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MOLECULAR CHARACTERIZATION OF *Micrococcus luteus:* A POTENTIAL SOURCE OF BIOSURFACTANT FOR ENHANCED REMEDIATION OF CRUDE OIL POLLUTED SITE

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ABSTRACT

This study assessed the biosurfactant producing potentials of culturable bacteria isolated from a humic freshwater ecosystem of Eniong River in the Niger Delta of Nigeria. Preliminary screening of the bacterial isolates using a hemolytic test, drop collapse test, oil spreading activity, and emulsification capacity assay revealed that 4 out of the 13 bacterial isolates were able to produce biosurfactant but isolate $EHSA_4$ was the best producer. Molecular characterization of the isolate using 16s rRNA sequencing techniques revealed that isolate $EHSA_4$ demonstrated 99% similarities to *Micrococcus luteus* AB023371. Plasmid profiling showed that biosurfactant production by *M. luteus* $EHSA_4$ is plasmid- mediated, as the bacterium lost its 7 kbp plasmid as well as its biosurfactant producing potential after curing. The ability of *M. luteus* $EHSA_4$ to enhance crude oil degradation by *B. subtilis* was also analyzed using monoculture of *B. subtilis* and a culture consortium comprising *B. subtilis* and *M. luteus* $EHSA_4$. Analysis revealed enhanced degradation of crude oil and its PAH component by *B. subtilis* cultured with *M. luteus* $EHSA_4$. Degradation by monoculture *Bacillus* sp resulted in 19.65% reduction of the total petroleum hydrocarbon (TPH) content from 20.3467 mg/l to 16.3082 mg/l within 30 days while 46.06 % reduction was recorded for the bacterial consortium with residual TPH of 10.9755 mg/l. Enhanced degradation of crude oil also affected the PAH load of residual oil. The total PAH content of the residual oil degraded by *Bacillus* sp alone was 2.4547 mg/l as against 2.1833 mg/l removed by *B. subtilis* and *M. luteus*$EHSA_4$ after 30 days. Naphthalene, anthracene, benzo(a)pyrene, acenapthylene and benzo(a)anthracene were the most degraded PAH suites. The potential of these bacterial populations can be explored for the remediation of crude oil-polluted environment and farmlands, in the oil-producing Niger Delta region of Nigeria

INTRODUCTION

Petroleum products are common environmental pollutants and they contain non-aromatic, mono-aromatic, and an extensive suite of polycyclic aromatic hydrocarbons (PAHs) which can be toxic to organisms and pose significant hazard to human health and the earth's ecology (Urum *et al.* 2005). To address the inherent challenges various remediation technologies have been developed, including microbial remediation technologies - which mainly depend on the use of microorganisms to degrade, transform, detoxify, or breakdown contaminants. Despite the advantages of microbial remediation, its efficiency is limited majorly by the limited availability of crude oil components (especially PAHs) to microbes, a condition attributed to its low solubility and strong and/or irreversible sorption to soil (Rockne *et al.* 2002; Essien *et al.* 2015). Nevertheless, many microorganisms are capable of producing by-products such as bio-surfactants that can emulsify or increase the availability of hydrophobic compounds. Bio-surfactants are amphiphilic molecules that reduce the surface and interfacial tension between and

within two liquids, or that between a liquid and a solid (Bodour and Miller-Maie 2002; Aparna *et al.*2011). Biosurfactants unlike other chemical surfactants, are synthesized by microbes like bacteria, fungi, and yeast. They function by dropping surface tension, stabilizing emulsions, promoting foaming, and are usually non-toxic and biodegradable.

Recently interest in biosurfactants has increased because of its diversity, flexibility in operation, and are more eco-friendly than chemical surfactants (Saharan *et al.* 2011; Eduardo *et al.*2011). It is therefore promising to isolate indigenous bio-surfactant producing microorganisms with the prospect of effective surfactant production. In this study, we scavenged for strong biosurfactant producing strains of bacteria from a humic freshwater ecosystem that can be used in enhanced bioremediation of crude oil polluted environments.

MATERIALS AND METHODS

Study area/ Sample Collection

The humic freshwater ecosystem of Eniong River is a tributary of the middle course of the Cross River located in the Niger Delta of Nigeria. The river lies between latitude $5^{o}12^{l}$ N – $5^{o}22^{l}$ N and longitude $7^{o}54^{l}$ E – $8^{o}2^{l}$ E and is characterized by intense coloration due to humic substances and possibly soluble iron (Essien *et al*. 2015; Abraham and Essien 2016). Sediment samples from the water body were collected using Shipek grab sampler. The samples were contained in amber bottles, stored in ice–packed chest cooler to preserve their quality, and then transported to the laboratory for analyses.

