Isolation and characterization of thermotolerant and halotolerant aerobic bacterial that produce biosurfactants and degrade petroleum hydrocarbons from produced water discharge point

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Abstract: Six thermo and halo-tolerant bacterial isolates from a produced water discharge area were evaluated for their ability to produce biosurfactants and degrade petroleum hydrocarbons simultaneously under elevated temperature and saline conditions. Phylogenetic analysis using 16S rRNA sequencing revealed that all the 6 bacterial isolates were homologous to the class Gammaproteobacteria. Growth and degradation studies over a time course were carried out using the produced water samples collected at discharge point and supplemented with 10% crude oil (Escravos light) as the sole carbon and energy source for microorganisms. At an elevated temperature of 48^0C and salinity level of 9016mg/L, the bacterial isolates were able to grow and produce highly biological active biosurfactants and when used as a mixed culture, the bacterial isolates were able to degrade petroleum hydrocarbons by reducing the residual total petroleum hydrocarbon (TPH) by 94.6% within 2 weeks of exposure. Our findings demonstrated that in-situ bioremediation using resident thermotolerant and halotolerant bacterial flora found at a produced water discharge area can be employed to degrade and eliminate the residual and accumulated TPH found within the produced water discharge area. This can reduce the threat of chronic pollution to the marine environment by petroleum hydrocarbons that otherwise accumulate to significant proportions with time if left untreated.
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Introduction

Produced water results from production of oil and gas hydrocarbons from underground oil reservoirs. When hydrocarbons are produced, the water component is separated from the oil and gas, treated and discharged into the sea for near-shore and offshore oil production operations. Produced water constitutes a source of chronic pollution to the marine environment since it is continuously discharged to the marine environment over a long period of time (Middleditch, 1984; Okoro, 2010). According to Middleditch, 1985 and Neff *et al* 1989, the chemical properties of produced water that could cause harmful effects to marine organisms includes its elevated salinity, altered ion ratios, low dissolved oxygen, heavy metals, biocides, petroleum hydrocarbons and other organics. The fate of these components when discharged into the environment will depend upon the chemical and physiochemical properties of the individual components, the properties of the receiving environment as well as the metabolic versatility of the biota in that environment (Neff, 1985).

Even though produced waters are treated by thermal treatment processes to remove the petroleum hydrocarbon and other organics, research has shown that the conventional thermal treatment processes does not completely remove the total petroleum hydrocarbon (TPH) especially the recalcitrant polycyclic aromatic hydrocarbon (PAH) (Okoro, 1999). As a result, natural biodegradation processes are required to augment the conventional mechanical treatment processes in order to reduce the residual TPH concentrations in the environment.

Recent investigations by Okoro (2010) showed that the temperature and salinity at the produced water discharge points ranged from 42^0C to 48^0C and 7033 -11012 mg/L respectively. This suggests that the resident microbial flora at produced water discharge points are likely to be dominated by thermo and halotolerant microorganisms.

The rate of biodegradation by the resident microbial flora is influenced by several factors such as nutrients, oxygen, pH value, temperature, salinity, composition, concentration and bioavailability of the contaminants as well as the history of the contaminated environment (Atlas, 1981). 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 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 limit 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.

In the present study, we evaluated the potential for indigenous microorganisms isolated from produced water discharge points 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), and synthesis of biosurfactants under extreme environmental conditions has been investigated by Cameotra and Makkar, 1998.

In the course of our investigation, biodegradation and biosurfactant production potential of 6 bacterial isolates from a produced water discharge area were evaluated with the aim of establishing whether the resident bacterial flora can produce potent biosurfactants and consequently degrade petroleum hydrocarbons at an elevated temperature and saline conditions that are associated with produced water discharge areas.

Materials and Methods

Sample collection:

Produced water samples were collected in sterile 500 ml glass Wheaton bottles within the 100 m circumference of the produced water discharge point from a nearshore oil production facility in Nigeria. The total circumference coverage at the discharge point of produced water is about 200m and is barricaded with a sieve barrier to prevent oil from penetrating the surrounding sea water.

Physico-chemical analysis of samples

Samples collected from produced water discharge points 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 a partition gravimetric method (Eaton *et al*, 1995) 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 the persulphate digestion method (Eaton *et al,* 1995) while ammonia nitrogen was determined by titrimetric method as described in Eaton *et al*, 1995. pH and temperature of the samples were measured on site 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 larger 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 and energy source and held in the lid of the petri-dishes. The dishes were wrapped with a masking tape to increase the vapor pressure within the petri-dishes and 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.

