Simultanious biosurfactant production and hydrocarbon biodegradation by the resident aerobic bacterial flora of oil production skimmer pit at elevated temperature and saline conditions

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Abstract: Six aerobic thermo and halotolerant bacterial isolates from an oil production skimmer pit were evaluated for their ability to produce biosurfactants and degradepetroleum hydrocarbons simultaneously under elevated temperature and saline conditions. Phylogenetic analysis using 16S rRNA sequencing revealed that the six bacterial isolates used in the study (SKP-1, SKP-2, SKP-3, SKP-4, SKP-5 and Skp-6) were most homologous to the gammaproteobacteria*Pseudomonas* sp. VS-1, *Pseudomonas aeruginosa* strain S2QPS8, *Serratia marcescens* strain A4, *Pseudomonas stutzeri,Pseudomonas stutzeri* strain RA10 and *Pseudomonas stutzeri* strain BOD-3 respectively. Using previously sterilized skimmer pit sample as the sole nutrient, carbon and energy sources and at an elevated temperature of 45^0C and salinity (Chloride) level of 6012mg/L, all the bacterial isolates in a mixed culture were able to grow, produce biosurfactants and degrade petroleum hydrocarbons simultaneously by removing about 92% of residual TPH in the skimmer pit within 2 weeks of exposure. This study suggests that in-situ bioremediation procedure using the resident aerobic bacterial flora of the skimmer pit that are thermotolerant and halotolerant can be developed to degrade the petroleum hydrocarbon contaminants in-situ. This bioremediation procedure can be a more attractive and cost effective option than the costly thermal treatment option that is currently in operation in the industry.

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Introduction:

Many hydrocarbon contaminated environment are characterized by extreme environmental conditions such as very low or elevated temperatures, highly acidic or alkaline pH, high saline concentrations and or high pressures. Indigenous hydrocarbon degrading microorganisms associated with such environments have been able to adapt to such extreme environmental conditions and play an important role in the biological treatment of these polluted extreme habitats(Margesin and Schinner, 2001, Muller *et al*, 1998, Niehaus *et al*, 1999 and White *et al*, 1999).

Oil production skimmer pit is one of such extreme environmental habitats because of its elevated temperature and saline conditions. Skimmer pit is arelatively cheap and simple short term storage of liquid oily wastes arising from oil production activities. The pits are dug on the ground and the base and the walls are lined with heavy oil impermeable material such as PVL, Polythene or oil resistant rubber sheeting to prevent ground water and soil contamination. The sheets are pre-lined with sands to prevent the plastic sheet from being punctured by sharp objects. As a result of frequent movement of hot liquids used for washing, flushing and cleaning of oil facility into the skimmer pit, its temperature is usually elevated ranging between 45 and 60^0C(Margesin and Schinner, 2001). Some residual chemicals and salts used in flushing the facility can also find its way into the skimmer pit. When the skimmer pit is filled up, the liquid waste are usually evacuated and treated by thermal means before disposal and this is usually an expensive process.

Most industrial operators are of the opinion that if the liquid wastes in the skimmer pit are treated biologically on the spot, it will reduce drastically the high cost of evacuation, transportation and thermal treatment and this idea motivated the conduct of the present study. Ideally if the indigenous microbial floras of the skimmer pit are capable of degrading the hydrocarbons under its elevated temperature and saline conditions, an appropriate bioremediation technology canbe applied to clean up the skimmer pit thus reducing the cost of evacuation, transportation and thermal treatment of the skimmer pit liquid wastes.

