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THE USE OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TO STUDY THE GENETIC VARIATION OF BIOSURFACTANT PRODUCING BACTERIA

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Biosurfactants are structurally diverse group of surface-active substances produced by micro-organisms. A total of 65 bacteria spp. were isolated from environmental samples and were identified using various biochemical tests. Eleven out of the 65 bacterial isolates with codes 1HC (*Bacillus cereus*), 2HC (*Corynebacterium*) 3C and 15HC (*Streptococcus* sp.), 5HC (*Micrococcus lentus*), 6C (*Pseudomonas aeruginosa*), 7HC (*Pseudomonas aeruginosa*), 10HC (*Bacillus* sp.), 12HC (*Eschericia coli*), 13HC (*Staphyloccocus aureus*) and 14HC (*Proteus* sp.) were screened for biosurfactant producing ability using the conventional methods: β haemolysis, methylene blue agar and emulsification index tests. These served as standards to screen the other 54 bacterial isolates for chromosomal and plasmid mediated ability for biosurfactant production. The RAPD shows that the genes coding for biosurfactant production occurs in the chromosomal DNA of some of these bacterial strains. The bands indicate the gene coding for biosurfactant production. The relatedness of these isolates was ascertained using PyElph software.

Keywords: Biosurfactant, Chromosomes, Plasmids, RAPD.

1. Introduction

Biosurfactants are amphiphilic surface active compounds produced by microorganisms (bacteria, fungi). They contain both hydrophobic and hydrophilic moieties that confer ability to accumulate between fluid phases such as oil/water, air/water, solid/liquid reducing the surface and interfacial tensions and forming emulsions (Desai & Banat, 1997). A need to replace chemical surfactants with biosurfactant in the fields of agriculture, cosmetic, food, pharmaceutical, environmental, industries and bioremediation turns the attention towards the microbial world, as they have ability to produce largely unexplored variety of metabolites, e.g. biosurfactant (Maier, 2003).

Screening of biosurfactant-producing bacteria from soils contaminated by hydrocarbons constitutes a powerful tool for the selection of strains with high emulsifying capacity. There are different screening methods for identifying biosurfactant producing bacteria. These include: β haemolysis test, Centriamide test (CTAB)/methylene blue test, oil displacement test and emulsification index test. It is however, difficult to detect the type of biosurfactant produced by the microbes using a single method owing to the chemical and functional properties. In view of this, it appears that combinations of screening methods are

needed to understand the ability of a single microbe in producing biosurfactant (Satpute *et al.,* 2008; Kiran *et al.,* 2010).

Biosurfactant producing microorganisms for biodegradative capabilities is important in microbial ecology especially with molecular techniques. The interest in this area has been catalyzed by the rapid advancement of molecular ecological methodologies. The ability of an organism to degrade a specific substrate is clear evidence that its genome harbors the relevant degrading gene (Jyothi *et al.,* 2012). However, to replace chemically synthesised surfactants with biosurfactant, the understanding of physiology, genetics and biochemistry of biosurfactant producing organisms is necessary. The genetics of producer organism is an important factor affecting the yield of all biotechnological products because the capacity to produce a metabolite is bestowed by the genes of the organisms. The bio-industrial production process is often dependent on use of hyper producing microbial strains even though cheap raw materials, optimized medium, culture conditions and different recovery processes have been employed. A product economy suggests that a production process often cannot be made commercially viable and profitable until the yield of final product by the producer organism is naturally high. As with other biosynthetic pathways, a valuable approach to the study of biosurfactant production is the use of mutants which might be naturally occurring or induced by transposition. Industrial production process is dependent on the availability of recombinant and mutant hyper producers if good yield are lacking from the natural producer strains. Moreover even if high yielding natural strains are available, the recombinant hyper producers are always required to economize further the production process and to obtain products with better commercially important properties (Altschul *et al*., 1997; Asubel *et al*., 1987; Birnbooim and Doly, 1979; Ferrara *et al*., 2006; Maidak *et al.*, 1997; Thompson *et al.*, 1997; Turner *et al*., 1999). The extraction of DNA from cells are of primary importance to the field of biotechnology to create genetically engineered organisms used for the production of beneficial products like insulin, antibiotics, hormones, biosurfactant, etc. Bacteria containing a plasmid harboring a gene of interest can be induced to produce large quantities of that gene. Plasmids used in genetic engineering are called vectors. Plasmids serve as important tools in genetic and biotechnology laboratories where they are commonly used to multiply or express particular genes. The microbial diversity of different species of bacterial producing biosurfactant from the environment is a useful measure of the variety of this same gene that is responsible for biosurfactant production. This microbial and phylogenic biodiversity we studied using the random amplified polymorphic DNA (RAPD).

1.1 Random amplified polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation. It involves the use of a single arbitrary primer in a PCR reaction resulting in the amplification of many discrete DNA products. This procedure detects nucleotide sequence polymorphism in a DNA amplification based assay using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA templates. If these priming sites are within an amplification distance of each other, a discrete DNA product is produced through thermo cyclic amplifiable distance. The polymorphisms between individuals result from sequence difference in one or both of the primer binding sites and are visible as the presence or absence of a particular RAPD band such polymorphisms thus behave as dominant genetic markers. The RAPD involves a target DNA sequence exponentially amplified with the help of arbitrary primers, a thermostable DNA polymerase, dideoxy nucleotide tri-phosphate and magnesium. The reaction involves repeated cycles each consisting of a denaturation, a primer annealing step and extention. The most crucial factors that need to be optimized in a RAPD reaction are the magnesium concentration, enzyme concentration, DNA concentration and annealing temperature of the primer (Sambrook and Russell, 2001).

The use of conventional methods: β haemolysis, methylene blue agar, emulsification index, drop collapsing method, oil displacement, etc in identifying biosurfactant producing isolates is extensive when

many isolates are involved. This is because not only one of these methods is conclusively used in the identification (Satpute *et al.,* 2008; Kiran *et al.,* 2010; Okore *et al.*, 2013; 2017a; 2017b) and as a result this study used the presence of chromosomes and plasmids to identify biosurfactant producing bacteria using RAPD method. The quantity of biosurfactant produced by microorganism is small considering the size of the microorganisms. The size of these organisms that produce biosurfactants can discourage large scale production hence the genetic study that leads to manipulation of genes for higher yields. The diversity of organisms that produce biosurfactant in the environment is a ready raw material for the production (Okore *et al.*, 2013; 2017). The relatedness of the producers and the diversity will encourage more searches for novel biosurfactant producers. The genetic study will determine where the gene coding for the biosurfactant production is conferred. This will encourage the manipulations of these genes for better biosurfactant production for large scale applications. In order to achieve these, the objectives are to isolate and identify bacteria from different environmental samples; to extract the chromosomes and plasmids of the isolates; to screen the isolates for biosurfactant production using RAPD; to identify the molecular sizes of the genes coding for the biosurfactant production; to study the relatedness of the bacterial isolates from the environmental samples.