Optimal growth temperatures were determined by incubating 250 ml samples of diluted produced water 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 measured by plate counts.

Biodegradation studies using produced water as the sole carbon and energy source:

Growth and degradation studies over a time course were carried out using the produced water samples collected at discharge point and supplemented with 10% crude oil (Escravos light) as the sole carbon and energy source for microorganisms. 250 ml of samples were inoculated

with 5 ml of already prepared starter cultures of mixed bacterial isolates from produced water discharge point grown on Mills *et al*., 1978 media and incubated at 48^0C in a rotary shaker for 2 weeks. At weekly intervals, 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 & #956;m; 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 medium by Rosenberg *et al*, 1988 medium were centrifuged at 7000xg for 30 mins 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. **Lipids 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 30 mins. 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 Comassie blue 9250 (0.16ml), percloric acid (5.15ml), add distilled water to make 200 ml. The reagent was stirred in a dark bottle overnight and filtered with Whatman No. 1. filter paper. Protein extract (0.5 ml) was added to 1ml cuvette + 0.5 mls of the reagent. The absorbance at 620 nm 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 (18,400DA) 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 sterilized by filtration before use. 100 ml of the minimal salt media was prepared in a 250 ml Erlenmeyer flask and 0.1% hydrocarbon substrate was inoculated followed by the addition of 1 ml of the bacterial inoculum from the already prepared nutrient broth and incubation for 48 hrs 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 genetic sequencing technique.

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 DNAs (2µl) of the bacterial 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, 10 µl Q solution, 1 µl dNTPs, 1 µl forward primer (EUB 27F), 1 µL, of reverse primer (EUB 1492R) and 0. 25µl, Toptag polymerase. 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 QubitFluorometer (Invitrogen). The PCR products were later sequenced at the University of Calgary Core DNA services. The 16s rRNA gene sequence homology was determined by performing a BLAST search using the National Center for Biotechnology Information (NCBI) database. The sequences can be found under the GenBank accession numbers JQ815397-JQ815402.

Bacterial Identification:

Sequences were compared with those in the National Centre for Biotechnology information (NCBI) database by BLAST searches. The sequences for each identified bacterial isolate were deposited in GenBank under accession numbers JQ815397 – JQ815402.

Results:

Physico-Chemical analysis of samples.

Physico-Chemical analysis was carried out on samples collected from produced water discharge points. The temperature and salinity level at the time of sampling were 48^{0}C and 9016mg/l respectively. Detailed results are shown in Table 1.

Determination of the optimal growth temperature of the bacterial isolates.

The bacterial isolates were grown on minimal salt broth medium of Mills *et al*, 1978 with crude oil as the sole carbon and energy source and incubated at various temperatures (32^{0}C, 48^{0}C and 55^{0}C) on a rotary shaker. The aim is to determine the optimal growth temperature of the bacterial isolates. All the bacterial isolates grew well at both 32^{0}C and 48^{0}C but poorly at 55^{0}C. Isolates CPW-1, CPW-3, CPW-5 and CPW-6 grew well at 48^{o}C but recorded optimal growth at 32^{0}C while isolates CPW-2 and CPW-4 which equally grew well at 32^{o}C recorded optimal growth at 48^{0}C. Detailed results are shown in Table 3.

Table 1.Physico-Chemical analysis of samples from produced water discharge point.

	Parameters measured	Concentration (mg/L)
1	Total Petroleum Hydrocarbon (TPH)	320
2	Salinity	9016
3	BOD-5	160
4	COD	210
5	Dissolved Oxygen	3
6	Ammonia	1.20
7	Phosphorus	2.80
8	Potassium	68
9	pH	7.2
10	In-Situ Temperature	48^{0}C

Isolation and Characterization of bacterial isolates from produced water discharge point.

Six bacterial isolates were isolated from produced water discharge points using the minimal salt formulation and procedure of Mills *et al,* 1978. The cultures were purified by the streak method and maintained in nutrient agar slants at low temperature (4^{0}C) till further identification. The stages adopted in bacterial identification involved bacterial genomic DNA extraction, purification and amplification by PCR and 16S rRNA sequencing. Agarose gel electrophoresis of PCR amplified DNA are shown in Fig. 1 while the identified bacteria isolates with their accession numbers are listed in Table 2.

