacterial species identified from the polluted soil samples were *Bacillus azotoformans*, *Bacillus larvae*, *Bacillus alvei*, *Bacillus coagulans, Lactobacillus casei, Corynebacterium xerosis, Corynebacterium kutceri, Enterobacter munditii, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Bacillus marinus, Enterococcus hirae, Enterococcus faecium.* These types of organisms were also isolated and identified from the previous studies done by scientists. Although places differ in the geographical

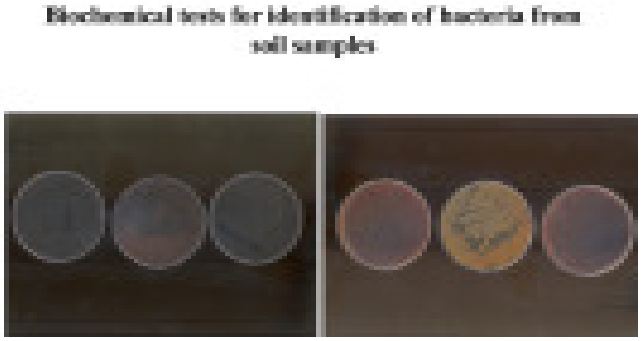

Fig. 15 : EMB test.

Fig. 16 : Mac Conkey's agar test.

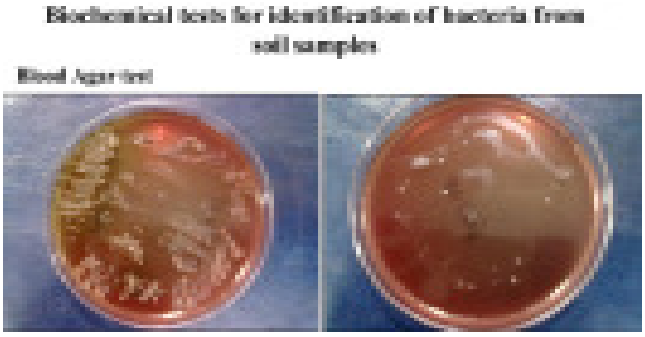

Fig. 17 : 17 alpha haemolysis.

Fig. 18 : Beta haemolysis.

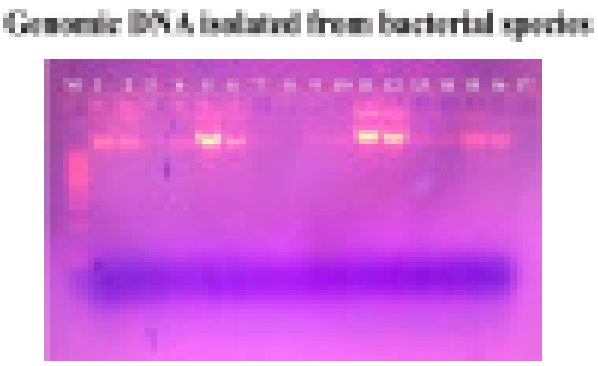

Fig. 19 : Genomic DNA bands on 2% agarose gel.

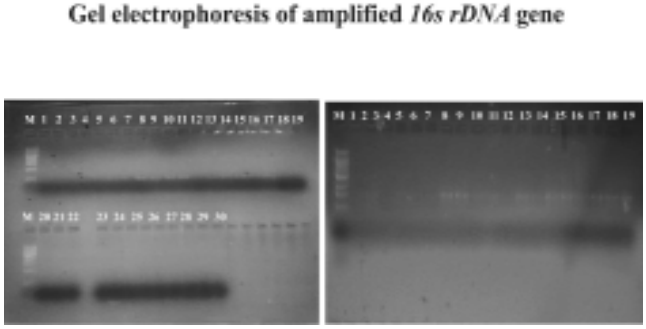

Fig. 20 : Colony PCR.

Fig. 21 : Amplification from genomic DNA.