Biodegradation is the metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances which are then integrated into natural biogeochemical cycles. The intensity of biodegradation is influenced by several factors such as nutrients, oxygen, pH value, temperature

composition, concentration and bioavailability of the contaminants as well as the history of the contaminated environment. Temperature plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism. Bioavailability and solubility of less soluble hydrophobic substances such as the aliphatics and polyaromatic hydrocarbons are temperature dependent. According to Margesin and Schnner 2001, a temperature increase affects the decrease in viscosity which in turn affects the degree of distribution and increase in diffusion rates of organic compounds, the reverse is the case with decreased temperature. Higher degradation rates are therefore expected at elevated temperatures. The increased volatilization and solubility of some hydrocarbons at elevated temperatures also affects toxicity and allows biotransformation with high substrate concentrations (Muller *et al*, 1998, Whyte *et al*, 1999). Microorganisms that grow optimally above 40^0C are designated as thermophiles. Most thermophiles known are moderate and show an upper temperature border of growth between 50 and 70 ^{0}C but optimum growth temperature of extreme thermophiles and hyper-thermophiles occurs at 70-80 and above 80^0C respectively (Margesium and Schinner, 2001). The use of thermophiles for biodegradation of hydrocarbons with low water solubility is therefore of interest as solubility and bioavailability are enhanced at elevated temperatures. It is expected that enhanced biodegradation of petroleum hydrocarbons is likely in skimmer pits because of its elevated temperature environments.

We also evaluated the potential for indigenous microorganisms found in skimmer pits to produce biosurfactants which can also enhance biodegradation. Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures which make them potential candidates for enhancing biodegradation and oil recovery (Desai and Banat, 1997). Biosurfactants have been used by several investigators to enhance the removal of hydrocarbons from oil contaminated environments (Huang *et al*, 2009, Okoro, 2009, Inakolu *et al*, 2004, Bovdoloi and Konwar, 2009).

In the present study, thermo and halo tolerant indigenous bacterial species were isolated from an oil production skimmer pit and their biodegradation and biosurfactant production potential were evaluated. The isolated bacterial species were further characterized by genetic methods using the 16S rRNA sequencing technique.

Materials and Methods:

Physico-chemical analysis of Skimmer Pit samples:

Skimmer pit samples were analyzed for total petroleum hydrocarbon (TPH), salinity, biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen, ammonia-nitrogen, phosphorus, potassium, pH and temperature. TPH was estimated by partition gravimetric method while salinity was measured as chloride by argentometric method as described in Eaton *et al*, 1995. The BOD and COD were also determined as described in Eaton *et al*., 1995. Phosphorus and Potassium were estimated by persulphate digestion method while Ammonia nitrogen was determined by titrimetric method as described in Eaton *et al*, 1995. pH and temperature of the samples were measured with digital Orion pH meters and thermometers respectively.

Isolation of hydrocarbon utilizing microorganisms

Hydrocarbon utilizing microorganisms were isolated with minimal salt media as described in Mills *et al*, 1978. The media plates contained in petri-dishes were inoculated with 0.1ml of serially diluted samples and inverted over sterile membrane filters moistened with crude oil (Escravos light) as the sole carbon source and held in the lid of the petri-dishes. The dishes were wrapped round with a masking tape so as to increase the vapor pressure within the petri-dishes and the plates were incubated at 32^{o}C for 6 days. At the end of incubation, developed colonies were isolated, sub-cultured and further purified for the purpose of identification.

Determination of optimal growth temperatures of hydrocarbon utilizing microorganisms from the skimmer pit

Optimal growth temperatures were determined by incubating previously sterilized 250 ml samples of skimmer pit inoculated with mixed bacterial culture from previous isolates in a temperature controlled rotary shaker for 7 days. At every 2 day interval, 0.1ml of the samples was withdrawn and population density of the bacteria determined by plate counts.

Biodegradation studies using the Skimmer pit as the sole carbon and nutrient source:

Growth and degradation studies over a time course were carried out by using the skimmer pit samples as the sole carbon and nutrient sources for microorganisms. 250ml of skimmer pit samples were inoculated with 5mls of already prepared starter cultures of mixed bacterial isolates from skimmer pits grown on Mills *et al*., 1978 media and incubated at

45^0C and 50^0C in a rotary shaker for 2 weeks. At every week interval, the residual hydrocarbon was extracted with methylene chloride and analyzed in a gas chromatograph.