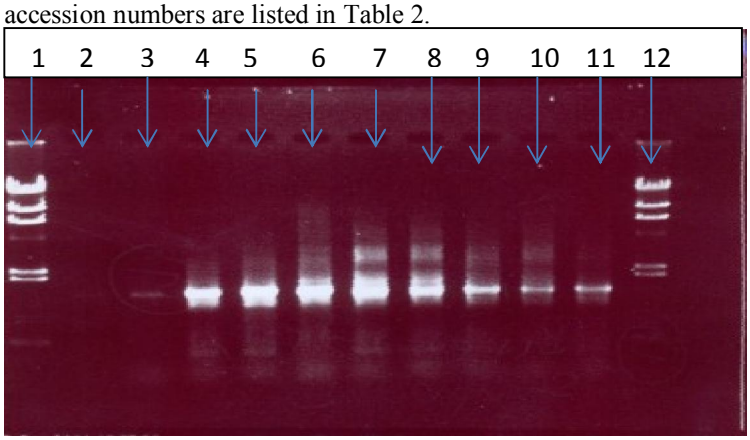

Fig. 1. Agarose gel electrophoresis picture of PCR amplified 16S rRNA genes (Lanes 1 & 12 = Lambda DNA Xhind111, 2 = Negative control, 3 = Unidentified bacterial isolate, 4 = *CPW-1*, 5 & 6 =CPW-2, 7 = *CPW-3*, 8 = *CPW-4*, 9 = *CPW-5, 10&11=CPW-6)*

Table 2. Assession numbers of 16S rRNA genes and nearest homolog of isolates from produced water discharge obtained in the study.

Isolate code number	GenBank Accession number	Closest GenBank Homolog	% Identity to nearest homolog	Class of nearest homology	Name of nearest homolog
CPW-1	JQ815397	FJ4497685.1	98	Gammaproteobacteria	*Pseudomonas* sp. VS-71
CPW-2	JQ815398	HQ844502.1	99	Gammaproteobacteria	*Pseudomonas aeruginosa* strain S2QPS8
CPW-3	JQ815399	JF441244.1	99	Gammaproteobacteria	*Serratia marcescens* strain A4
CPW-4	JQ815400	JF419326.1	99	Gammaproteobacteria	*Pseudomonas* sp. MM15
CPW-5	JQ815401	EU391389.1	97	Gammaproteobacteria	*Pseudomonas aeruginosa* strain SG-1
CPW-6	JQ815402	AJ31229.1	98	Gammaproteobacteria	*Pseudomonas stutzeri*

Table. 3. Determination of optimal growth temperatures of different bacterial isolates from produced water discharge point.

Isolate Code No.	Name of closest homolog	Incubation period in days/Bacterial Population at 32^0C (cfu/ml x 10^4)				Incubation period in Days/Bacterial Population at 48^0C (cfu/ml x 10^4)				Incubation period in Days/Bacterial Population at 55^0C (cfu/ml x 10^4)			
		0	**2**	**4**	**6**	**0**	**2**	**4**	**6**	**0**	**2**	**4**	**6**
CPW-1	*Pseudomonas* sp. VS-71	0.026	38	76	54	0.015	18	24	28.6	0.018	0.008	0.001	0.006
CPW-2	*Pseudomonas aeruginosa* strain S2QPS8	0.028	36	64	48	0.028	38	88	74	0.012	0.002	0.003	0.004
CPW-3	*Serratia marcescens* strain A4	0.026	13	46	75	0.011	3.40	18	33.50	0.034	0.004	0.002	0.003
CPW-4	*Pseudomonas* sp. MM15	0.011	18	48	45	0.021	38	75	63	0.016	0.008	0.002	0.006
CPW-5	*Pseudomonas aeruginosa* strain SG-1	0.034	11	48	57	0.018	16	24	36	0.012	0.08	0.06	0.02
CPW-6	*Pseudomonas stutzeri*	0.018	22	56	55	0.0012	12	36	28	0.003	0.043	0.002	0.0011

Biodegradation studies.