area, bacteria remained almost similar. Vasileva-Tonkova and Gesheva (2003) had shown the presence of *Corynebacterium* sp. in antartic soils polluted with hydrocarbons. Bahig *et al*. (2008) showed the presence of species of *Bacillus, Micrococcus, Staphylococcus, Pseudomonas*, *Eschershia, Shigella, Xanthomonas, Acetobacter*, *Citrobacter, Enterobacter, Moraxella* and *Methylococcus in* agriculture soil irrigated with waste water from industries at egypt. Manoharan *et al*. (2010) also found the presence of *Staphylococcus* sp., *Micrococcus* sp. *and Pseudomonas* sp. in indigenous soils contaminated with petroleum. Das and Chandran (2010) showed nine bacterial strains, namely, *Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium* sp., *Micrococcus roseus, and Corynebacterium* sp. are capable of degrading crude oil. Okerentugba and Ezeronye (2003) also showed the potential of *Chromobacterium, Flavobacterium, Bacillus, Vibro, Citrobacter, Enterobacter, Micrococcus, Klebsiella, Planococcus, Pseudomonas* and *Camplobacter* species in degradation of petroleum. Austin *et al*. (1977) examined 99 strains of petroleum degrading bacteria, isolated from Chesapeake Bay water and sediment, by numerical taxonomy procedures. Eighty-five percent of the petroleum degrading bacteria examined in this study were defined at the 80 to 85% similarity level within 14 phenetic groups. This groups were identified as *actinomycetes* (mycelial forms, four clusters), *coryneforms*, *Enterobacteriaceae*, *Klebsiella aerogenes*, *Micrococcus* spp. (two clusters), *Nocardia spp.* (two clusters), *Pseudomonas spp.* (two clusters), and *Sphaerotilus natans.*

This similarity in bacterial species surviving in different geographical environment suggests that it is the nutrition availability which makes bacterial species to thrive in particular environment. Contaminants are often potential energy sources for bacteria. They survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche. They have broad range of enzymes that enable them to degrade many chemicals (Madigan *et al*., 1998). They are omnipresent, and are capable of rapid growth when provided with nutrients and conditions favorable for metabolism and cell division. They are involved in catalysis and synthesis of organic matter in the aquatic and terrestrial environments. Many substances, such as lignin, cellulose, chitin, pectin, agar, hydrocarbons, phenols, and other organic chemicals, are degraded by them. The rate of decomposition of organic compounds depends upon their chemical structure and complexity and upon environmental conditions (Madigan *et al*., 1998). Hence, in present studies bacterial species found in automobiles

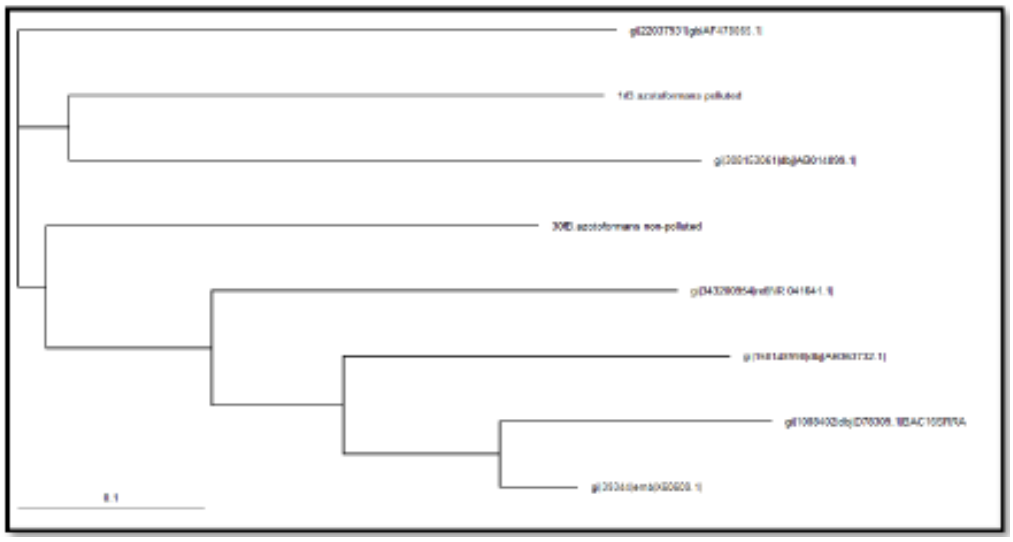

Fig. 22 : Phylogenetic trees showing evolutionary relationship of polluted and non-polluted site *B. azotoformans* with 16s rDNA sequences available in the NCBI database.