Gas chromatographic analysis of the hydrocarbon:

1µl of the extracted oil was injected by an auto-injector (7683B series, Agilent Technologies, Santa Clara, CA) into a gas chromatograph (7890N series, Agilent) that was connected to a mass-selective detector (5975C inert XL MSD series, Agilent). The gas chromatograph was equipped with an HP-1 fused silica capillary column (length 50 m, inner diameter 0.32 mm, film thickness 0.52 μm; J&W Scientific) with helium as the carrier gas. The GC-MS system was operated as described by Agrawal *et al*, 2012.

Biochemical Characterization of Biosurfactants produced by the bacterial isolates:

Previously grown pure microbial cultures on Rosenberg *et al*, 1988 medium were centrifuged at 7000g for 30mins to separate the cells from the supernatants. The supernatants were then precipitated with hydrochloric acid as described in Umeji *et al*, 2010 and analyzed for the following;

a. **Lipid analysis using thin layer chromatography:** Precoated silica gel (20x 20cm) plates with petroleum ether, diethylether and acetic acid (90:1:1) as developing solvents. After air drying, the plates were stained with 5% sulphuric acid in 95% ethanol followed by heating at 150^0C for 30mins. The RF values of developed spots were calculated and compared with values of standard compounds in similar solvents as described in Kates (1972).

b. **Protein analysis:** The protein content of cell extracts was determined using the method of Bradford (1976). Reagents used included Coomassie blue 9250 (0.16ml), percloric acid (5.15ml), add distilled water to make 200ml. The reagent was stirred in a dark bottle overnight and filtered with Whatman No. 1. Filter paper. Protein extract (0.5ml) was added to 1ml cuvette + 0.5 ml of the reagent. The absorbance at 620nm was read against the reagent blank made up of 0.5ml water + Coomassie reagent. The concentration of the protein was extrapolated from the standard curve prepared with bovine serum albumin as the standard.

c. **Carbohydrate analysis:** The carbohydrate content of the biosurfactant was estimated using the anthrone method as described by Spiro (1966).

SDS Polyacrylamide gel (12%) electrophoresis:

To determine the molecular weight of proteins. After development, the gel was stained with Coomassie brilliant blue solution and allowed to stay overnight and photographed. Standard protein markers used include; Lysozyme (egg white) 14,000 DA, Beta-lactoglobulin, Bovine milk (18,400 DA) and egg albumin (45,000DA).

Petroleum products and other hydrocarbons used as emulsifier products

Crude oil was obtained from SPDC, Nigeria while Kerosine and Diesel oil were obtained from NNPC, Nigeria. Olive oil was purchased from a local supermarket in Nigeria while the rest of hydrocarbon substrates used were purchased from Merck chemicals.

Hydrocarbon substrate specificity of crude biosurfactants;

The ability of the bacterial isolates to grow on both pure and mixed hydrocarbon substrates as sole carbon source were tested on a liquid minimal salts media of Mills *et al,* 1978. All the substrates except the highly flammable ones were autoclaved before use, the flammable ones such as n-alkanes, and kerosene were sterilised by filtration before use. 100 ml of the minimal salt media was prepared in a 250ml Erlenmeyer flask and 0.1% hydrocarbon substrate was inoculated followed by the addition of 1ml of the bacterial inoculum from the already prepared nutrient broth and incubation for 48hrs at room temperature. Emulsion turbidity was measures as described in Rosenberg *et al*, 1979.

Determination of emulsification activity:

The standard emulsification assay of Rosenberg *et al,* 1979 was used in the determination of emulsification activity of the bacterial cultures used for the studies. The samples to be tested (0.5-0.1ml) were introduced into a 125ml flask containing TM buffer (20mM Tris-HCL) pH (7.0), 10mM, $MgS0_4$ to a final volume of 7.5ml and then 0.1ml of a 1:1 (v/v) mixture hexadecane and 2-methylnaphthalene was added. The samples were incubated at 30^0C with reciprocal shaking (160 strokes/min) for 1hr. Turbidity was then determined in a Klett-Summerson photometer (fitted with green filter). One unit of emulsifying activity per millilitre is defined as the amount of biopolymer that yielded 100 Klett units in the assay mixture. Emulsion turbidity was directly proportional to the amount of biopolymer produced.