A mixed bacterial culture comprising of six isolates obtained from produced water discharge points were subjected to biodegradation tests using previously sterilized produced water (with 10% crude oil) and diluted with seawater 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 reduced the TPH from its initial value of 4800ppm at week 0 (Control) to 260ppm at week 2 (96.4% reduction). GC chromatograms of biodegraded oils are shown in Fig. 2.

Biochemical characterization of biosurfactants produced by bacterial isolates.

Preliminary and partial biochemical characterization of crude biosurfactants produced by the bacterial isolates showed that all the bacterial isolates with the exception of CPW-3 produced the glycolipid class of biosurfactants that are notable for the Pseudomonas species. Detailed results are shown in Table 4.

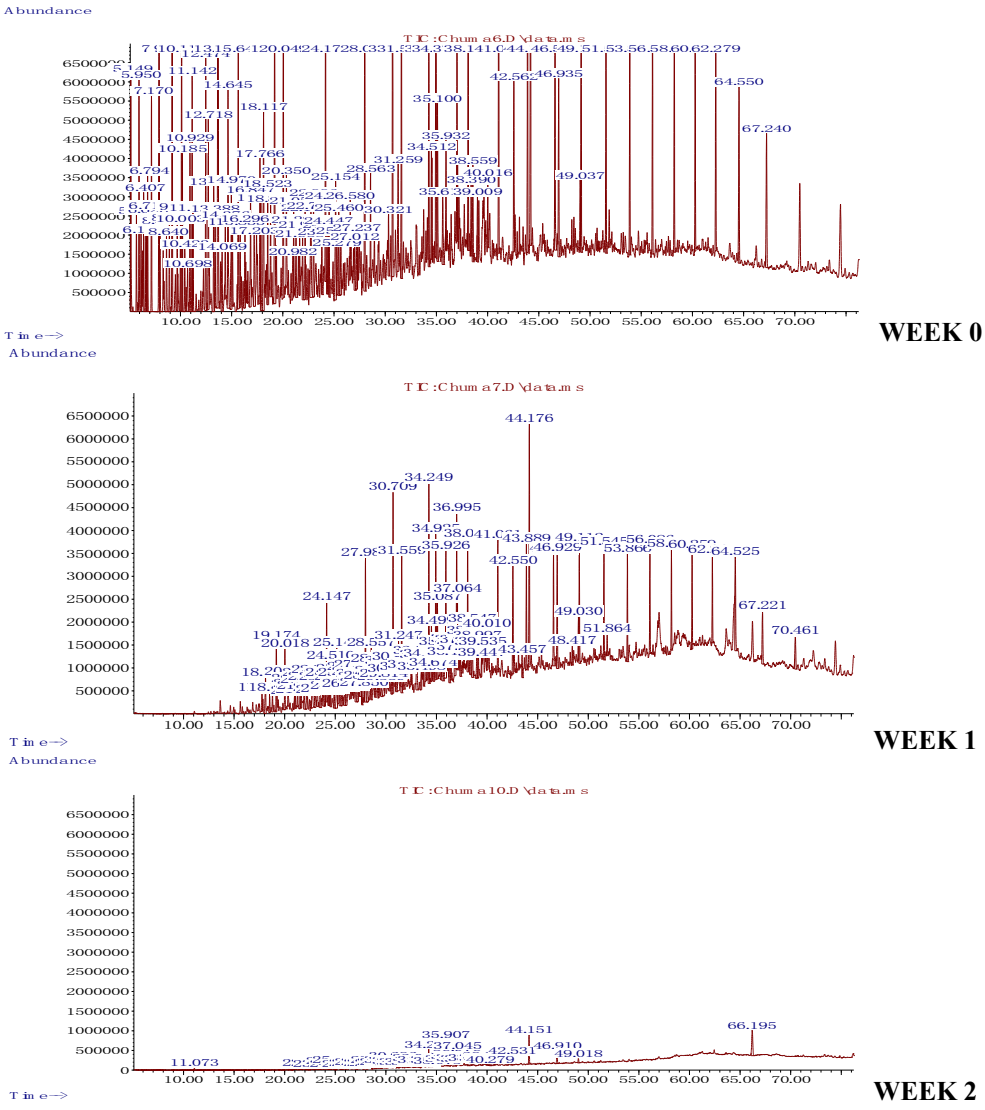

Fig. 2. Biodegradation of petroleum hydrocarbon by mixed bacterial culture isolates from untreated produced water (Residual petroleum hydrocarbon; Week 0 = 4800ppm, Week 1 = 1200ppm, Week 2 (260ppm).