2. Materials and Methods

2.1 Sampling

Soil samples were collected from near diesel pump (DI), petrol pump (PSMI), kerosene pump (KI), paint industry (PSI), cassava mill (CAI), palm oil mill (POI), abattoir (ABAI), zinc industry (ZI), automobile workshop (AUI); water samples from swimming pool (SWI), fish pond (FI) and Otamiri River (OTI). These samples were immediately taken to the Microbiology laboratory of Federal Polytechnic Nekede Owerri for analysis.

2.2 Isolation of Microorganisms From the Environmental Samples Using Serial Dilution

1g of each of the soil samples were collected and placed into a sterile test tube. Thereafter, 9 ml of sterile water was added to each of the sample test tubes. Serial dilution of the samples was carried out and 10^2 to 10^{10} was plated in triplicates by spread plate method on nutrient agar medium and incubated under aerobic condition at 37^{o}C for 24 h. After 24 h of incubation, the organisms on the plate were re-incubated in nutrient agar slant in bijou bottles and refrigerated at 4^{o}C for further biochemical test

2.3 Screening for Biosurfactant Production

2.3.1 Emulsification Index (E_{24})

Several colonies of pure culture were suspended in test tubes containing 2 ml of nutrient broth after 24-48 h of incubation, 2 ml of hydrocarbon (diesel, petrol, crude oil, kerosene) and vegetable oil were added respectively to each tube. Then the mixtures were vortexed at high speed for 2 min and allowed to stand for 24 h. The emulsification index (E_{24}) is the height of the emulsified layer (cm) of the liquid/aqueous-column divided by total height (cm), multiplied by 100 (Okore *et al.*, 2013; 2017a; 2017b).

$$\text{Emulsification index E24} = \frac{\text{Height of emulsion layer x 100}}{\text{Total height}}$$

2.3.2 Screening for Biosurfactant Production Using Methylene Blue Agar

2.3.2.1 Preparation of methylene blue agar plate

28 g of nutrient agar was weighed and poured into a 1000 ml conical flask, 1 L of water was poured into the conical flask and then was swirled to homogenize. It was then autoclaved at 121^{o}C. After autoclaving, the medium was brought out and was allowed to cool to cheek bearable temperature. 50 ml of methylene blue was collected and poured into the conical flask containing the nutrient agar and was swirled to homogenize. Thereafter methelyene blue agar was poured into Petri dishes and allowed to solidify (Okore *et al*., 2013; 2017a; 2017b).

2.3.2.2 Inoculation of the isolate onto the methylene blue agar plates

The isolate on the nutrient agar slant was collected using sterilized wireloop and streaked on the surface of the methylene blue plates. The methylene blue agar plates were incubated at 30^{o}C for 48 h. The occurrence of defined clear zone around the colonies is an indication of biosurfactant production.

2.3.3 Screening for biosurfactant producing microbes using βhaemolysis

2.3.3.1 Preparation of blood agar

Biosurfactant producing microorganism can be screened using β heamolysis assay. 28 g of nutrient agar was weighed and poured into a 1000 ml conical flask, 1 L of water was poured into the conical flask and then was swirled to homogenize. It was then autoclaved at 121^{o}C for 15 minutes at 151bs pressure. Thereafter, the nutrient media was brought out and was allowed to cool to cheek bearable temperature. 5 ml of whole blood was collected from a donor and poured into the flask containing the nutrient agar, and shaken to homogenize. The blood agar was then poured into sterile Petri-dishes and allowed to solidity (Okore *et al*., 2013; 2017a; 2017b).

2.3.3.2 Inoculation of the isolate onto the blood agar plate

The isolates on the nutrient agar slant were streaked on blood agar plates. The blood agar plates were incubated at 30^{o}C for 72 h. The occurrence of define clear zone around the colonies is an indication of β-heamolytic activity.

2.3.3.3 Biochemical tests for identification of bacterial isolates

The biochemical test used for identification of isolates include: Gram staining, Catalase test, Indole test, Oxidase test, Citrate utilization test, Sugar fermentation test.

2.4 Extraction of Chromosomal DNA by Boiling Method

Chromosomal DNA of the test isolates was extracted using modified procedure of Sambrook and Russell, (2001) and Ogbulie and Nwakanma, (2015). This was carried out by pipetting 1.5 ml of the overnight cultures of each isolate into pre labeled 1.5 ml eppendorf tubes. The broth culture was mixed by votexing and transferred into another labeled eppendorf tube, then centrifuged at 10,000 rpm for 2 min. The supernatant was discarded by decanting and tube blotted on a paper towel provided. There after 1ml of sterile distilled water was added this was vortexed and centrifuged again at 10,000 rpm for 5min. The supernatant discarded by decanting and tube blotted again on a paper towel then addition of 1ml of sterile distilled water again. This was vortexed and centrifuged at 10,000 rpm for 5 min and the supernatant

discarded as stated above. There after 200 µL of sterile distilled water was added and vortexed to homogenize the pellets then boiled at 100^{o}C for 10 min. After boiling, vortex and centrifuge at 10,000 rpm for 5 min. Then transfer the supernatant into another pre-labelled eppendorf tube by gentle aspiration using a micropipette.

2.5 Screening for Plasmid DNA by Tris EDTA NaOH SDS (TENS) Method

The plasmid DNA of the test isolates was extracted using TENS modified procedure of Sambrook and Russell, (2001) and Ogbulie and Nwakanma, (2015). This was carried out by pipetting 1 ml of the overnight culture into pre labeled eppendorf tubes, mixed by votexing and then centrifuged at 13,000 rpm for 2 min. The supernatant then discarded by decanting gently leaving a little of the broth which will be vortex at high speed until the plasmid is suspended in the broth. Then 300 µL of Tris EDTA NaOH (TENS) solution was added into the broth and mixed by inverting the tubes until it becomes slimy (indicating cell lysis). There after 150 µL of sodium acetate was added and vortexed for 10 sec, centrifuged at 13,000 rpm for 5 min and supernatant transfered into 1.5 ml eppendorf tubes. Then 900 µL of ice cold absolute ethanol (99% ethanol) was added, vortexed and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and white pellets observed. Then 1000 µL of 70% ethanol (ice cold) was added, centrifuge at 13,000 rpm for 5 min, and supernatant discarded as earlier mentioned. This step was repeated again and the resultant dry pellets redissolved in 40 µL of Tris EDTA (1XTE). The extracted plasmid DNA samples were subjected to agarose gel electrophoresis as described by Sambrook and Russel, (2001) and Ogbulie and Nwakanma, (2015). The gel was observed for presence of bands corresponding to plasmid DNA.