Identification of Bacterial isolates by 16S rRNA sequencing.

DNA extraction and amplification.

Genomic DNA was extracted from 42.5 ml aliquot of the samples using MP Biomedical FastDNA technique as described in the fast DNA Kit, Catalog #6540-400.

PCR amplification and purification for pure microbial cultures cultures

Extracted genomic DNA (2µl) of the bacterial pure cultures were amplified through PCR (94 °C, 7 min;then 30 cycles of 94 °C 10 s, 60 °C 1.30 s, 72 °C 90 s; 72 °C; final hold at 4 °C) using 25µl of nuclease free water and 23 µL of PCR Master Mix comprised of (5 µL PCR buffer, 5 µL Corral load, 10µl Q solution, 1µl dNTPs, 1µl primer forward(EUB 27F), 1µL, primer reverse (EUB 1492R) and 0. 25µl, toptag). PCR product was verified on a 0.7% agarose gel and purified with a QIAquick PCR Purification Kit (Qiagen). The concentrations of the PCR products were determined on a Qubit Fluorometer (Invitrogen). The PCR products were sequencedat the University of Calgary core DNA services.

Bacterial Identification:

Sequences were compared with those in the National Center for Biotechnology information (NCBI) database by BLAST searches and the sequences for each identified bacterial isolate were deposited in Genebank under accession numbers JQ815397-JQ815404

Results:

Physico-chemical analysis;

Physico-chemical analysis of skimmer pit samples showed an in-situ temperature of 40-45^{0}C during sampling and relatively high salinity and TPH concentrations as shown in Table 1. It is obvious that these parameters may vary during rainy season since the skimmer pit is an open pit. From the available data, it can be advanced that the skimmer pit environment can sustain microbial growth and proliferation; it can also be selective because of its elevated salinity and temperature conditions.

Table 1. Physico-Chemical analysis of Skimmer pit samples.

	Parameters measured	Concentration (mg/L)
1	Total Petroleum Hydrocarbon (TPH)	4100
2	Salinity	6012
3	BOD-5	220
4	COD	240
5	Dissolved Oxygen	4
6	Ammonia	2.60
7	Phosphorus	4.50
8	Potassium	120
9	pH	6.9
10	In-Situ Temperature	40-45^{0}C

Isolation and Characterization of Bacterial isolates from oil production skimmer pit.

By using the minimal salt procedure of Mills *et al*, 1978, 6 different bacterial cultures were isolated from skimmer pit samples. The cultures were purified by streaking method and maintained in nutrient agar slants at low temperature (4^{0}C) for further identification. The stages adopted in bacterial identification involved bacterial genomic DNA extraction, purification and amplification by PCR and genetic sequencing. The agarose gel electrophoresis picture of PCR amplified DNA are shown in Fig. 1. While the identified bacterial isolated with their accession numbers are shown in table 2.

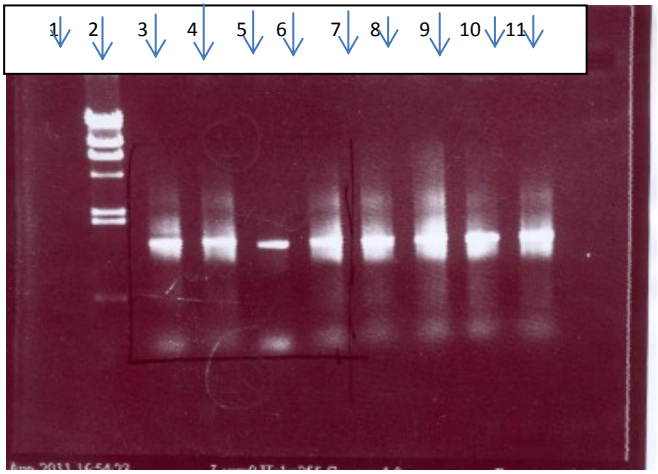

Fig. 1. Agarose gel electrophoresis picture of PCR amplified DNA showing bands that verified the presence of 16S rRNA gene in the bacterial isolates from oil production skimmer pit (Lanes 1 = Negative control, 2 = HIND 111 Ladder, 3 = *SKP-1*, 4 = *Same as 3*, 5 = *SKP-2*, 6 = *SKP-3*, 7 = Same as 6, 8= SKP-4, 9 = *SKP-5*, 10 = *SKP-6*, 11 = Negative control).