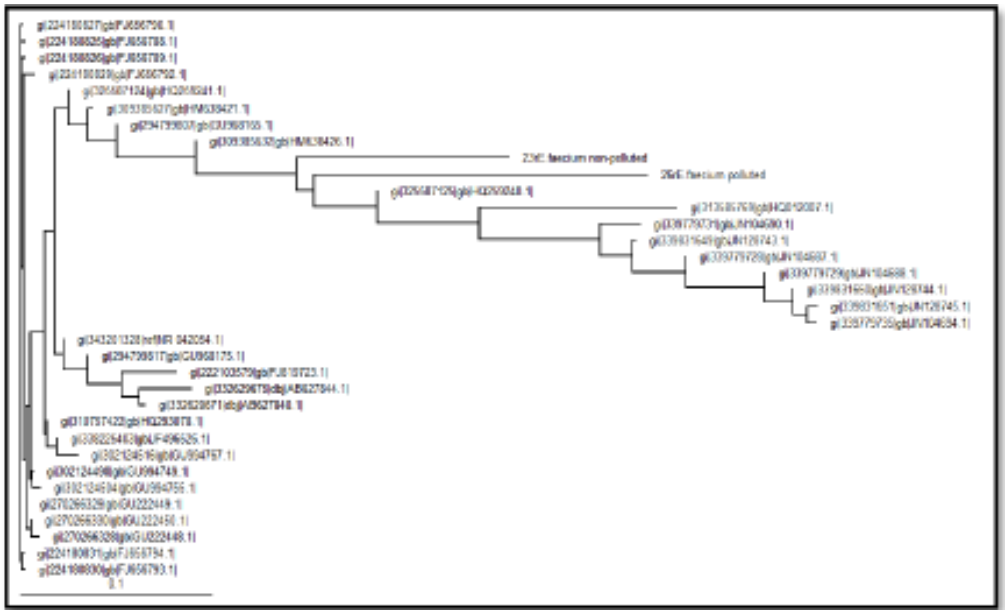

Fig. 23 : Phylogenetic trees showing evolutionary relationship of polluted and non-polluted site *E. faecium* with 16s rDNA sequences available in the NCBI database.

polluted soil were more than that of Saurashtra University campus soil. This result suggests that increase in pollutants of soil is directly proportional to the bacterial population.

Majority of genetic evolutionary/taxonomic work is based on aligning DNA sequences and then reconstructing the phylogeny based on predefined evolutionary models (Woese, 1987; Ludwig *et al*., 1998; Kolaczkowski and Thornton, 2004). Many conserved genes like *gyrase-II*, *pthA*, *rpo*, *nuoB*, *petC* and *16s rDNA* are used as phylogenetic markers for identification and isolation of bacterial populations (Palmieri *et al*., 2010). Among them the use of *16S rDNA* gene was done to study bacterial phylogeny and taxonomy was done because it is the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the *16S rDNA* gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the *16S rDNA* gene (1,500 bp) is large enough for informatics purposes. It has properties, which predestine it as a universal phylogenetic marker (Patel, 2001). Hence, multiple sequence alignment of *16s rDNA* gene sequences was done with the sequences available in the NCBI data base and phylogenetic trees were generated. Remarkable

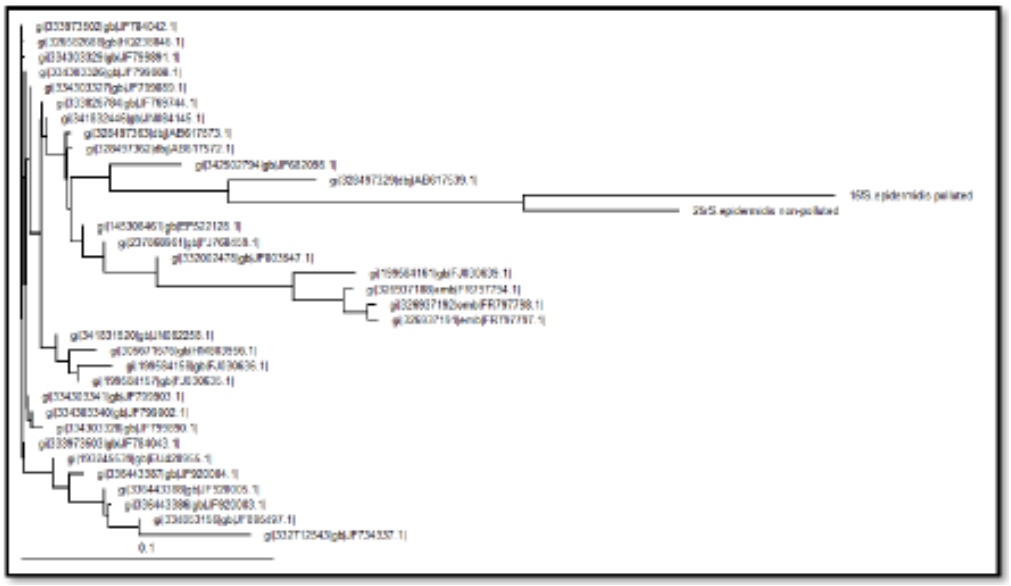

Fig. 24 : Phylogenetic trees showing evolutionary relationship of polluted and non-polluted site *S. epidermidis* with *16s rDNA* sequences available in the NCBI database.