Table 4. Biochemical Characterization of Bio-surfactants

Isolate code number	Name of closest homolog	Biochemical composition of the Bio-surfactant			Class of Bio-surfactant Produced
		Protein (µg/ml)	Carbohydrate (µg/ml)	Lipid (µg/ml)	
CPW-1	*Pseudomonas* sp. VS-71	0	160	7.60	**Glycolipids**
CPW-2	*Pseudomonas aeruginosa* strain S2QPS8	0	220	13.50	**Glycolipids**
CPW-3	*Serratia marcescens* strain A4	6.80	0	14.20	**Lipopeptide**
CPW-4	*Pseudomonas* sp. MM15	0	160	12.60	**Glycolipids**
CPW-5	*Pseudomonas aeruginosa* strain SG-1	0	110	23.50	**Glycolipids**
CPW-6	*Pseudomonas stutzeri*	0	86	12.30	**Glycolipids**

Emulsifying properties of crude biosurfactants on various hydrocarbon substrates

Emulsifying properties of the various biosurfactants produced by the bacterial isolates were evaluated and the biosurfactants were found to have wide substrate specificity emulsifying wide range of hydrocarbons which include; aliphatic hydrocarbons ranging from pentane to octane. Aromatic hydrocarbons which were emulsified include; benzene, toluene, zylene, b-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 5.

Biological activity of crude biosurfactants

The biosurfactants produced by the bacterial isolates showed relatively high biological activity in all the 6 isolates with an optimal pH range of 7.01-7.12. The highest biological activity of 48.50µ/ml was however observed in the biosurfactants produced by isolate CPW-3. Detailed results are shown in table 6.

Discussion:

Physicochemical analysis of samples showed TPH and Salinity levels of 320mg/L and 9016mg/L respectively and an in-situ temperature of 48^0C, indicating considerable TPH in produced water at the point of discharge. It is also expected that as a result of the elevated saline and temperature condition at the point of discharge of produced water, most of the resident bacterial flora that thrives in that environment will be thermotolerant and halotolerant.

We discovered in the course of our investigation that the bacterial flora at the point of discharge of produced water were dominated by homologs closely related to Pseudomonas.

In the determination of optimal growth temperature of the bacterial isolates using temperature ranges of 32^0C, 48^0C and 55^0C, it was observed that homologs closely related to *Pseudomonas* sp. VS-71, *Serratia marcescens* strain A4, *Pseudomonas aeruginosa strain SG-1 and Pseudomonas stutzeri* grew well at both 32^0C and 48^0C, but poorly at 55^0C. The optimal growth temperature was however at 32^oC. On the contrary, homologs closely related to *Pseudomonas* aeruginosa strain S2QPS8 and *Pseudomonas sp. MM15* grew better at 48^0C than at 32^0C. Generally all the bacterial isolates grew well at both 32^0C and 48^0C but poorly at 55^0C suggesting that the isolates were moderate thermophiles.

Table 5. Emulsifying properties of crude biosurfactants produced by thermo and halotolerant isolates from produced water discharge point.

Hydrocarbon Substrates		Bacterial Isolates and their various emulsion turbidities (in KU)					
ALKANES		**CPW-1**	**CPW-2**	**CPW-3**	**CPW-4**	**CPW-5**	**CPW-6**
1	**n-Pentane**	61	46	51	65	56	34
2	**n-Hexane**	32	42	65	48	33	56
3	**Cyclohexane**	23	62	54	33	61	47
4	**Decane**	44	38	32	24	68	54
5	**Pentadecane**	66	85	48	71	33	73
6	**Hexadecane**	56	55	56	76	62	54
7	**Octadecane**	51	48	22	45	38	56
Aromatics							
8	**Benzene**	100	97	120	76	120	88
9	**Toluene**	75	130	75	120	86	120
10	**Xylene**	50	74	80	130	180	110
11	**Buthyl benzene**	85	53	61	40	110	75
12	**Octyl benzene**	78	48	65	55	102	54
Hydrocarbon Mixtures (1:1)							
13	**Hexadecane + Methylnaphthalene**	230	210	160	245	240	260
14	**Benzene + Cyclohexane**	110	120	80	65	102	98
15	**Toluene + Cyclohexane**	85	160	210	44	58	65
	Others						
16	**Olive oil**	220	180	140	150	230	320
17	**Kerosine**	110	230	260	230	220	280
18	**Diesel oil**	360	310	320	220	380	340
19	**Crude oil**	430	480	560	580	540	450

