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IDENTIFICATION OF THE BACTERIAL COMMUNITY RESPONSIBLE FOR DECONTAMINATING ELEME PETROCHEMICAL INDUSTRIAL EFFLUENT USING 16S rDNA PCR DENATURING GRADIENT GEL ELECTROPHORESIS

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ABSTRACT
Identification of bacteria community responsible for decontaminating Eleme petrochemical industrial effluent using 16S PCR denaturing gradient gel electrophoresis (DGGE) was determined. Gene profiles were determined by extracting DNA from bacterial isolates and amplified by polymerase chain reaction (PCR) using 16S rDNA eubacterial primers with a GC clamp. PCR amplified DNA was separated by Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Comparative evaluation of primers for PCR amplification of DNA revealed that V6V8F and V6V8R primers were most suitable for amplification DNA. Denaturing gradient gel ratio of 20% : 80% was most suitable for differentiation of bacterial profiles. Sequencing and phylogenetic analysis of DGGE products were successful displaying 88% -100% similarity and homology to Roseococcus sp. LW5(FM956480.1). Dendograms and similarity matrix revealed 81.5% -100% from sampling points.

Key words: *Petrochemical industrial effluent; polymerase chain reaction; Denaturing gradient gel electrophoresis (DGGE); Deoxyribonucleicacid (DNA);Primers*

1. INTRODUCTION
The Company processes natural gas liquids, which occur in association with crude oil, made up of paraffins and olefins that can be combined to form desired petrochemicals. These products that serve as raw materials in the downstream industries for the production of plastics and other related products come out as crystalline thermophilic resins in the form of pellets. Additives, including antioxidants, stabilizers are added to polymers just before pelletizing to improve its resistance and quality. These activities generate different forms of wastes, which eventually end up in the environment with or without treatment. The Eleme Petrochemical industrial effluent take various forms which include; processed wastewater (raw effluent);clarified water (effluent undergoing treatment); retention pond gate(treated waste water) and receiving river. So also are the wastewaters generated as a result of office or domestic activities in the company (domestic wastewater or sewage)[1].
Studies on Eleme Petrochemical effluents have revealed that although effluents from Eleme Petrochemical Company Limited generally contain relatively low concentrations of pollutants in the water and sediment, accumulation of these pollutants overtime can be fatal to aquatic and human life [2].
The unabated accumulation of these pollutants overtime in aquatic and terrestrial environments may be fatal to wildlife and man. This therefore makes imperative the need for early resolution of the problem of treatment for Eleme petrochemical effluent[2].This dire need in recent times has found microorganisms quite instrumental. Microorganisms have been implicated in showing strong ability to bio-treat petrochemical effluents. The potentials of microorganisms to catabolize and metabolize xenobiotic compounds have been recognized as potentially effective means of toxic and hazardous wastes disposal.

2. MATERIALS AND METHODS
Eleme Petrochemical Company Limited (EPCL) is situated in Eleme, Rivers state in the oil-rich Niger Delta area of Nigeria. It was established by the Federal

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Government of Nigeria in 1988.The major feedstock used in the company is delivered to it in liquid form via pipeline from the liquefied natural gas (LNG) plant located at Obiafu/Obrikom in Rivers state. The feedstock is free from methane, but composed of ethane, propane and butane with minor quantities of pentane and heavier hydrocarbons. The major products of the company are low density and linear low density polyethylene (LLDPE), polypropylene (PP), vinyl chloride monomer, butane and mixture of other olefins. Effluents are usually treated with sulphuric acid, caustic soda, alum, urea, Di-ammonium phosphate, anionic polyelectrolyte and calcium hypochlorite. Thereafter, the treated effluent is directly discharged into receiving river bodies.

The Eleme River in Eleme kingdom took its source at Oyigbo and flows down Agbonchia farm settlement, Njuru, Okerewa and Aluto at which point the petrochemical effluent is discharged into it before entering into tidal creek by NNPC housing estate Aleto and flows down to Okrika. The river passes through sparse vegetation and its course flows across many roads and as such receives storm water runoffs from roads too.

2.1 Sample collection

Samples were collected once a month between July 2007 and March 2008.Water samples were collected with a 2- liter plastic hydro-bios water sampler and transferred to clean 2-liter polyethylene containers and 250ml capacity borosilicate glass bottles as described by [1]. The effluent samples include the process wastewater(PWW) (untreated effluent), clarified water(CW),retention pond gate (RPG),which is the industrial effluent that has undergone both chemical and biological treatment to eliminate or reduce waste contents to acceptable levels and the receiving river(RR) of Eleme Kingdom. These were collected in polyethylene containers and borosilicate bottles of the same capacity [1]. They were rinsed several times with water or effluent samples at the point of collection. The samples were transported to the laboratory using iced packed coolers after appropriate labeling.

2.2. DNA extraction from petrochemical industrial effluent

Zymoclean™ Gel DNA Recovery Kit (catalog nos.D4001, D4002, D4007&D4008) was used for extraction of DNA from effluent samples. Effluent samples were filtered using microfunnel™filter unit with super ^{R} membrane (0.24μm). The effluent samples were collected from the processed wastewater (PWW), clarifier (CW), retention pond gate (RPG) and receiving river(RR) from Eleme petrochemical industrial effluent. The effluent was filtered in triplicates of 200ml each. After filtration, the filter membrane was aseptically removed. Using sterile forceps, the filter membrane was picked and inserted into a water bead tube. Four milliliters (4ml) of bead solution was added to the water bead tube and vortexed for 30seconds.Five hundred micronlitre (500μl) of solution WD1 was added to each bead tube and vortexed for 30seconds to mix. The water beads were then secured firmly on a flatbed vortex pad with tape. It was then vortexed at maximum speed for 10minutes taking care to make sure the tape does not loose. After vortexing, it was centrifuged at 2500×g for 1minute.The supernatant was then transferred to a clean 15ml centrifuge tube (about 3.2ml of supernatant was transferred).Six hundred microlitres of solution WD2 was then added to the supernatant and vortexed for 5 seconds. It was then incubated at 4^{o}C for 5 minutes, the tubes were then centrifuged for 4minutes at 2500xg.Avoiding the pellet, the entire volume of supernatant was transferred into another clean 15ml centrifuge tube(approximately 4ml was recovered).Eight milliliter(8ml) of solution WD3 was added to the supernatant and vortexed for 5 seconds. The supernatants were then loaded into 50ml spin filter tubes and centrifuged at 2500xg for 2minutes.The flow through was discarded. Three millilitres of solution WD4 was added and centrifuged for 3minutes at 2500×g, flow through discarded. It was re-centrifuged for 5 minutes at 2500×g and being careful not to splash liquid on the filter basket, the spin filter was placed into a new 50ml collection tube provided .Three millilitres of solution WD5 was directly added to the center of the filter membrane and tubes re-centrifuged for 2minutes at 2500×g.The spin filter were then discarded. The DNA in the tube were then stored at -20^{o}C.