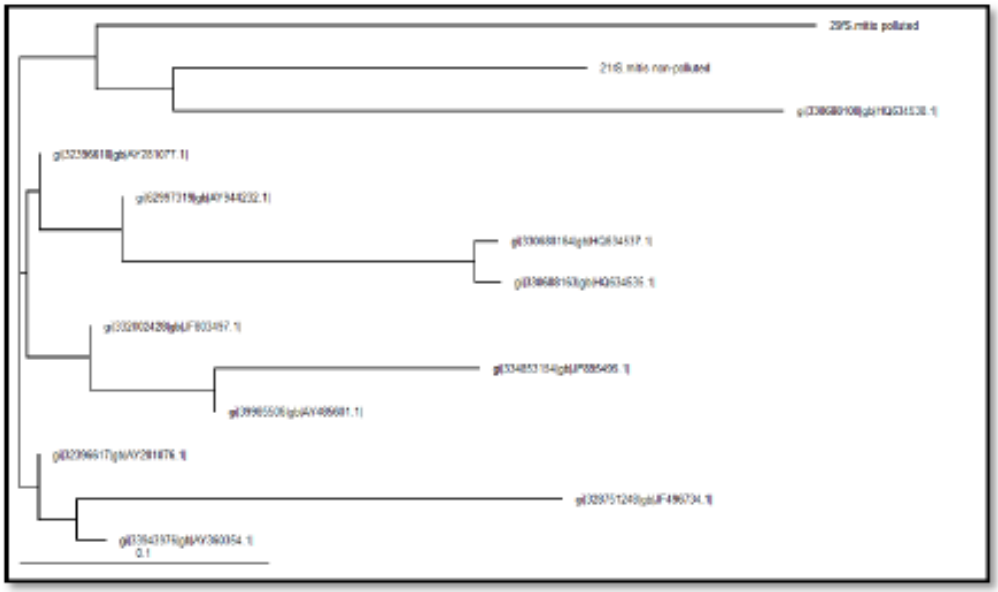

Fig. 25 : Phylogenetic trees showing evolutionary relationship of polluted and non-polluted site *S. mitis* with *16s rDNA* sequences available in the NCBI database.

changes in the clade of neibour-joining tree of bacterial species found in polluted and non-polluted area helped to study the evolutionary pattern among the bacterial species due to change in environmental condition by pollution. Further, pairwise alignment between *16s rDNA* sequences of bacterial species isolated from polluted and non-polluted area helped to study the nucleotide diversity among the species using DnaSP software. Nucleotide diversity is a measure of genetic variation. It is usually associated with other statistical measures of population diversity and is similar to expected heterozygosity. This can be calculated by examining the DNA sequences directly or may be estimated from molecular marker data. Once the nucleotide diversity has been estimated, it can be found out whether adaptation has potentially played any role in influencing these sequence changes.

Tajima's test, or D-test statistic (Tajima, 1989) test is the neutral theory of molecular evolution (Kimura, 1983). This was done to study the evolutionary changes among the organisms due to adaptation at molecular level. This test suggests that vast majority of molecular differences that arise through spontaneous mutation do not influence the fitness of the individual. A corollary to this theory is that the genomes evolve primarily through the process of