2.6 RAPD PCR Assay

The PCR assay was carried out at the Biotechnology laboratory of the Department of Biotechnology in Federal University of Technology Owerri, Imo State. This was done using thermal cycler model 006 A & E UK. The assay was carried out using RAPD primer, OPA8, FIRE POL PCR master mix, composed of DNA polymerase, 12.5 mM $MgCl_2$, 1 mM of dNTPs and dye. The cycling conditions to be used include initial heating at 95^{o}C for 5 min; followed by 40 cycles of 95^{o}C for 1 min, extension temperature of 72^{o}C for 2mins, final extension temperature at 72^{o}C for 10 min.

2.7 The PCR Products Will be Analyzed using Gel Electrophoresis

The PCR products were analyzed using Gel electrophoresis as described by Sambrook and Russell, (2001) and Ogbulie and Nwakanma, (2015). This was carried out by preparing 1.5% agarose, and 100 ml of 1X Tris Boric EDTA (TBE) buffer added. This was dissolved by boiling/heating for 3-5 min using microwave oven. It was allowed to cool to 50^{o}C and add 10 µL of ethidium bromide then gently mix by swirling. This was then poured into electrophoresis tank with comb in place to obtain a gel thickness of about 4.5 mm (avoiding bubbles). It was allowed to stand for 20 min to polymerize/solidify. The comb was gently removed and the tray placed in the electrophoresis tank. There after 1X TBE buffer was poured into the tank ensuring that the buffer covers the surface of the gel. Then about 15-20 µL of the sample (PCR product of the 54 samples) were mixed with 2 µL of the loading dye and the mixture fully loaded into the wells created by the comb with marker (standard) in lane 1 followed by the controls: 1HC (*Bacillus cereus*), 2HC (*Corynobacterium* sp.), 3C and 15HC (*Streptococcus* sp.), 5HC (*Micrococcus lentus*), 6C (*Pseudomonas aeruginosa*), 7HC (*Pseudomonas aeruginosa*), 10HC (*Bacillus* sp.), 12HC (*Eschericia coli*), 13HC (*Staphyloccocus aureus*) and 14HC (*Proteus* sp.). The electrode was then connected to the power pack ensuring that the negative terminal is at the well side where the samples are loaded. The electrophoresis was then run at 60-100 V until the loading dye has migrated to the end or about three-quarter of the gel field, before turning off and disconnecting the power.

The resultant gel lanes will be observed using UV-transilluminator.

2.8 Molecular Detection and Similarities of Test Isolates

The relatedness of the isolates was detected and confirmed using Py Elph software tool for gel image analysis. The distant lades of the phylogenetic tree were created depicting the relatedness of the individual isolates.

3. Result

Table 1. Biochemical tests of bacterial spp. isolated from environmental samples and the standard for Random Amplified Polymorphic DNA assay

S/N	SAMPLE CODE	MEDIA	MORPHOLOGICAL CHARACTERISTICS	GRAM STAIN	CATALASE	INDOLE	OXIDASE	CITRATE	SUGAR FERMENTATION TEST				POSSIBLE BACTERIA	**CODE for the bacterium used as the standards**
									SLOPE	BUTT	GAS	H_2S		
1	DI-1, OTI-4, OTI-6, CAI-1, AUI-3, POI- 1, SWI- 1, SWI- 5, KI- 2, PSI-2, PSMI-2, ZI-4, FI-7	N.A	Milkfish flat elongated non mucoid colony	-ve rod	-	-	+	-	R	Y	+	-	*Proteus* spp.	FI-7 = 14HC
2.	DI-4, ZI-2, AUI-1, ABAI-1, SWI-3, FI-3, PSI-6, PSMI-5, KI-7	N.A	Bluish green pigmented non mucoid colony	-ve rod	-	-	+	-	R	R	-	-	*Pseudomonas aeruginosa*	PSMI-5 = 6C KI-7 = 7HC
3.	FI-4	N.A	Orange pigmented flat non-mucoid colony	-ve rod	-	-	+	+	R	Y	+	-	*Pseudomonas* spp.	
4.	DI-2, ABAI-3, ZI-3, CAI-2, AUI-4, POI-2, SWI-2, PSI- 4, PSMI-3, OTI-1, KI-6	N.A	Milkish raise non mucoid colony	+ve cocci	+	-	-	-	R	Y	+	-	*Staphylococcus aureus*	KI-6 = 13HC
5.	PSI- 1, PSI-8, ABAI-4	N.A	Milkish flat non mucoid colony with zone of clearance	+ve cocci in chains	-	-	-	-	Y	Y	+	-	*Streptococcus* spp.	PSI-8 = 15HC ABAI = 3C
6.	OTI- 2, FI-6	N.A	Milkish flat mucoid colony	-ve rod	-	+	-	-	Y	Y	+	-	*Escherichia coli*	FI-6 = 12C
7.	KI -4, DI-5	N.A	Milkish raised needle pointed colony	+ve rod	+	-	-	-	R	R	-	-	*Corynebacterium*	DI-5 = 2HC
8.	DI-3, PO-3, ABAI-2, FI-1, KI-5, PSI-7,	N.A	Milkish enlarged non mucoid colony with irregular shaped edges	+ve cocci	+	-	-	+	R	Y	+	-	*Micrococcuss* spp.	
9.	ZI-1, CAI-3, SWI-4, SWI-6	N.A	Golden yellow pigmented raised non-mucoid colony	+ cocci	+	-	-	+	Y	Y	-	-	*Micrococcuss lentus*	SWI-6 = 5HC
10.	OTI-3, ZI-6, KI-1, PSMI-1, DI-6	N.A	Milkish flat with irregular shaped non mucoid colony	+ve rod	+	-	-	+	R	Y	+	-	*Bacillus* spp.	DI-6 = 10HC
11.	OTI-5, ZI-5, FI-2, FI-8 PSI-3, PSI-5, PSM1-4,	N.A	Milkish flat non mucoid colony with rough edges	+ve rod	+	-	-	-	Y	Y	-	-	***Bacillius cereus***	**FI-8 = 1HC**
12.	AUI-2, FI-5,	N.A	Milkish flat rhizoid non mucoid colony	+ve rod	-	-	-	+	R	Y	+	-	*Bacillus subtilis*	

The *Proteus* spp. are gram negative Proteobacteria rod and a total of thirteen *Proteus* spp. were isolated from the environmental samples. There was a total of ten *Pseudomonas aeruginosa and Pseudomonas* spp. isolated, eleven *Staphylococcus aureus,* three *Streptococcus* spp., two *Escherichia coli,* two *Corynebacterium,* ten *Micrococcuss* spp. and *Micrococcuss lentus,* fourteen *Bacillus* spp., *Bacillus cereus* and *Bacillus subtilis* isolated from the environmental samples.