2.3. PCR amplification of 16S rDNA for petrochemical industrial effluent bacterial isolates

Different regions of the 16S rDNA were amplified with different primers in order to determine the primers that provided the best DGGE differentiation of the microbial community responsible for decontaminating petrochemical industrial effluent.

2.3.1 V3 PCR Amplification

One set of primers amplifying the variable V3 region(V3F and V3R) as described by [3] was used to amplify 16S rDNA from the petrochemical industrial effluent bacterial isolates. The V3 region of the 16S rDNA was amplified using V3 Reverse primer(518R-primer V3 16s MWG-Biotech,5'-ATT ACC GCG GCT GCT GG-3') and V3 Forward primer(338F-primer,5'-ACT CCT ACG GGA GGC AGC AG-3') with a GC clamp(CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC G)[4] in order to increase DGGE separation. Amplification was performed in a programmable heating thermocycler(C1000 Thermal Cycler).

The reaction mixture(50µl total volume for each sample) for the PCR was composed as follows: 25µl of 2×master mix(2×mm),0.2µl of forward and revise primers,1.5µl of template DNA and 23.3µl of nuclease free water.PCR amplification was performed as follows:5 min at 94^0C to denature template DNA,10 cycles decreasing by 1^0C each round from 66^0C primer annealing,20 cycles at 56^0C for amplimer extension,3 min at 72^0C,and then the final extension was carried out at 72^0C for 10 min.

The PCR product was analyzed by electrophoresis on 1% TAE agarose (Agarose 3:1,Melford Laboratories Ltd., Ipswich,UK) gel containing ethidium bromide (0.2µg/ml) in 1x TAE running (40Mm Tris base,20mM acetic acid,1mM EDTA) at 75 volt for about 90 min. Samples contained 10µl of PCR product and 2µl of loading dye (Promega).The gel was viewed under a UV transilluminator (GelDoc XR, Biorad) and the gel pictures were recorded by using the GelDoc system (The Quantity one 4.6.5 Basic software, USA).

2.3.2 16*S PCR Amplification*

One set of primers amplifying the 16S rDNA fragments as described by [5] was also used. The 16S rDNA fragments were amplified with primers 16S forward primer(5'-AGA GTT TGA TYM TGG CTC AG-3') and 16S reverse primer(5'-ACG GYT ACC TTG TTA CGA CTT-3'). Amplification was performed in a programmable heating thermocycler (C1000 Thermal Cycler).

The reaction mixture(50µl total volume for each sample) for the PCR was composed as follows: 25µl of Dream Taq™Green PCR Master Mix(2x),2µl of forward and revise primers,1µl of template DNA and 21µl of nuclease free water and 1µl of $MgCl_2$.PCR amplification was performed as follows:3 min at 95^0C for initial denaturation of template DNA,1 cycle; 30s at 95^0C for denaturation, 30s at 5^0C for primer annealing,1min at 72^0C for extension,25-40 cycles;and15 min at 72^0C for the final extension, I cycle .

The PCR product was analyzed by electrophoresis on 1% TAE agarose(Agarose 3:1,Melford Laboratories Ltd., Ipswich, UK) gel containing ethidium bromide (0.2µg/ml) in 1x TAE running (40Mm Tris base,20mM acetic acid,1mM EDTA) at 75 volts for about 90 min. Samples contained 10µl of PCR product and 2µl of loading dye (Promega).The gel was viewed under a UV transilluminator (GelDoc XR, Biorad) and the gel pictures were recorded by using the GelDoc system (The Quantity one 4.6.5 Basic software, USA).

2.3.2 V6-V8 PCR Amplification

One set of primers amplifying the V6-V8 region of 16S rDNA was also used as described by [6].The V6-V8 region was amplified with primers V6V8 forward primer(5'-AGC AGT AGG GAA TCT TCA-3') and V6V8 Reverse primer(5'ATT TCA CCG CTA CAC ATG-3') with a GC clamp(CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC G) [4]in order to increase DGGE separation. Amplification was performed in a programmable heating thermocycler (C1000 Thermal Cycler).

The reaction mixture(50µl total volume for each sample) for the PCR was composed as follows: 25µl of Dream Taq™Green PCR Master Mix(2x),2µl of forward and revise primers,1µl of template DNA and 20µl of nuclease free water and 2µl of $MgCl_2$.PCR amplification was performed as follows:2 min at 95^0C for initial denaturation of template DNA,2 cycles; 30s at 95^0C for denaturation, 35 cycles;40s at 56^0C for primer annealing,1hr at 72^0C for extension, and 5 min at 72^0C for the final extension.

The PCR product was analyzed by electrophoresis on 1% TAE agarose (Agarose 3:1,Melford Laboratories Ltd., Ipswich, UK) gel containing ethidium bromide (0.2µg/ml) in 1x TAE running (40Mm Tris base,20mM acetic acid,1mM EDTA) at 75 volts for about 90 min. Samples contained 10µl of PCR product and 2µl of loading dye (Promega).The gel was viewed under a UV transilluminator (GelDoc XR, Biorad) and the gel pictures were recorded by using the GelDoc system (The Quantity one 4.6.5 Basic software, USA).