Table 2. Accession numbers of 16S rRNA genes and nearest homolog of isolates from skimmer pit samples obtained in the study

Isolate Code	GeneBank Accession Number	Closest GeneBank Homolog	% Identity	Class of nearest Homology	Name of nearest Homology
SKP-1	JQ815397	FJ497685.1	99	Gammaproteobacteria	*Pseudomonas* sp. VS-1
SKP-2	JQ815398	HQ844502.1	99	Gammaproteobacteria	*Pseudomonas aeruginosa* strain S2QPS8
SKP-3	JQ815399	JF441244.1	99	Gammaproteobacteria	*Serratia marcescens* strain A4
SKP-4	JQ815402	AM905852.2	99	Gammaproteobacteria	*Pseudomonas stutzeri*
SKP-5	JQ815403	JN585674.1	99	Gammaproteobacteria	*Pseudomonas stutzeri* strain RA10.
SKP-6	JQ815404	JN565980.1	100	Gammaproteobacteria	*Pseudomonas stutzeri*strain BOD-3

Determination of optimal growth temperatures of the bacterial isolates

The bacterial isolates used in the present study were grown and incubated at various temperatures on minimal salt broth with crude oil as the sole carbon and energy source to determine their various optimal temperatures for growth. The incubation temperatures used were 32^0C (Room temperature), 45^0C (Skimmer pit temperature) and 55^0C (thermophylic temperature).All the bacterial isolates grew well at 32^0C, and 45^0C but poorly at 55^0C. Isolates most homologous to *Pseudomonas* sp. VS-1, *Pseudomonas stutzeri*, *Pseudomonas stutzeri* strain RA10 and *Pseudomonas stutzeri* strain BOD-3 recorded their optimal growth temperatures at 45^0C while the isolates most homologous to *Pseudomonas* sp. strain S2QPS8 and *Serratiamarcescens strain A4*had their optimal growth temperatures at 32^0C. Detailed results are shown in table 2.

Table. 2. Determination of optimal growth temperatures of different bacterial isolates from skimmer pit

Sample Code	Name of nearest homolog	Incubation period in days/Bacterial Population at 32^0C (cfu/ml x 10^4)				Incubation period in Days/Bacterial Population at 45^0C (cfu/ml x 10^4)				Incubation period in Days/Bacterial Population at 50^0C (cfu/ml x 10^4)			
		0	2	4	6	0	2	4	6	0	2	4	6
SKP-1	*Pseudomonas* sp. VS-1	0.026	33	76	54	0.015	48	84	68.60	0.018	0.008	0.001	0.006
SKP-2	*Pseudomonas aeruginosa* strain S2QPS8	0.018	46	84	68	0.028	18	28	14	0.022	0.002	0.005	0.004
SKP-3	*Serratia marcescens* strain A4	0.026	13	46	75	0.011	3.40	28	33.50	0.034	0.008	0.002	0.004
SKP-4	*Pseudomonas stutzeri*	0.011	48	88	45	0.021	38	112	63	0.016	0.008	0.002	0.006
SKP-5	*Pseudomonas stutzeri* strain RA10.	0.034	11	48	57	0.018	36	64	46	0.011	0.08	0.06	0.05
SKP-6	*Pseudomonas stutzeri* strain BOD-3	0.023	12	73	46	0.012	08	80	58	0.020	0.08	0.03	0.01

Note: Skimmer pit temperature during the period of sampling ranged from 40- 45^0C.

Biodegradation studies

The mixed bacterial populations from skimmer pit comprising of SKP-1, SKP-2, SKP-3, SKP-4, SKP-5 and SKP-6 were subjected to biodegradation tests using previously sterilized skimmer pit sample as the sole carbon and nutrient source. At every weekly interval, samples were collected and the residual hydrocarbon analyzed by gas chromatograph. It was observed that after 2 weeks of exposure, the mixed bacterial populations from skimmer pit reduced the TPH from its initial value of 4000ppm at week 0 to 330ppm at week 2 (91.7% reduction). The GC chromatograms of the biodegraded oil are shown in Fig. 2.