Table 2. Emulsification index screening of the standards used for biosurfactant using hydrocarbon (petrol, diesel, kerosene, crude and vegetable oil)

S/N	HYDROCARBON	PETROL			DIESEL			KEROSENE			CRUDE OIL			VEGETABLE OIL		
	ISOLATE	Total length (cm)	Emulsified length (cm)	Emulsification index (E_{24})%	Total length (cm)	Emulsified length (cm)	Emulsification index (E_{24})%	Total length (cm)	Emulsified length (cm)	Emulsification index (E_{24})%	Total length (cm)	Emulsified length (cm)	Emulsification index (E_{24})%	Total length (cm)	Emulsified length (cm)	Emulsification index (e_{24})%
1	*Bacillus cereus*	2.5	1.0	40.00	3.2	1.2	37.50	3.0	1.4	46.66	2.5	1.2	48.00	2.1	1.1	52.38
2	*Corynebacterium* spp.	2.3	0.9	39.13	2.8	1.2	42.85	2.6	1.3	50.00	2.8	1.3	46.42	2.2	1.0	45.45
3	*Streptoccoccus* spp.	2.5	0.9	36.00	2.8	1.3	46.42	2.8	1.3	46.42	2.7	1.3	48.14	3.1	1.2	38.70
4	*Micrococcus luteus*	2.4	1.0	41.66	2.6	1.3	50.00	2.7	1.3	48.14	3.2	1.4	43.75	2.4	1.0	41.66
5	*Pseudomonas aeruqinoa*	2.5	1.0	40.00	2.9	1.3	44.82	3.0	1.4	46.66	3.0	1.4	46.66	2.1	1.1	52.38
6	*Bacillus subtilis*	2.5	1.0	40.00	2.9	1.3	44.82	2.7	1.4	51.85	3.0	1.3	43.33	1.8	0.9	50.00
7	*Escherichia coli*	2.3	0.8	34.78	2.7	1.3	48.14	2.9	1.5	51.72	2.8	1.4	50.00	2.5	1.0	40.00
8	*Staphyloccoccus aureus*	2.1	0.7	33.33	2.8	1.2	42.85	2.8	1.3	46.42	3.1	1.4	45.16	2.1	1.2	57.14
9	*Proteus* spp	2.5	1.0	40.00	2.8	1.2	42.85	2.7	1.2	44.44	2.9	1.4	48.27	2.7	1.0	37.04

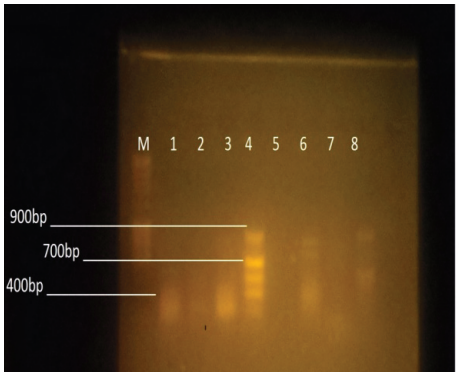
The isolates used as standards showed high E24 using the different hydrocarbons. The highest E24 using diesel as the hydrocarbon is 50% recorded by *Micrococcus lentus*. *Corynebacterium* (50%), *Bacillus subtilis* (51.8%) and *E.coli* (51.72%) recorded high E24 using kerosene. The E24 recorded using crude oil are *Streptococcus* sp. (48.14%), *E.coli* (50%), *Proteus* sp. (48.27%). The *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* recorded high E24 (52.38%, 52.38% and 57.14% respectively) using vegetable oil.

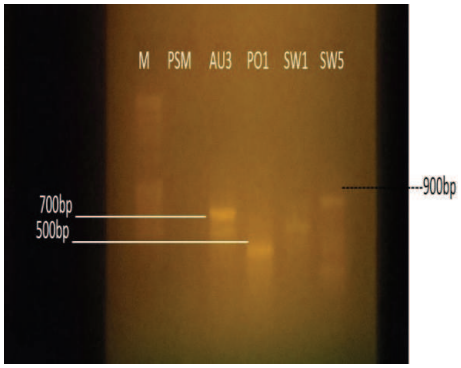
Table 3. Screening of the standards for biosurfactant production

Isolates	Samples	Bacteria	β Haemolysis	Methylene blue agar
1	FI-8	*Bacillus cereus*	+	-
2	DI-5	*Corynebacterium*	+	-
3	ABAI-4	*Streptococcus* spp.	+	+
4	SWI-6	*Micrococcus lentus*	+	+
5	PSMI-5	*Pseudomonas aeruginosa*	+	-

6	KI-7	*Pseudomonas aeruginosa*	+	+
7	DI-6	*Bacillus* spp.	+	+
8	FI-6	*Escherichia coli*	-	+
9	KI-6	*Staphylococcus aureus*	+	-
10	FI-7	*Proteus* spp.	+	-
11	PSI-8	*Streptococcus* spp.	-	+

The βhaemolysis screening test 9 (81.8%) of the isolates used as standard produced zones of clearance around the colony and for the methylene test 6 (54.5%) isolates, showed clear halos in methylene blue agar plate.





Figures 1 and 2. RAPD profile of biosurfactant producing *Proteus* spp. isolated from environmental samples. Legend: M- 100 bp marker, 1=DI-1 , 2=OTI-4, 3= OTI-6 , 4=ZI-4 , 5= CAI-1, 6=KI-2, 7= PSI-2, and 8=Standard=14HC, -PSMI-2 , -AU-3, - POI-1, -SWI-1, and –SWI-5.

Table 4. Isolates and chromosome sizes of *Proteus* spp.

Isolates	DI-1	OTI-4	ZI-4	CAI-1	KI-2	PSI-2	14HC	PSMI-2	AUI-3	POI-3	SWI-1	SWI-5
No. of bands	4	4	9	-	8	-	9	-	7	5	6	9
Band size (base pair)	400	400	900	-	800	-	900	-	700	500	600	900

From Fig 1, Fig 2 and Table 4 only *Proteus* spp. isolated from cassava mill soil, soil from paint industry and soil from near petrol pump showed no bands this maybe because the genes coding for biosurfactant production is plasmid mediated. There were only five isolates that showed bands which indicate that the genes coding for biosurfactant production are present in their chromosome, they are *Proteus* spp. isolated from DI, (Diesel soil), OT4 (Otamiri river sample), ZI4 (soil from zinc industry), (kerosene pump soil), AUI (automobile workshop soil), POI (palm oil mill soil), SWI (swimming pool sample). The sizes of these bands vary from 400 base pairs to 900 base pairs.