Table 6. Biological activity of biosurfactants produced by thermo and halotolerant bacterial isolates

Isolate code	Biological activity (u/ml)	Optimal pH
CPW-1	48.30	7.06
CPW-2	38.20	7.11
CPW-3	42.30	7.12
CPW-4	42.50	7.01
CPW-5	43.20	7.10
CPW-6	36.40	7.20

Margesin and Schinner, 2001 and Muller *et al*, 1988 have advanced that higher biodegradation rates are expected at higher temperatures because solubility and bioavailability of hydrocarbons are enhanced at elevated temperatures. The microbial isolates used in the present study when used as a mixed culture reduced the residual TPH in the sample by 94.6% within 2 weeks of exposure at a temperature of 48^0C. A similar study was conducted by Lugowski *et al*, 1997 using a mixed culture of thermophyllic aerobic bacteria comprising predominantly of *Pseudomonas* species that were used to detoxify hydrocarbon contaminated effluent stream at 42^0C. Kumar *et al,* 2008 have also investigated biosurfactant production and hydrocarbon degradation by thermotolerant *Pseudomonas* species at a temperature of 45^0C and salinity levels of up to $6g/l^{-1}$.

It was observed in the course of our investigation that homologs closely related to *Pseudomonas stutzeri* species were among the dominant bacterial flora at the produced water discharge point. Many investigators have implicated psychrophyllic and mesophyllic *Pseudomonas stutzeri* species in hydrocarbon biodegradation (Stringfellow and Atkin 2005, Olanira *et al*, 2008, Tandich 2011, Alonso-Gutirez *et al*, 2009) but to the best of our knowledge, our investigation was the first to implicate thermotolerant and halotolerant homologs closely related to *Pseudomonas stutzeri* species in biodegradation and biosurfactant production.

On the biosurfactant production ability of the bacterial isolates, it was observed that all the bacterial isolates produce biosurfactants with high biological activity at elevated temperature and saline conditions that are prevalent at the produce water discharge points. Partial biochemical characterization of biosurfactants produced by the bacterial isolates showed that the 5 bacterial isolates that belonged to the Pseudomonad group produced glycolipid class of biosurfactants that are common with the group while the homolog closely related to *Serratia marcescens* produced the lipopeptide biosurfactant that are associated with *Serratia marcescens*. It is also interesting to note that all the biosurfactants produced by the bacterial isolates showed wide substrate specificity ranging from normal alkanes to aromatics and complex hydrocarbon mixtures. The highest biological activity of 48.30 µ/ml was recorded with the biosurfactant produced by homologs closely related to *Pseudomonas sp. VS-71* which was closely followed by the other homologs closely related to *Pseudomonas aeruginosa* strain SG-1 (43.20 µ/ml). Some investigators have recorded biosurfactant production by mesophyllic *Pseudomonas aeruginosa and Pseudomonas stutzeri* species (Olaniran et al, 2008, Onabasili and Aslim, 2009 and Celik et al, 2008) but to the best of our knowledge, our work was the first to implicate thermotolerant and halotolerant *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* isolates from produced water in biosurfactant production and petroleum biodegradation.

Conclusion:

It is noted generally that simultaneous hydrocarbon biodegradation and biosurfactant production by oil field bacteria that has been demonstrated by several investigators (Kummar *et al*, 2008, Okoro, 2009 and Manif *et al*, 2011) can be applied to eliminate chronic hydrocarbon pollutants that are associated with marine produced water discharges and this is a major focus of our study. In conclusion therefore, our study has demonstrated that the resident microbial flora found at the produced water discharge area in an oil producing facility in Nigeria were both thermotolerant and halotolerant. These bacterial isolates produce biosurfactants and are capable of rapid degradation of hydrocarbon in a mixed culture under elevated temperature and saline conditions. This implies that a simple bioremediation approach using resident microbial flora can be employed to degrade the residual TPH found within the produced water discharge area at its elevated saline and temperature conditions..

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