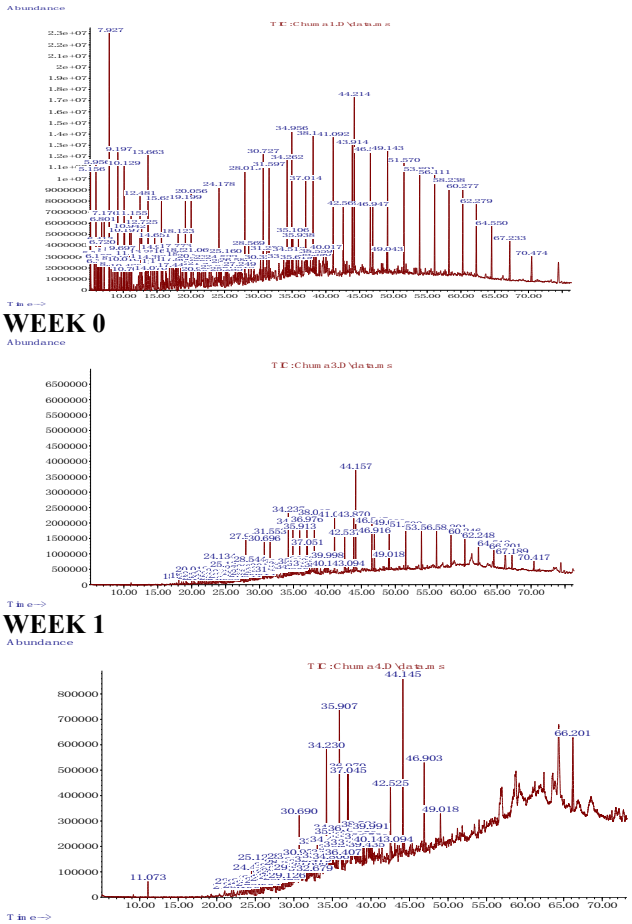

Fig. 2. Biodegradation of petroleum hydrocarbon by mixed bacterial culture isolates from Skimmer pit (Residual petroleum hydrocarbon; Week 0 = 4000ppm, Week 1 = 1100ppm, Week 2 (330ppm)

Biochemical characterization of Biosurfactants produced by the bacterial isolates;

Preliminary biochemical characterization of crude biosurfactants produced by the bacterial isolates showed that the following bacterial isolates namely; SKP-1, SKP-2, SKP-4, SKP-5 and SKP-6 had considerable concentrations of carbohydrates and lipids with no traces of protein and were therefore tentatively classified as glycolipids. *Isolate SKP-3* had both protein and lipid component without any traces of carbohydrate and was tentatively classified as lipoproteins. Detailed results are shown in table 3.

Hydrocarbon substrate specificity of crude biosurfactants.

Emulsifying properties of the biosurfactants produced by the bacterial isolates were evaluated and the various biosurfactants were found to have a very wide substrate specificity emulsifying a wide range of hydrocarbons which include aliphatic hydrocarbons ranging from pentane to octane. Aromatic hydrocarbons which were also emulsified include Benzene, Toluene, Xylene, Buthyl benzene and Octyl benzene. Mixtures of hydrocarbon compounds which were emulsified include; Crude oil, Olive oil, Kerosine, Diesel oil, Hexadecane + 2-methyl naphthalene, Benzene + cyclohexane and Toluene + cyclohexane. Detailed results are shown in table 4.

Biological activity of crude biosurfactants:

The biological activity assay on the biosurfactants produced by the bacterial isolates showed relatively high biological activity of all the biosurfactants produced with optimal pH range of 7.01-7.35. The highest biological activity of 47.50 u/ml was observed in the biosurfactants produced by isolate SKP-3. Detailed results are shown in Table 5.