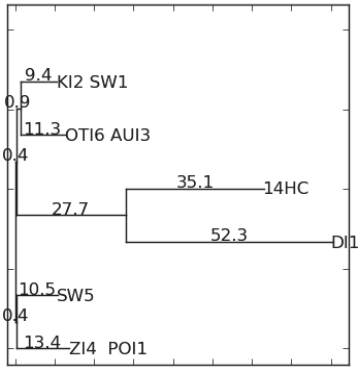
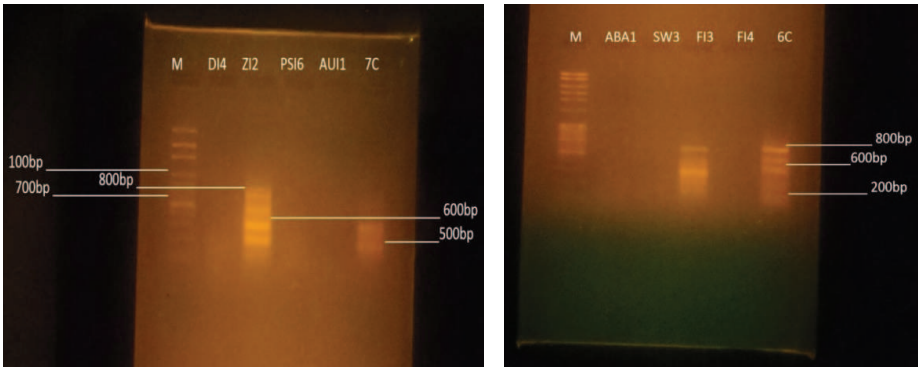


Figure 3. Dendrogram/Phylogenetic tree showing the relatedness of the *Proteus* spp from environmental samples

The KI2 and SWI are the same *Proteus* spp., OTI6 and AUI3 are also same specie but KI2 and SWI share a close reationship OTI6 and AUI3. ZI4 and POI are same specie. SW5 has a closer relation to ZI4 and POI but a different spece.

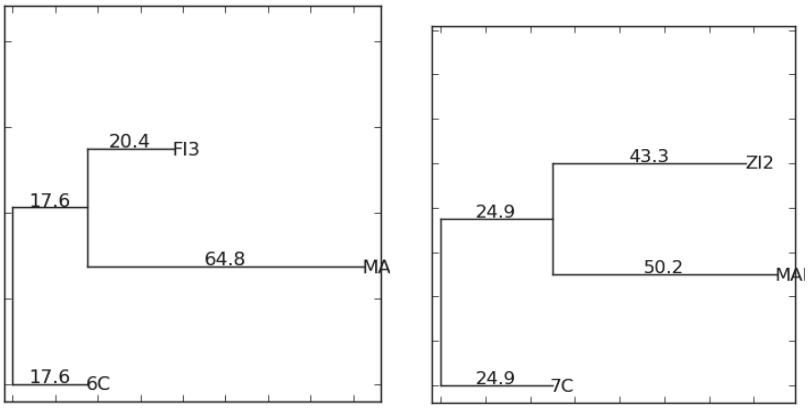


Figures 4 and 5. RAPD profile of biosurfactant producing *Pseudomonas aeruginosa* isolated from environmental samples
Legend: M= 100 bp marker, - D1-4, - ZI-2, - PSI-6, -AUI-1, and Standard-7C, -ABA-1, -SW-3, - FI-3, -FI-4, and Standard=6C

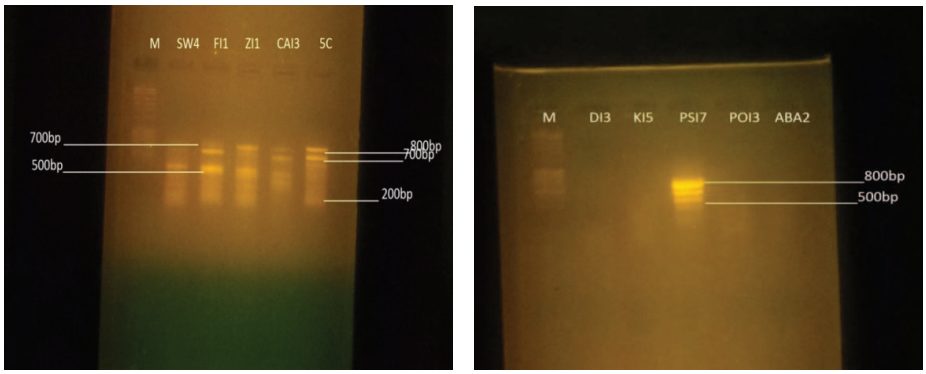
Table 5. Isolates and chromosome sizes of *Pseudomonas aeruginosa*

Isolates	DI-4	ZI-2	PSI-6	AUI-1	7C	ABAI-1	SWI-3	FI-3	FI-4	6C
No. of bands	-	8	-	-	5	-	-	8	-	8
Band size (base pair)	-	800	-	-	500	-	-	800	-	800

The Fig 4, Fig 5 and Table 5 showed that only the *Pseudomonas aeruginosa* isolated from soil of zinc industry (ZI) and fish pond water sample (FI) had bands ranging from 500 to 800 base pairs.



Figures 6 and 7. Dendrogram/Phylogenetic tree showing the relatedness of the *Pseudomonas aeruginosa* strains from environmental samples.
FI3 and ZI are sub-strains of the *Pseudomonas aeruginosa* strains 6C and 7C respectively and share distant relationship.



Figures 8 and 9. RAPD profile of biosurfactant producing *Micrococcus lentus* and *Micrococcus* spp. isolated from environmental samples
Legend**:** M= 100bp marker, -SW-4, -FI-1, -ZI-1, -CAI-3, and Standard-5C, -DI-3 , - KI-5, -PSI-7, -POI-3, and –ABA-2

Table 6. Isolates and chromosome sizes of *Micrococcus lentus* and *Micrococcus* spp

Isolates	SW-4	FI-1	ZI-1	CAI-3	5C	DI-3	KI-5	PSI-7	POI-3	ABA-2
No. of bands	5	7	8	8	8	-	-	8	-	-
Band size (base pair)	500	700	800	800	800	-	-	800	-	-

From the Fig 8, Fig 9 and Table 6, *Micrococcus lentus* and *Micrococcus* spp. isolated from swimming pool water sample, fish pond, soil of zinc industry, cassava mill soil and soil from paint industry showed bands for biosurfactant production. These bands ranged from 500 to 800 base pairs.