2.4. Denaturing gradient gel analysis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was performed using a Bio-Rad D-Code Universal Mutation Detection System. All DGGE reagents were from Severn Biotechnology Ltd, Worcestershire, UK. Two different denaturing gradients 30%:50% and 20%:80% urea-formamide [7,16] were used to make

the denaturing solutions for DGGE gel in order to select the most appropriate denaturing gradient for microbial community decontaminating petrochemical industrial effluent.

Before making the gel,0% denaturant solution and 100% denaturant solution were prepared. Zero percent denaturant solution contained 40% acrylamide/bis,50X TAE buffer, and deionised water while 100% denaturant solution contained urea,50X TAE, 40% acrylamide/bis and deionised formamide (see appendix for constitution).

This analysis was started from preparing the sealing gel:1.5 ml of 0% denaturant solution,13.5µl of 10% ammonium persulphate (APS),and 1.35µl of N,N,N,N-tetramethyl-ethylenediamine (TEMED) were mixed and then 750µl was pipetted into each corner of the glass plates to seal it. For the 30%:50% denaturant gradient after 30min of sealing of plates, high denaturant solution(7.5 ml of 0% denaturation solution,7.5ml of 100% denaturant solution,150µl of DGGE dye,135µl of 10%APS and 13.5µl of TEMED) and low denaturant solution (10.5ml of 0% denaturing solution,4.5ml of 100% denaturing solution,135µl of 10% APS and 13.5µl of TEMED) were added into the glass plates using a gradient maker (Bio-Rad model 385).The 20%:80% denaturant gradient was prepared with high denaturant solution(3ml of 0% denaturing solution,12ml 100% denaturation solution,150µl of DGGE dye,135µl of 10%APS and 13.5µl of TEMED) and low denaturant solution(12ml of 0% denaturing solution,3ml 100% denaturing solution,135µl of 10%APS and 13.5µl of TEMED) added into plates also using the gradient maker (Bio-Rad model 385).After insertion of the 16-well comb on each glass plates, the gel was left at room temperature for 1hr to solidify before storing overnight at 4^0C.

Samples containing equal amounts of PCR amplicons (20µl of PCR products +20µl of loading dye) and marker (10µl of ladder +10µl of loading dye) were loaded into the gel wells. Electrophoresis was run in 1x TAE buffer at 60^0C,for 20 min at 20V and subsequently for 16 h at 60 V [8].After electrophoresis, the gels were stained for 30min with 2ul of Gelstar diluted in 20µl if 1x TAE. Stained gels were visualized in a GelDoc System (Bio-Rad).

2.5 Excision, re-amplification and purification of DGGE bands

DGGE bands were carefully excised on an UV trans-illuminator with a sterile scalpel and subsequently transferred to a sterile1.5 ml tube (Eppendorf tube) containing 20µl of TE buffer and stored at 4^0C overnight to allow diffusion of the DNA. The samples were then centrifuged at 12,000 rpm for 10min. One microlitre of the supernatant was subsequently used for re-amplification of the DNA fragment. The re-amplification of DGGE products used the same PCR reaction mixture and conditions as stated above but without a GC-clamp. Electrophoresis was run at 75V for 1 h using 5 µl of re-amplified products on a 1% TAE agarose gel containing ethidium bromide(0.2µg/ml) to check product recovery and to estimate product concentration. The gel was again viewed and images recorded using the GelDoc system.

Distinct bands were cut out of the gel with sterile scalpel into sterile 1.5ml tubes and kept at -20^0C until use. Cut DNA were purified using a Zymoclean Gel DNA Recovery Kit (The Epigenetic Company; Cambridge, UK).The instructions of the kit were modified as follows:500µl of ADB buffer added to each volume of gel. The sample was incubated at 55^0C for 30min.The melted agarose solution was added into Zymo-spin column and put into a collection tube before centrifugation for 30seconds at 13,000 rpm. The collection tube was then emptied. Wash buffer(200µl) was added to the column and centrifuged for 30sec at 13,000rpm.The step was repeated thrice. The Zymo-spin column was then placed into a new 1.5ml (Eppenndorf tube) and 10µl of nuclease-free water was added directly to the column matrix and re-centrifuged to elute the DNA. Finally, the purified DNA product was kept at -20^oC until use.

2.6 DNA sequencing

Ten microlitre(10µl) of eluted DNA was diluted in 10µl of nuclease free water, while the 16S V6V8F primer was prepared with 2pmol/µl concentration and sent to MWG Company in Germany (Eurofins MWG, Operon Sequencing Department, Anzinger Str.7a, 85560 Ebersberg, Germany) for sequencing. The result of DNA sequences were analyzed by using BLAST (Basic Local Alignment Search Tool) search programme.[17]

3. RESULTS

The results presented here deals with comparative evaluation of primers for pcr amplification of DNA from petrochemical industrial effluent isolates

Plates 1-3 shows the results of three different sets of primers(16SF & 16SR; V3F & V3R & V6-V8) stained

on 1% agarose gel to determine the primers that will provide the best DGGE differentiation of bacterial isolates from petrochemical industrial effluent. V6V8F & V6V8R primers enabled all the 16S rDNA fragments of bacterial isolates to be amplified (plate3) with the expected size of 500bp.V3F&V3R had 200bp size (plate2) and 16S and 16SR (plate1) could not enable the isolates to all be amplified.