Table 3. Partialbiochemical characterization of bio-surfactants

	Bacterial Isolate	**Biochemical composition of the Bio-surfactant**			**Class of Bio-surfactant Produced**	Name of nearest homolog
		Protein (µg/ml)	**Carbohydrate (µg/ml)**	**Lipid (µg/ml)**		
1	**SKP-1**	0	260	15.60	**Glycolipids**	*Pseudomonas* sp. VS-1
2	**SKP-2**	20.30	160	33.50	**Glycolipids**	*Pseudomonas aeruginosa* strain S2QPS8
3	**SKP-3**	16.50	0	14.80	**Lipoproteins**	*Serratia marcescens* strain A4
4	**SKP-4**	0	180	12.20	**Glycolipids**	*Pseudomonas stutzeri*
5	**SKP-5**	0	110	26.50	**Glycolipids**	*Pseudomonas stutzeri* strain RA10.
6	**SKP-6**	0	240	13.50	**Glycolipids**	*Pseudomonas stutzeri* strain BOD-3

Table 4. Hydrocarbon substrate specificity of crude biosurfactants produced by high temperature tolerant bacterial isolates from crude oil production skimmer pit.

Hydrocarbon Substrates		**Emulsion Turbidity of Bacterial Isolates (KU)**					
ALKANES		***SKP-1***	***SKP-2***	***SKP-3***	***SKP-4***	***SKP-5***	***SKP-6***
1	**n-Pentane**	41	36	56	55	14	36
2	**n-Hexane**	32	48	55	68	36	53
3	**Cyclohexane**	28	65	44	33	52	41
4	**Decane**	54	38	31	14	75	88
5	**Pentadecane**	86	110	48	77	34	43
6	**Hexadecane**	55	65	96	36	48	62
7	**Octadecane**	23	48	42	41	33	78
Aromatics							
8	**Benzene**	110	67	120	86	28	130
9	**Toluene**	35	120	65	130	46	86
10	**Xylene**	56	54	80	110	130	108
11	**Buthyl benzene**	75	43	41	46	160	140
12	**Octyl benzene**	78	28	65	35	80	110
Hydrocarbon Mixtures (1:1)							
13	**Hexadecane + Methylnaphthalene**	130	210	160	240	110	140
14	**Benzene + Cyclohexane**	85	110	80	65	88	76
15	**Toluene + Cyclohexane**	45	160	220	41	76	48
	Others						
16	**Olive oil**	120	80	140	53	43	130
17	**Kerosine**	110	220	260	130	120	210
18	**Diesel oil**	320	310	360	220	85	320
19	**Crude oil**	560	480	560	380	310	510

Table 5. Biological activity of biosurfactants produced by high temperature tolerant bacterial isolates from oil production skimmer pit.

	Code number of Isolate	Biological activity (u/ml)	Optimal pH
1	SKP-1	45.30	7.06
2	SKP-2	38.60	7.21
3	SKP-3	47.50	7.12
4	SKP-4	32.10	7.01
5	SKP-5	41.20	7.35
6	SKP-6	36.40	7.01

Discussion:

Chemical analysis of skimmer pit samples used in the present study revealed that it contained substantial micronutrients that can sustain microbial growth and proliferation even though at anelevated salinity and temperature conditions. The use of thermophiles for biodegradation of hydrocarbons with low water solubility such as crude oil is of interest as solubility and thus bioavailability are enhanced at elevated temperatures (Margesin and Schinner, 2001). Temperature plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism because bioavailability and solubility of less soluble hydrophobic substances such as aliphatic and aromatic hydrocarbons are temperature dependent according to Margesin and Schinner (2001). Muller *et al*, 1988 and Margesin and Schinner (2001) also advanced that temperature increase affect the decrease in viscosity thereby affecting the degree of distribution and increase in diffusion rates of organic compounds. This means that higher biodegradation rates are expected at elevated temperatures as was observed in our studies.