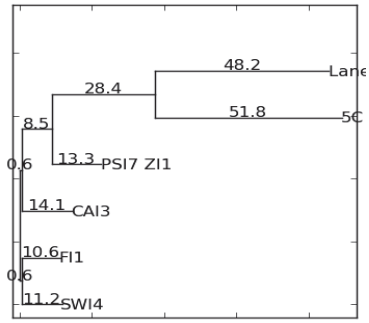
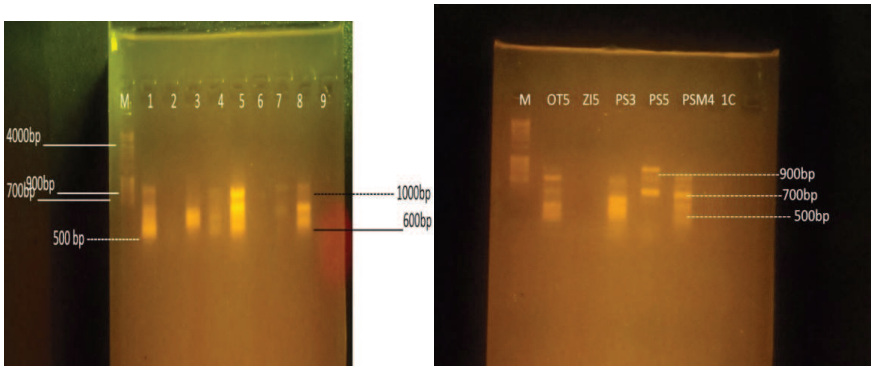


Figure 10. Dendrogram/Phylogenetic tree showing the relatedness of the *Micrococcus lentus* and *Micrococcus* spp from environmental samples

These *Micrococcus* spp. (SW14, FI-1, CAI-3) share very close relationship while these two sub specie PSI-7 and ZI-1 are distant from the three species SW14, FI-1 and CAI-3.

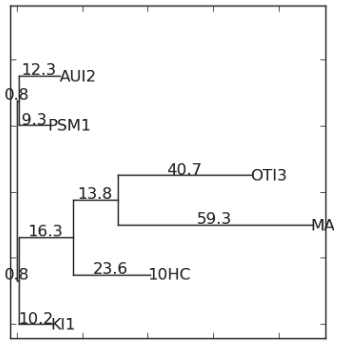


Figures 11 and 12. RAPD profile of biosurfactant producing *Bacillus* spp. *Bacillus subtilis* and *Bacillus cereus* isolated from environmental samples.
Legend: M= marker, 1=OTI-3, 2=ZI-6, 3=KI-1, 4=PSMI-1, 5=AUI-2, 6=FI-2, 7=FI-5 8=10HC (standard), -OTI-5- , - ZI-5, - PSI-3, -PSI-5, - PSM-4 and -1C (standard)

Table 7. Isolates and chromosome sizes of *Bacillus* spp. *Bacillus subtilis* and *Bacillus cereus*

Isolates	OTI-3	ZI-6	KI-1	PSMI-1	AUI-2	FI-2	FI-5	10HC	OTI-5	ZI-5	PSI-3	PSI-5	PSM-4
No. of bands	5	-	11	9	10	-	10	10	8	-	8	9	8
Band size (base pair)	500	-	1100	900	1000	-	1000	1000	800	-	800	900	800

The Fig 11, Fig 12 and Table 7 showed that only the *Bacillus* spp. *Bacillus subtilis* and *Bacillus cereus* isolated from zinc industry soil and fish pond water sample had no bands for biosurfactant production. The others showed bands sizes ranging from 500-1100 base pairs meaning that biosurfactant production is chromosome mediated.



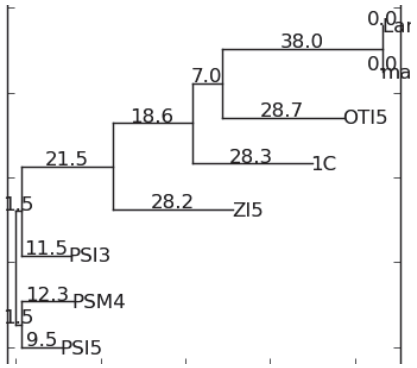


Figure 13. Dendrogram of *Bacillus* spp. and , *B. subtilis* and **Figure 14.** Dendrogram of *B. cereus*

The *Bacillus* spp. are KI-1, PSMI and AUI2 which is a *Bacillus subtilis.* All the three species share a close relationship. OTI3 is a very distant sub sub specie.

These are the strains of the *Bacillus cereus* PSI5, PSM4 and PSI3. ZI5 is a sub strain while OTI5 is a sub sub strain and very distant to the others.

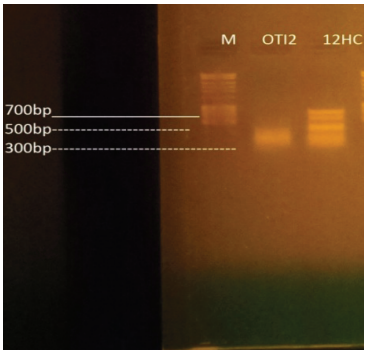


Figure 15. RAPD profile of biosurfactant producing *E.coli* isolated from environmental samples.
Legend: M= marker, -OT12, 12HC=Standard

Table 8. Isolates and chromosome sizes of *E.coli*

Isolates	OTI-2	12HC
No. of bands	5	7
Band size (base pair)	500	700

The Fig 15 and Table 8 showed that the Otamiri river sample showed chromosomal band size of 500 base pair.

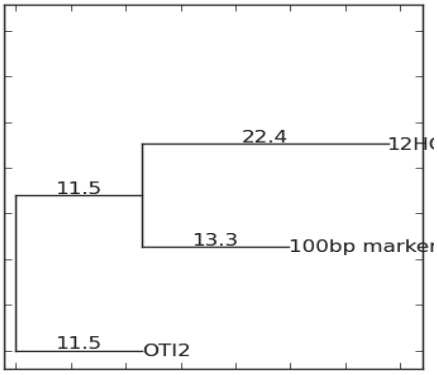


Figure 16. Dendrogram of *E.coli*

From Fig 16 we see that OTI2 is a strain of *E.coli* while 12HC is sub strain and they share distant relationship.