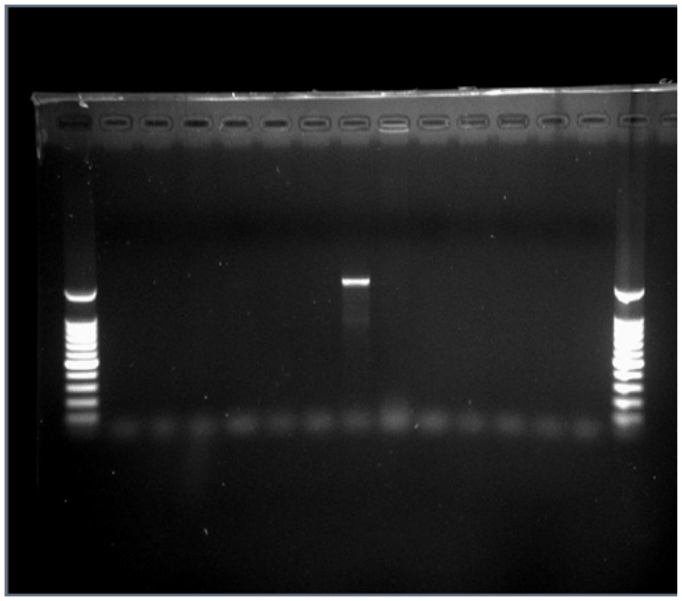

Plate 1: Ethidium bromide-stained 1% agarose gel showing product size using 16SF and 16SR primers for amplification of 16S region of I6S Rdna of bacterial isolates from petrochemical industrial effluent.

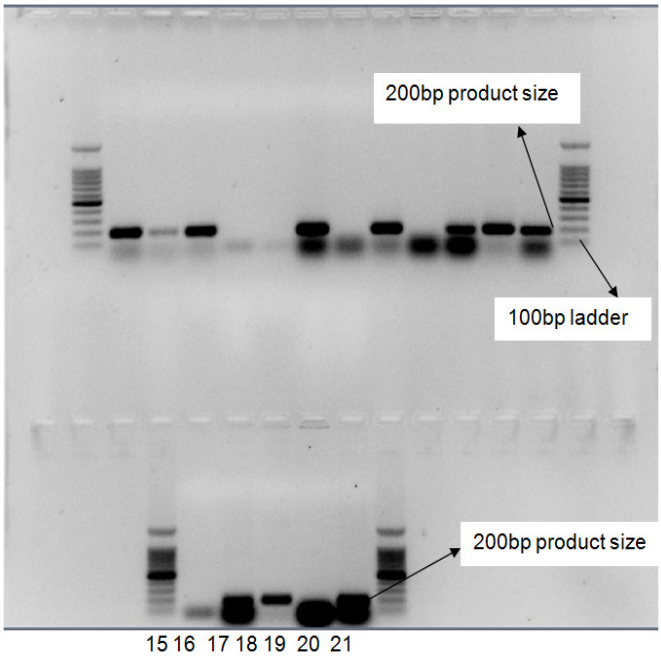

Plate 2: Ethidium bromide-stained 1% agarosegel showing product size using V3F &V3R primers for amplification of V3 region of 16S rDNA of isolates from petrochemical industrial effluent.

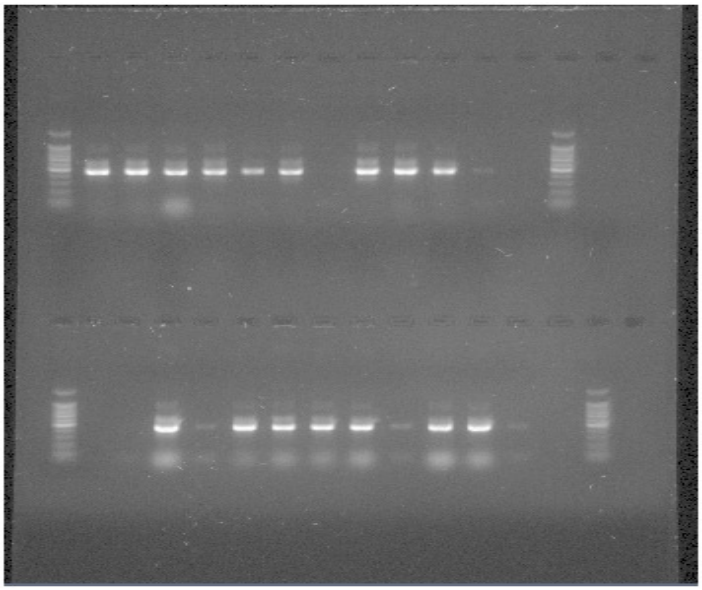

Plate 3: Ethidium bromide-stained 1% agarose gel showing product size using V6-V8F& V6-V8R primers for amplification of V6-V8 region of 16S rDNA of isolates from petrochemical industrial effluent

3.1 Denaturing gradient gel analysis (DGGE) of 16s rDNA of bacterial community from petrochemical industrial effluent

Plates 4-7 shows the DGGE profiles of the PCR products different primers obtained from bacterial community from petrochemical industrial effluent using two denaturing gradients. Plate 4 shows PCR products from GCV3F and V3R primers run on 20% to 80% denaturing gradient, plate 5, PCR products of GCV6V8F and V6V8R primers run on 30% to 50% denaturing gradient and plates6 and 7, PCR products from GCV6V8F and V6V8R primers on 20%-80% denaturing gradient. Plates 6 and 7 had a better DGGE differentiation pattern as compared to plates 4 and 5.

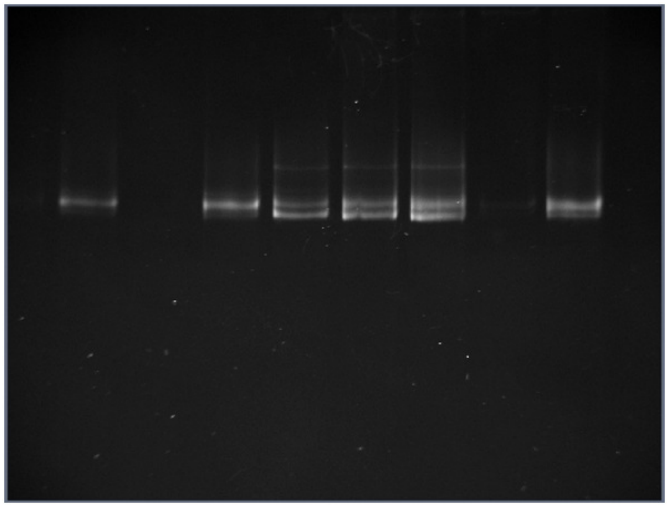

Plate 4: 20%:80% DGGE fingerprints of PCR- amplified region using GCV3F and V3R of 16S rDNA from effluent bacterial communities. Lanes:1-3,effluent samples from river; 4-6 effluent from clarifier, and 7and 8samples from retention pond gate.