The skimmer pit that was used in the present study had an elevated temperature that varied from 40-45^{0}C and salinity concentration of 6012mg/L. These peculiar environmental conditions were able to sustain the growth and proliferation of some of the six bacterial isolates that were used in the studies. All the six bacterial isolates used in our studies grew well at both 32^{0}C and 45^{0}C but poorly at 50^{0}C suggesting they are thermotolerant but moderate thermophiles. The 6 bacterial isolates when used as a mixed culture were able to degrade about 92% of the residual TPH of the skimmer pit within 2 weeks of exposure at 45^{0}C and at a salinity (Chloride) level of 6012mg/L.

Lugowski *et al* (1997) developed a mixture of thermophylic aerobic bacteria comprising predominantly of *Pseudomonas* species that were used to detoxify hydrocarbon contaminated effluent stream at 42^0C and this suggests that some *Pseudomonas* species can be thermotolerant. All the 6 bacterial isolates were able to grow well at a high salinity level of 6012mg/L suggesting that they are also halotolerant.

Another interesting aspect of the present study is the production of biosurfactant by the 6 bacterial isolates at the elevated temperature and saline conditions of the skimmer pit. All the 6 bacterial isolates used in the present study produced biosurfactants with high biological activity and wide substrate specificity. The highest biological activity of 47.50µ/ml was recorded with isolate SKP-3 which is homologous to *Serratia marcescence* strain A4 at a pH optimum of 7.2. The biosurfactants produced by the 6 bacterial isolates exhibited wide substrate specificity and emulsified a wide range of substrates from alkanes to aromatics to complex hydrocarbon mixtures. The highest emulsion turbidity in all the biosurfactants tested was recorded with crude oil.

Several authors have implicated both psychrophylic and mesophylic pseudomonas species in biodegradation and biosurfactant production (Javis and Johnson, 1949, Atlas, 1981, Desai and Banat, 1997, Guero-Santos *et al*, 1984, Song *et al,* 2006) but report on the involvement of thermotolerant and halotolerant *Pseudomonas* species in biodegradation and biosurfactant production is scanty in literature. Some investigators like Kummer *et al*, 2008 have isolated halotolerant and thermotolerant *Pseudomonas* species from oil contaminated soils that are capable of producing biosurfactants and degrade hydrocarbon at a temperature of 45^0C and salinities up to 6%. Margesin and Schinner (2001) have also investigated the biodegradation and bioremediation of hydrocarbons under extreme environmental conditions while Cameotra and Makkar (1998) investigated the synthesis of biosurfactants under extreme environmental conditions. Our investigation revealed that simultaneous production of biosurfactants and biodegradation of hydrocarbons can be achieved in an extreme environmental condition such as that of skimmer pit by the resident microbial flora that were able to adapt to such harsh environmental conditions. *Serratiia* mercescens have also been implicated by many investigators in petroleum hydrocarbon biodegradation (Ijah, 1998, Okoro, 1999, Wongsa *et al*, 2004,) and biosurfactant production (Roldan-Carrilo *et al*, 2011, Anyanwu *et al*, 2011) but our work was the first to implicate the halotolerant and thermotolerant isolates that are homologous to*Serratia marcescens* in both biodegradation and biosurfactant production.

Simultaneous hydrocarbon biodegradation and biosurfactant production by oil field bacteria as demonstrated by Okoro, 2009, 2010 and Mnif *et al*, 2011 can be used to gradually eliminate environmental hydrocarbon pollutants and we have further demonstrated in the present study that rapid elimination of hydrocarbon pollutants from the skimmer pit can also be achieved even at its elevated saline and temperature conditions using resident bacteria that both produce biosurfactants and degrade petroleum hydrocarbons simultaneously.

Conclusion:

The present study was able to establish that the 6 bacterial isolates from skimmer pit used in the present study were able to carry out biodegradation and biosurfactant production simultaneously at the skimmer pits' elevated saline and temperature conditions. This is an indication that insitu-biodegradation procedure can be carried out successfully by the resident microbial flora of the skimmer pit. From the economic point of view it will be cheaper to carry out an in-situ bioremediation procedure on the skimmer pit to remove the petroleum hydrocarbon contaminants than to adopt the very costly thermal treatment processes that involves costly evacuation of wastes, transportation and offsite thermal treatment.

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