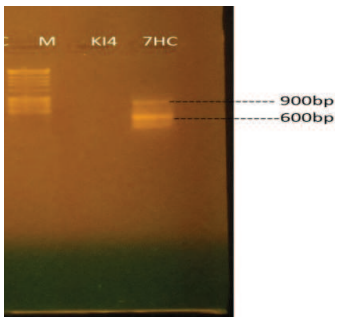


Figure 17. RAPD profile of biosurfactant producing *Corynebacterium* sp. isolated from environmental samples. Legend: M=marker, -K14, 2HC=standard

The *Corynebacterium* sp. (K14) showed no bands. The gene coding for biosurfactant production is not conferred on the chromosome of the organism it maybe plasmid mediated.

Table 9. Isolates and chromosome sizes of *Corynebacterium* sp.

Isolates	KI-4	2HC
No. of bands	-	9
Band size (base pair)	-	900

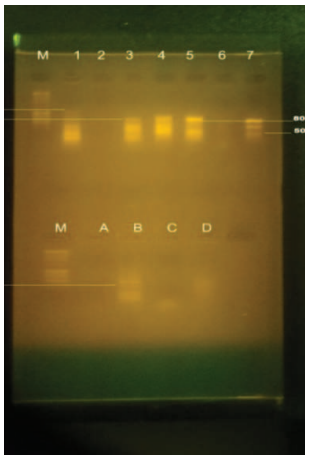


Figure 18. RAPD profile of biosurfactant producing *Staphylococcus aureus* isolated from environmental samples. Legend: M=marker, 1=DI-2, 2=OTI-1, 3=ZI-3, 4=CAI-2, 5=PSI-4, 6=SWI-2, A=PSM-3, B=AUI-4, C=POI-2, D=ABAI-3, 7= (13HC standard)

Table 10. Isolates and chromosome sizes of *Staphylococcus aureus*

Isolates	DI-2	OTI-1	ZI-3	CAI-2	PSI-4	SWI-2	PSM-3	AUI-4	POI-2	ABAI-3	13HC
No. of bands	10	-	8	9	9	-	-	7	-	-	8
Band size (base pair)	1000	-	800	900	900	-	-	700	-	-	800

The Fig 18 and Table 10 showed chromosomal band sizes of the *Staphylococcus aureus* isolated from diesel soil, zinc industry soil, cassava mill soil, soil from paint industry and automobile workshop soil as 1,000, 800, 900 and 700 base pairs respectively.

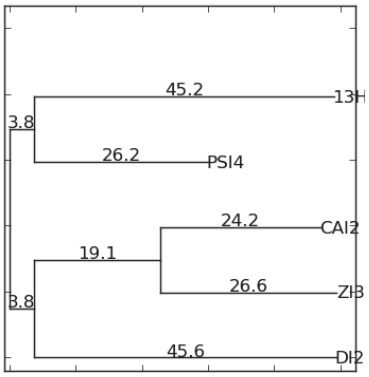


Figure 19. Dendrogram of *Staphylococcus aureus*

The DI2 and PSI4 are two sub strains of *Staphylococcus aureus* and the share very distant relationship. ZIB and CAI2 share a closer relationship and are sub sub strains.

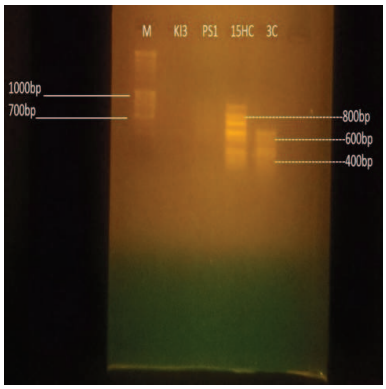


Figure 20. RAPD profile of biosurfactant producing *Streptococcus* spp. isolated from environmental samples
Legend: M- 100bp marker-PSI-1, -KI-3, 15HCand 3C-Standards.

Isolates and chromosome sizes of *Streptococcus* spp.

Isolates	KI-3	PSI-1	15HC	3C
No. of bands	-	-	8	6
Band size (base pair)	-	-	800	600

From the Fig 20 and Table 5 the *Streptococcus* spp. isolated from kerosene soil sample and soil from paint industry showed no bands for biosurfactant production.

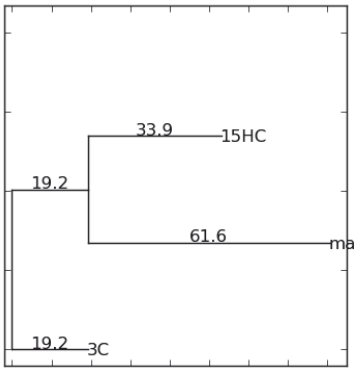
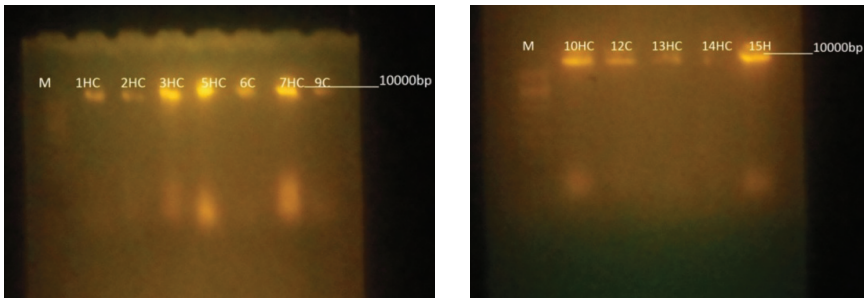


Figure 21. Dendrogram of *Streptococcus* spp

The 3C is a *Streptococcus* specie while 15HC is a sub specie and both share a distant relationship.

Plasmid Profiles of the Biosurfactant Producers



Figures 22 and 23. Agarose gel eletrophoresis of plasmid DNA from isolates used as standards

Key:
1HC = *Bacillus cereus*
2HC = *Corynebacterium*
3HC = *Streptococcus* spp.
6C = *Pseudomonas aeruginosa*
7HC = *Pseudomonas aeruginosa*
9C = *Bacillus subtilis*
10HC = *Bacillus* spp.
12C = *Escherichia coli*
13HC = *Staphylococcus aureus*
14HC = *Proteus* spp.
15H = *Streptococcus* spp.

All the isolates used as standards have common band of plasmid DNA size 10,000 bp which are important in biotechnology as a tool for genetic manipultion.