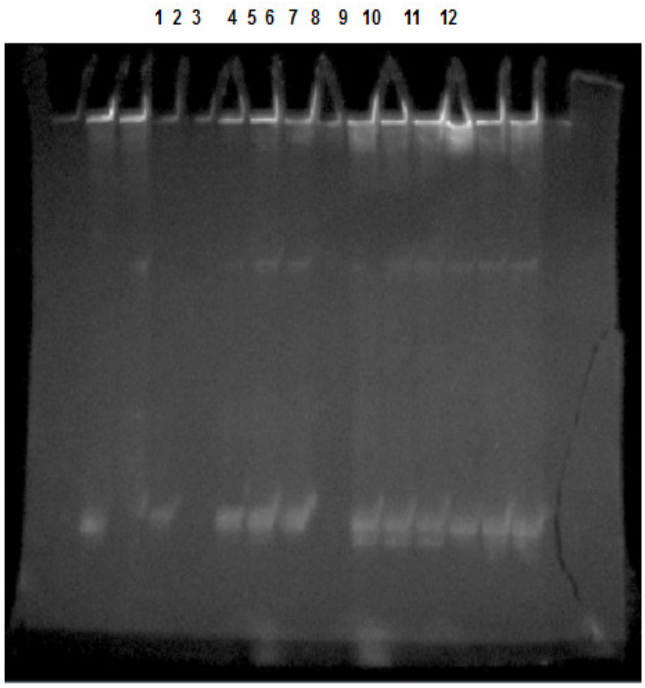

Plate 5: 30%: 50% DGGE fingerprints of PCR- amplified region using GCV6 –V8F and V6-V8R of 16S rDNA from effluent bacterial communities. Lanes: 1-3, effluent samples from clarifier; 4-6samples from river; 7-9, samples from processed water and lanes 10-12, samples from retention pond gate.

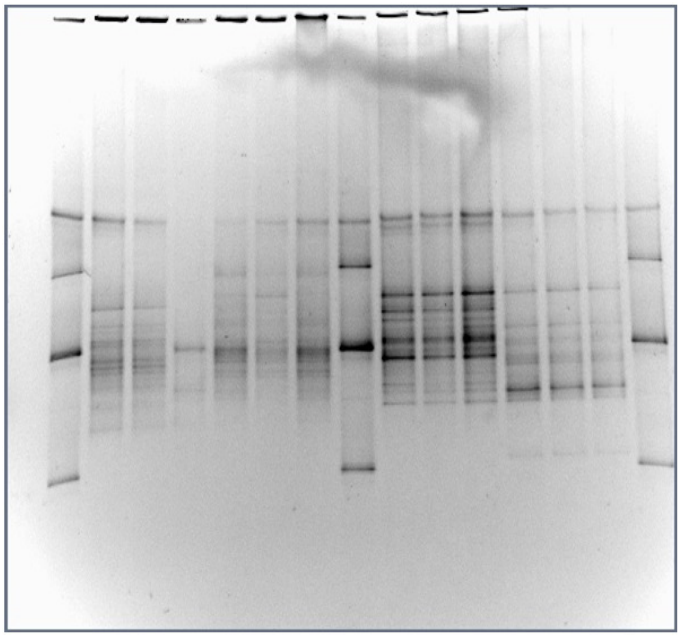

Plate 6: 20%:80% DGGE fingerprints of PCR- amplified region GCV6 –V8F and V6-V8R 16S rDNA from effluent bacterial communities. Lanes:1, 7 and 15,marker;2-4,effluent samples from clarifier;5,6 and 8samples from river;9-11,samples from processed water and lanes 12-14,samples from retention pond gate.

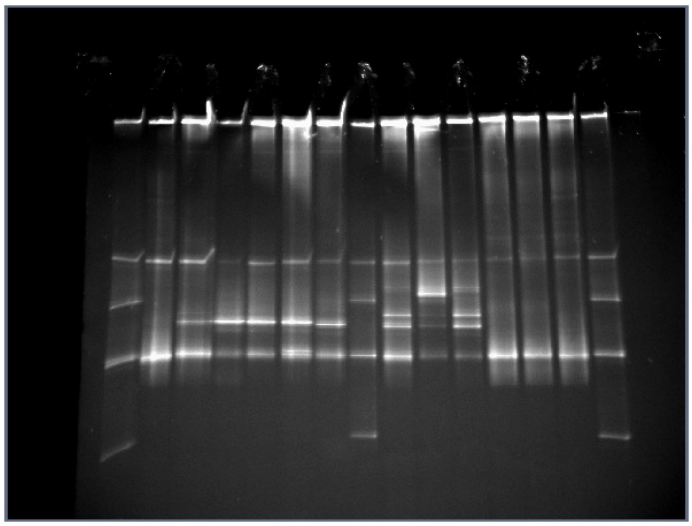

Plate 7: 20%:80% DGGE fingerprints of PCR- amplified region GCV6 –V8F and V6-V8R 16SrDNA of bacterial isolates from petrochemical industrial effluent.Lanes:1, 7 and 15, marker;2 ,*Enterobacter*;3, *Bacillus*;4, *Bacillus*;5, *Bacillus*;6, *Bacillus*; 8, *Enterococcus*; 9, *Lactobacillus*;10, *Enterococcus*;11, *Pseudomonas*; 12, *Streptococcus*;13, *Staphylococcus* and14, *Staphylococcus*.

3.2 Re-amplified PCR products (V6V8F and V6V8R) of DGGE Bands for Sequencing

Plates 8 and 9 shows the images of PCR products on1% ethidium bromide agarose gel of bacterial community from petrochemical industrial effluent from DGGE bands excised for sequencing. Fig 8 depicts excised bands of isolates from clarifier and receiving river while Fig 9 depicts cut bands from process wastewater and retention pond gate. They all had the required base pair of 500.