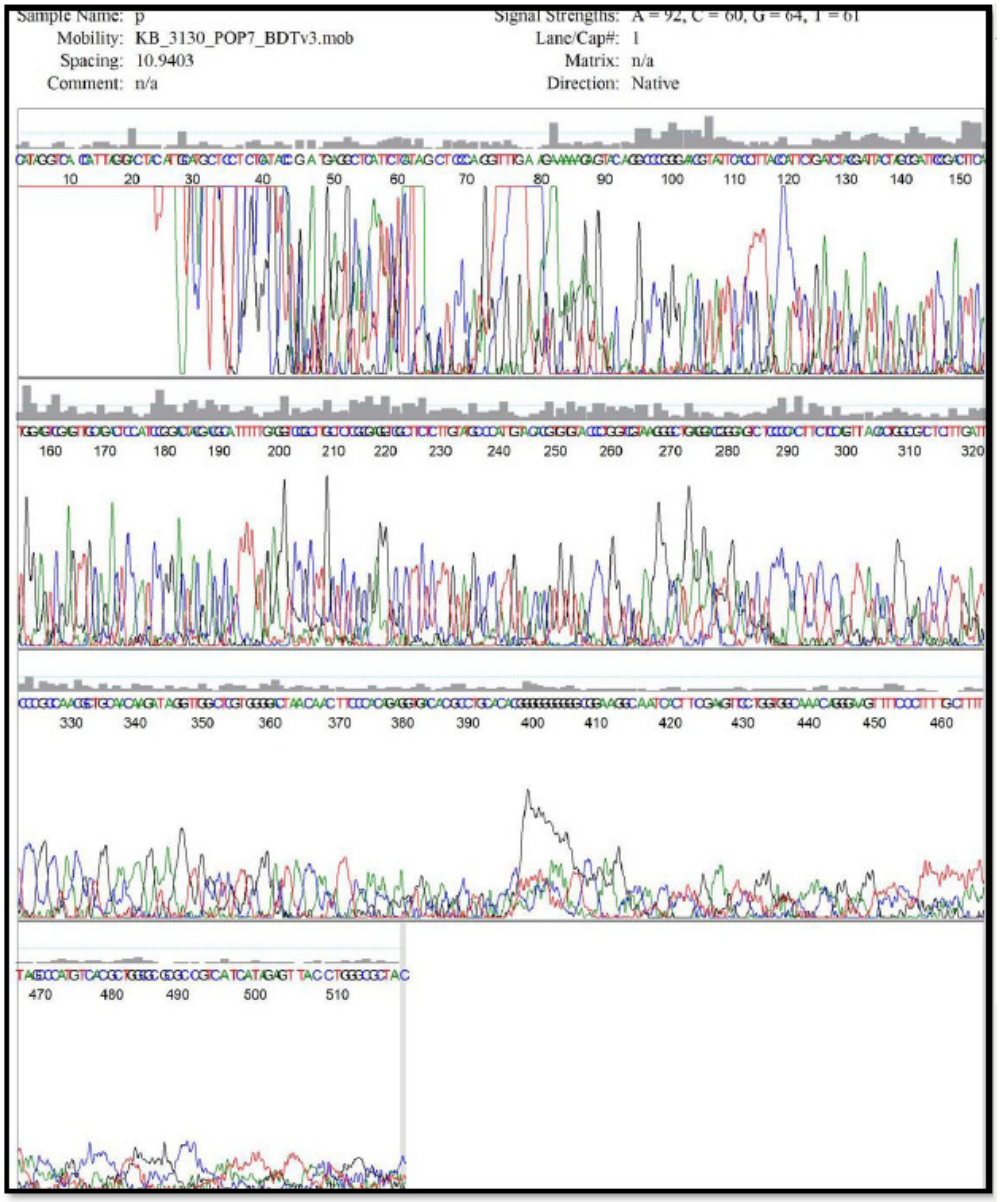

Fig. 26 : Chromatogram of *16s rDNA* sequence.

genetic drift. This statistic is used to monitor diversity within or between ecological populations, to examine the genetic variation in related species or to determine evolutionary relationships. Tajima's D statistic compared the difference between two estimates of the amount of nucleotide variation, one being simply the number of segregating sites (Watterson, 1975) and the other one being the average number of pairwise differences (Nei and Li, 1979; Tajima, 1983).

From present studies, it was concluded that different bacterial species showed wide variations among the polluted and non-polluted samples studied from the selected area than that of closely related sequences derived from the database. Nucleotide variations and significant changes in the è and D values of the sequences in comparison with the related sequences derived from the nucleotide database suggested that the pollution might have lead bacterial species towards adaptation at morphological and genetic level. The same genus and species surviving in the different areas (Polluted and non-polluted) showed differences in clade and phylogenic relationships for *16s rDNA* gene sequences. The analysis of DNA polymorphisms of *16s rDNA* sequences provided insights into the evolutionary forces acting on populations and species due to change in environmental condition by pollution. Nucleotide diversity in the *16s rDNA* gene sequence of bacterial species isolated from polluted and non-polluted soil samples was determined. Although

sequences data for the *16s rDNA* gene is highly conserved in different microorganisms and has been shown to be very accurate for identification of bacteria at genus and species level, it showed variation at genetic level in bacterial species surviving in different environmental conditions. Previous studies has shown that the regions on the *16s rDNA* are quite conserved and others are variable (Kullen *et al*., 2000). These variable regions might help microorganisms to adapt the environmental changes due to pollution. However, further analysis is required to prove this fact.

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