4. Discussion

The application of biosurfactant and biosurfactant producing bacteria in environmental technologies (bioremediation) has been studied by many researchers (Carrilo *et al*., 2003; Frielo *et al*., 2001; Saimmai *et al*., 2012; Tambekar and Gadakh, 2013). Both organic and inorganic contaminants can be removed through different processes in which biosurfactants are involved. There is great need for manipulations of these plasmids for greater yield to be made use of in environmental and industrial manipulations (Padmapriya and Rajeswari, 2011; Tamura *et al.*, 2007; Lee *et al*., 2005). The guidelines and regulation should be formulated for use of biosurfactants in different sectors. Genetically improved strains can be made by having better knowledge of genes involved in biosurfactant synthesis (Kumaar *et al.,* 2004; Morikawa *et al.*, 1992; Dubey and Juwarkar, 2004; Habe and Omori, 2003). According to Lorenz and Wackernagel, (1994) and Smith *et al.*, (2008) genes transfer can happen within species, different species and genera and can be incorporated into chromosomes and plasmid DNA. This transformation may result in genetic adaptation of bacterial species to stringent environmental conditions like high temperature, salinity and pH environment. Therefore extensive genetic studies are required on biosurfactant producing microorganisms.

This current study on chromosomal and plasmid DNA mediated biosurfactant producing ability, and determining their sizes is a contribution to the knowledge of molecular studies of biosurfactant

production. The sizes of the chromosomes ranged from 400 to 1,000 base pairs while the plasmid size of all the isolates used as standards is 10,000 base pairs. These genes can be manipulated using recombinant DNA technology to produce clones that can degrade xenobiotc compounds that are recalcitrants. Almost all the isolates showed bands indicative of chromosomal mediated biosurfactant production ability with sizes ranging from 400 base pairs to 1,000 base pairs except *Proteus* spp.: CAI-1, PSI-2, PSMI-2 isolated from cassava mill soil, paint industry soil, soil from near petrol pump respectively; *Pseudomonas aeruginosa*: SWI-3, FI-4, DI-4, PSI-6 and AUI-1 isolated from swimming pool water sample, fish pond water sample, soil near diesel pump, soil from paint industry and automobile workshop soil respectively; *Micrococcus* spp.: DI-3, KI-5, POI-3 and ABA-2 isolated from soil near diesel pump, soil near kerosene pump, palm oil mill soil and abattoir soil sample respectively. *Bacillus* spp.: ZI-6 and FI-5 isolated from zinc industry soil and fish pond water sample respectively. *Bacillus cereus*: ZI-5 isolated from zinc industry soil. *Corynebacterium* sp.: KI-4 isolated from soil near kerosene pump; *Staphylococcus aureus*: OTI-1, SWI-1, POI-2 and ABAI-3 isolated from Otamiri river water sample, swimming pool water sample, palm oil mill soil and abattoir soil sample respectively. *Streptococcus* spp.: KI-8 and PSI-1 isolated from soil near kerosene pump and soil from paint industry.

Padmapriya, and Rajeswari, (2011) worked on *Proteus inconstans* from which a plasmid with 1.8 kb was isolated and was cured by acridine orange. Biodegradation and biosurfactant activities were totally inhibited. Hence, they determined that biosurfactant production was purely plasmid mediated. Lee *et al*., (2005) worked with *Bacillus subtilis* C9 that effectively degrades aliphatic hydrocarbons up to chain length of C19 and produces a lipopeptide-type biosurfactant, surfactin. They obtained this transformable surfactin producer by genetic manipulation; the sfp gene cloned from *B. subtilis* C9 was integrated into the chromosome of *B. subtilis* 168, a non-surfactin producer by homologous recombination. The transformants reduced the surface tension of the culture broth from 70 mN/m to 28 mN/m. The transformants readily degraded n-hexadecane, although the original strain did not. The srfA operon is required for the non-ribosomal biosynthesis of the cyclic lipopeptide surfactin. The srfA operon is composed of the four genes, srfA A, srfA B, srfA C and srfA D. In their study, 32 kb of the srfA operon was amplified from *B. subtilis* C9 using a LA-PCR and ligated into PindigoBAC536 vector. The ligated plasmid was then transferred into *Escherichia coli* DH10B. The transformant exhibited a reduced surface tension in a culture broth. Dubey and Juwarkar, (2004) in their study on determination of genetic basis for biosurfactant production in distillery and curd whey wastes utilizing *Pseudomonas aeruginosa* strain BS2. *Pseudomonas aeruginosa* strain BS2 has been demonstrated to have an ability to produce potent biosurfactant, an ecofriendly substitute to synthetic surfactants from distillery and whey wastes and capable of reducing the pollution load of these wastes in the range of 85-90%. They determined the basis for identification of the genes responsible for biosurfactant production from wastes, by the presence of plasmid and its profile using suitable plasmid screening technique as strain BS2 produced excessive slime in Luria Burnetti broth, which interfered with the migration and detection of plasmid. Among the several methods, alkaline lysis method was the most suitable which aided in recovery of slime-free cell lysate and resulted in the formation of a discrete band of plasmid in agarose gel. Plasmid profile study demonstrated that plasmid had high molecular weight of 32.08 × 106 Da and possessed the genetic determinants for antibiotics (chloramphenicol, tetracycline and sulphonamide) and heavy metal salt (mercuric chloride) resistance and were used as markers in curing experiment. They determined the role of megaplasmid in biosurfactant production by curing of megaplasmid at highest sublethal doses of acridine orange (100 g/ml) and mitomycin-C (15 g/ml). The results indicated that only mitomycin-C treatment resulted in 28% of cell population which turned sensitive towards marker antibiotics and heavy metal salt due to loss of megaplasmid, which was further confirmed by agarose gel electrophoresis. The comparative analysis of biosurfactant production potential of cured cells with that of wild cells in both the wastes showed that the cured cells had similar potential capability of biosurfactant production as of wild strain which illustrates that genes responsible for biosurfactant production in distillery and whey wastes utilizing strain BS2 were not plasmid borne but resided on the chromosome where they are more stable.

5. Conclusion and Recommendation

The bacterial isolates from different environmental samples showed biosurfactant producing ability. From the result of the screening test, the gene coding for the biosurfactant production was present in some of the chromosomal DNA of the isolates. An alternative and eco-friendly method of remediation technology of environments contaminated with pollutants is the use of biosurfactants and biosurfactant producing microorganisms. The diversity of biosurfactants make them attractive group of compounds for potential use in a wide variety of industrial and biotechnological applications. Due to their biodegradability and low toxicity, they are very promising for use in the environmental biotechnologies. In spite of many laboratory based success in biosurfactants production and its immense commercial applications, the production of biosurfactant at a plant scale remains a challenging issue as the composition of final product is small. The understanding and thorough investigation of genetic mechanisms of newly identified biosurfactant producers can help in finding of these strains in gene expression in specific environment, which can help in highlighting the substrate obligations. The advances in genomics and proteomics in field of microbiology may lead to finding complete insight into the functions and diversity of biosurfactants.

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