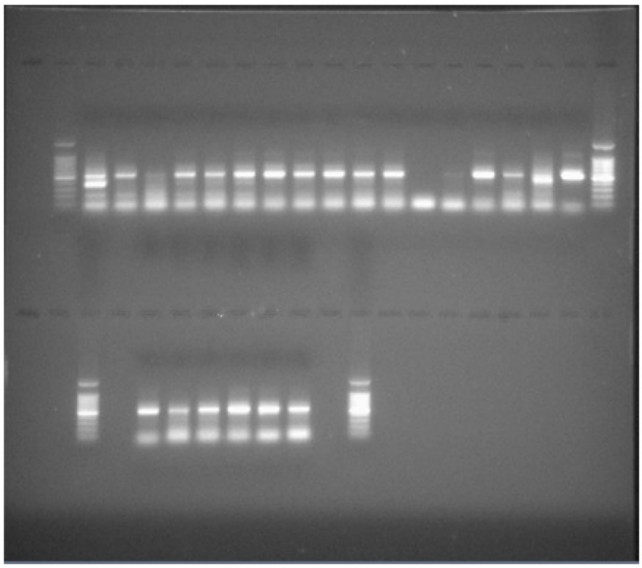

Plate 8: V6-V8 region of PCR products of bands excised from DGGE fingerprints of petrochemical industrial effluent stained with ethidium bromide on 1% agarosegel.Lanes: 1, 19, 20 and 27, 100bp DNA ladder; 2-16 bacteria community in clarifier; 17, positive control; 18, blank; 21-26, bacterial community from receiving river.

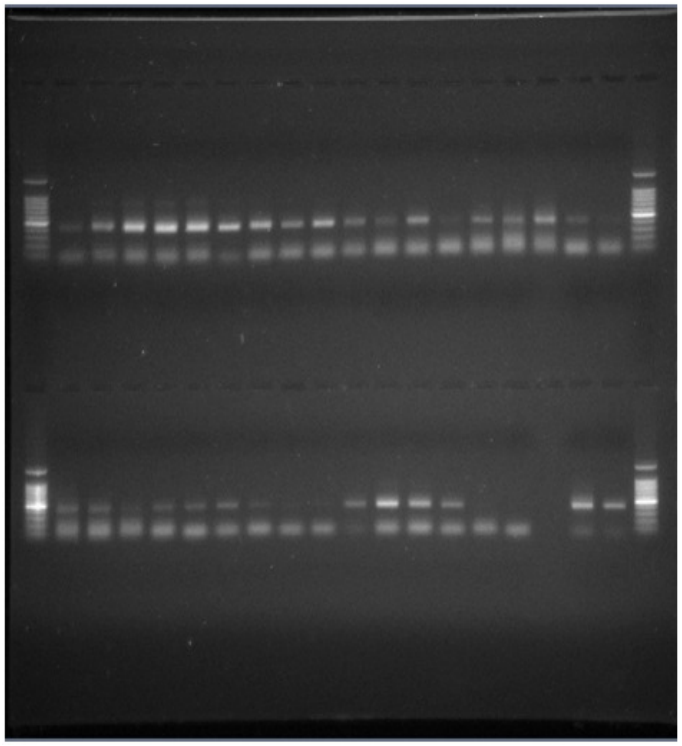

Plate 9: V6-V8 region of PCR products of bands excised from DGGE fingerprints of petrochemical industrial effluent stained with ethidium bromide on 1% agarose gel.Lanes:1,20,21 and 39,100bp DNA ladder; 2-19, 22-25 bacterial community in process wastewater;26-36,bacterial community from retention pond 37,positive control and38 blank .

3.3 Dendrograms and similarity matrices of DGGE gels of petrochemical industrial effluent and bacterial isolates

Figure 1 shows the dendogram and similarity matrixes for bacterial community in petrochemical industrial effluent .

3.4: Comparative sequence analysis for DGGE bands obtained from petrochemical industrial effluent bacterial community

Tables 1 and 2 depicts the results of basic local alignment search tool(BLAST) for DGGE bands excised from the various sampling points petrochemical industrial effluent and receiving river. The similarities of the bacterial 16S rDNA sequences with published data ranged from 97% to 100%.The organisms retrieved from process wastewater samples (Table1) included the following bacterial species: *Enterococcus durans*, *Enterococcusfaecalis*, *Pseudoho-bacterincheonensis*, *Roseococcus*sp and uncultured bacilli. Retrieved bacterial species from Clarifier include; *Lactobacilluscasei* and *Staphylococcus equorum*; Retention pond gate with the organisms *Staphylococcus equorum*, uncultured freshwater bacterium and uncultured *Lactococcus*. Receiving river had *Providenciavermicola, Enterococcusfaecium*, *Lactococcuslactis* and *Lactobacillusplantarum* (Table 2)

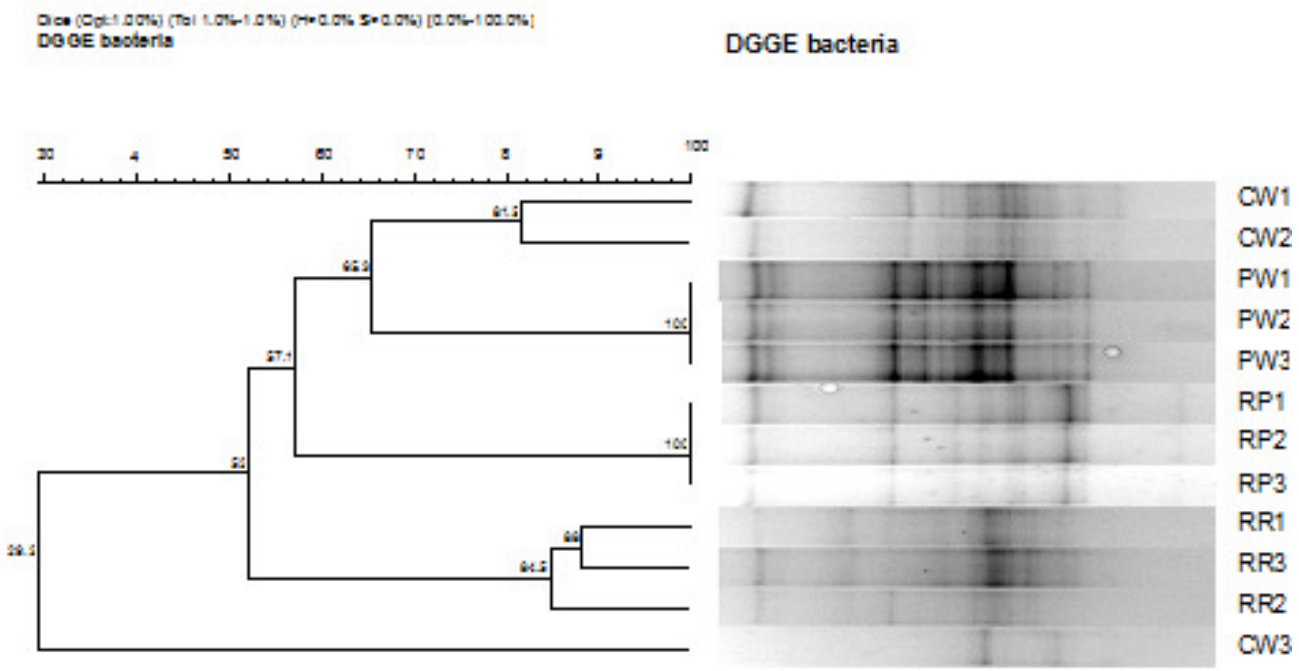

Figure 1: Dendrogram showing the relationship between bacterial communities from the different sampling points of petrochemical industrial effluent and receiving river.

Key: CW-Clarified water; PW- Process wastewater; RP-Retention pond gate; RR- Receiving river

Table1: Sequence similarity of excised DNA fragments of process wastewater bacterial community from the bacterial 16S rDNA clone library.

Sample ID	Closest Relative	Accession number	% Similarity	Sequence
PWW	*Enterococcus*sp Enrichment Culture clone AVCTGRB10A	HM346202.1	99%	CGAGCACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGT TAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAA AGCC ACTAACTACGTGCGAGCAGCCGCGGTAATAAAGCTGGCT
	Uncultured Bacilli Bacterium Clone MS166A1_D09	EF700564.1	99%	AGAAAGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAA CTGT TCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCGAGCAGCCGC GGT AATAAAACC
	Enterococcusdurans Strain R0-37	HQ603863.1	99%	GCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGG AAAC AGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTT CGG GTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCAC CAA GGCCACGATGCATAACCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAAACACA GCC
	Enterococcus faecalis Strain 45689	JF903802.1	99%	TATAATGCAGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAAG AGT GGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTG GAAA CAGGTGCTAATACCGTATAACAATGGACA
	*Rhodobacter*sp AM1R2	JN597282.1	97%	GGTCTTTCTTTCGGCTGGATCCACACAGGTGCTGCATGGCTGTCCTCAGCTCGTGTC GTG AGATGTTCGGTTAAGTCCGGCAACGAGCGCAACCCACGTCCCTAGTTGCCAGCATTC AGT TGGGCACTCTAGGGAAACTGCCGATGATAAGT
	*Roseococcus*sp LW5	FM956480.1	100%	TGGTGCCCGCAAGGGAACGACAAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTC GTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTCTATTTGCCAGCATGT TTG GGTGGGCACTCT
	PseudohobacterIncheonensis Strain KOPR1	GU322906.1	97%	GGTCTTTCTTTCGGCTGGATCCACACAGGTGCTGCATGGCTGTCCTCAGCTCGTGTC GTG AGATGTTCGGTTAAGTCCGGCAACGAGCGCAACCCACGTCCCTAGTTGCCAGCATTC AGT TGGGCACTCTAGGGAAACTGCCGATGATAAGT

Key: PWW-Process wastewater

Table1: Sequence similarity of excised DNA fragments of clarified water and retention pond bacterial community from the bacterial 16S rDNA clone library.

Sample ID	Closest Relative	Accession number	% Similarity	Sequence
CW	*Lactobacilluscasei* Strain 095	JN 560879.1	99%	GCTATACATGCAGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAAG AGTGGCGAACGGGTGAGTAACACGTGGGGAACCTGCCCATCAGAAGGGGATAACACTTGG AAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGA AAGGCGCT
	Staphylococcusequorum Strain SS13	AY126195.1	99%	GATAGGTACCGTCAGATGTGCACAGTTACTTACACATTTGTTCTTCCCTAATAACAGAGT TTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATT GCGGAAGATTCCCTACTGCTGCCTCCCGTAGGACGACTTC
RP	Uncultured Freshwater Bacterium clone 27PVC	JF277898.1	100%	CTGCACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC CGCAACGAGCGCAACCCTTGCCATCAGTTGCTACGAAAGGGCACTCTGATGGGACTGCCG GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGC TACACACGTCATACAA
	Uncultured *Lactococcus*	GQ365748.1	100%	TAGTTACCGTCACTTGATGAGCTTTCCACTCTCACCAACGTTCTTCTCTACCAACAGAGT TTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGACTTTCGTCCATT GCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAACGGTGCG
	Staphylococcusequorum strain SDMRI I	AY126195.1	99%	GATGTGAACAGTTACTTACACATTTGTTCTTCCCTAATAACAGAGTTTTACGATCCGAAA ACCTTCTTCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCT GCCTCCCGTAGG

Keys: CW-Clarified water; RPG-Retention pond gate;

Table2: Sequence similarity of receiving river bacterial community of 16S rDNA from 16S rDNAclone library

RR	Uncultured Bacilli Bacterium Clone MS211A1_H04	EF710074.1	99%	AGAAGTCTGACCGAGCACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT GTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAG CCACGGCTAACTACGTGCGAGCAGCCGCGGTAATTGT
	Providenciavermicola Strain W4	JN225414.1	98%	AGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGATGCTAATATCATCAACGATTG ACGTTACCGACAGAAAAAGCACCGGCTAACTCCGTGCGAGCAGCCGCGGTAATTATGG
	Enterococcus faecium	AB681208.1	99%	GAGTCTGACCGAGCACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGT TAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCC ACGGCTAACTACGTGCGAGCAGCCGCGGTAATAGAAG
	Lactococccuslactis	AB602805.1	100%	GTAGTTACCGTCACTTGATGAGCTTTCCACTCTCACCAACGTTCTTCTCTACCAACAGAG TTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGACTTTCGTCCAT TGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGCCCGTGCCCCCGCC
	Lactobacillusplantarum strain N149	JN792134.1	97%	ATACGTGACAGTTACTCTCACATATGTTCTTCCTTAACAACAGAGTTTTACGAACCGAAA ACCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGGGGAAGATTCCCT ACTGCTGCCTCCCGTAGG

Key: RR- Receiving water

4. DISCUSSION

Metagenomic DNA was extracted from bacterial isolates from petrochemical industrial effluent and bacterial community present in the effluent. The validity of this study largely depended on the extraction of representative metagenomic DNA of the microbial communities from the petrochemical industrial effluent. However, there are difficulties associated with effluent DNA extraction such as incomplete lysis, DNA adsorption to effluent surfaces, co-extraction of enzymatic inhibitors, yield loss, and degradation or damage of the DNA [9].

There is paucity of information regarding the extraction of DNA from petrochemical industrial effluent using Ultraclean ™ Water DNA extraction Kit(0.22µm). However, other extraction methods have been used to extract DNA from samples of varied origin as reported by [10].Plate 1 shows that PCR using 16SF and 16R primers and 1µl of DNA as template was not successful as only one product was observed when analyzed on agarose gels, suggesting that the primer could not enable the amplification of bacterial community from petrochemical industrial effluent. The primer V3F and V3R amplified the V3 region of the 16S rDNA giving a base pair size of 200 as seen in Fig2 using the same amount of DNA template. However, this did not agree with [8] who reported a basepair of 500 using same primer for detection of bacterial pathogens.

Strong PCR bands however were observed as shown in plate3 using V6V8F and V6V8R primers and fragments were of the expected size of 500bp. The primers were subsequently used for DGGE analysis with GC clamp added to the forward primers as described by[4].The result indicates that V6V8F and V6V8R primers are the best for DGGE differentiation of bacterial community from petrochemical industrial effluent.

The bacterial diversity examined by a number of bands and the migration position in a DGGE gel has been used to examine bacterial diversity,for example in sausages[3] in anaerobic sludge blanket granules [12],bacterial diversity in soil[13] and dairy wetland wastewater effluent [14],however researches on DGGE bacterial community in petrochemical industrial effluent is not well documented. In this study, the application of total bacterial DGGE was used to study the diversity of bacterial communities in petrochemical industrial effluent. This approach exploited the discriminatory power of DGGE to differentiate DNA molecules on the basis of differences in their sequences [15].

Results were obtained from two different denaturant gradients in the DGGE gels (Plates 4-7) to assess the best differentiation gradient for DGGE of bacterial community in the effluent. A 20-80% denaturant gradient ratio using GCV6V8F and V6V8R primers was optimal for the differentiation of bacterial community from petrochemical industrial effluent whereas a 30-50% denaturant gradient ratio using GCV3F and V3R had bands migrating out of the gels indicating that there was a poor differentiation. This however does not agree with [7] who reported bacterial community differentiation in cheese using the same denaturant ratio.

The results obtained in this study also show high bacterial diversity as revealed by DGGE. The DGGE patterns showed some strong bands with strong intensity which represents a wide variety and dominant bacterial species in effluent samples. These dominant bacterial species are well adapted and play the important role of biodegradation of the effluent. The profiles obtained by the DGGE agreed with the

results obtained by traditional methods which isolated an array of bacteria from petrochemical industrial effluents of similar composition.

DGGE band patterns from high diversity ecosystems such as marine sediments are very complex and difficult to interpret [16].Therefore, the application of advanced statistical methods is crucial. In this study, a similarity matrix, based on the presence and absence of individual bands, was calculated for the various sampling points and receiving river of petrochemical industrial effluent (fig 1).These DGGE band patterns reveal the differences in the bacterial community structures between the four sampling points. The differences between the four sampling points are considerable. For instance, the sample from process wastewater (PW) contains less similar bacteria with that of retention pond gate (57%).The reason could be due to the different treatments applied on the effluent at different stages. The 100% similarity of the bacterial community in the same effluent sampling points further proves this. Also the DGGE band patterns of isolates using the similarity matrix shows that there was a high bacterial diversity indicating the changes on the microbial community structure attributed to the process of biological wastewater treatment of the petrochemical industrial effluent plant of study. The similarities of the bacterial 16S rDNA sequences with published data ranged from 97% to 100% (Tables 18 and19). The sequence result revealed the presence of bacterial species of *Pseudomonas, Enterococcus, Lactobacillus, Rhodobacter, Staphylococcus, Lactococcus, Roseococcus* and uncultured bacterium. The results of the sequencing further proved the presence and ability of these organisms to biodegrade petrochemical industrial effluent into less environmentally hazardous products. The results of this study are interesting not only for the contribution to knowledge on the micro flora of petrochemical industrial effluent but also because the working application of the whole approach may represent a tool of utmost importance in ecological studies. Moreover,this kind of approach can play an important role in bioremediation of petroleum refinery and petrochemical wastewaters.

5. REFERENCES

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APPENDIX: DGGE PROTOCOL NOTES

PREPARATION OF REAGENTS – 0% and 100% denaturant solutions

	0% solution	100% solution
2 x 250ml work bench		
50X TAE	4ml	-
Urea	-	80g
deionised water	156ml	-
Fume cupboard		
40% acrylamide/bis	40ml	40ml
Formamide (deionised)	-	80ml
Make up to 200 ml (deionised H_2O)	-	-
Total Volume	200ml	200ml