Figure 1: Satellite Image of the Study area – Eniong River showing the sample stations

Isolation of Surfactant Producing Bacteria

The enrichment culture technique reported by Saravanan and Vijayakumar (2012) was adopted. Precisely 10 g of the sediment sample was inoculated into a conical flask containing 100 ml of sterile Mineral Salt Medium [K_2HPO_4 – 6g, NaCl – 12g, KH_2PO_4 – 6g, $(NH_4)_2SO_4$ – 6g, $MgSO_4.7H_2O$ – 2.6g, $CaCl_2.2H_2O$ – 0.16g, per liter (pH 7.0 $\pm$ 0.2)] (MSM) and enriched with 1% crude oil as carbon source. This was incubated at 28 °C in shaker incubator (100 rpm) for 7 days. After 7 days of incubation, the samples were serially diluted using sterile water and plated on Nutrient agar (NA). The plates were incubated at 28 ^{o}C for 48 hours. Discrete colonies that emerged were sub-cultured by streak method to derive mono-cultures (Cheesbrough, 2006). The pure cultures obtained were transferred into stock bottles containing freshly prepared NA slants and incubated at 30 $\pm$ 2^{o} C for 18 to 24 hours before storage at 4^{o}C for screening for bio-surfactant producing potential.

Screening of Biosurfactant Producing Potential of Bacteria and Molecular Characterization of the Best Surfactant Producer

Biosurfactant producing potential of the isolates was screened using a hemolytic test (Youssef *et al.* 2004), drop collapse test (Plaza *et al*. 2006), oil spreading activity **(**Youssef *et al.* 2004), and emulsification capacity %EC_{24} (Cooper and Goldenberg 1987). Preliminary identification of the bio-surfactant producing bacterial strain was done based on their cultural and biochemical characteristics as recommended by Chessbrough (2006) and Brenner *et al*. (1982). This revealed a Gram positive, cocci in pairs, and catalase positive bacterium (isolate $EHSA_4$) as the best producer. Confirmation was carried by the 16S rRNA gene sequence analysis.

DNA was extracted from a pure culture using a salting-out procedure described by Miller *et al.* (1988). The extracted DNA was amplified by polymerase chain reaction using primers and PCR conditions adapted from Tuleva *et al*. (2009), with forward primer 12F1 (5' CGTGCTTAACACATGCAA 3') and the reverse primer 1390R1 (5' GCCACCGGCTTCGGGTGTTTA 3'). The PCR was performed in the following mixture: approximately 100 ng bacterial DNA, 10 pmol of each primer, 0.2 $mmol^{-1}$ of each dNTPs, 1x supplied PCR buffer (including 1.25 mmol $l1^{-1}$ $MgCl_2$, 0.5 U ExPrimeTaq (Genet Bio, Chungnam, Korea) and ddH_2O up to a final volume of 25µl. The cycling conditions were as follows: initial denaturation at 95 ^oC for 5 min; 30 cycles denaturation at 95 ^oC for 45 sec., annealing at 58 ^oC for 45 sec. and extension at 72 ^oC for 90 sec.; final extension at 72 ^oC for 5 min. The obtained amplified products were electrophoresed using 2% agarose gel and stained with ethidium bromide. DNA sequencing was performed by Sanger (dideoxy) sequencing Technique to determine the nucleotide sequence of the isolate using automated PCR cycle-Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems. This nucleotide sequence obtained was subjected to BLAST analysis by direct blasting on *http://blast.ncbi.nlm.nih.gov*.

Plasmid Profiling of the Best Surfactant Producing Isolate ($EHSA_4$)

The method described by Maniatis *et al.* (1982) and Kraft *et al*. (1988), was employed to extract and cure the plasmid from the strongest bio-surfactant producing isolates. Precisely 1.5 ml of 24 hours' test organism was centrifuged for 1 minute and the supernatant gently decanted, leaving about 50 - 100µl together with cell pellet. Thereafter, 300µl of TENS was added and the suspension mixed by inverting tubes 3 – 5 times until the mixture became viscous. This was followed by addition of 150 µl of 3.0M sodium acetate pH 5.2, vortexed to mix completely, and then spun for 5 minutes in micro-centrifuge to pellet the cell debris and chromosomal DNA. The supernatant was transferred into a fresh tube; mixed with 900µl of ice-cold absolute ethanol, and spun for 10 minutes to pellet plasmid DNA.

The supernatant was discarded while the pellet was rinsed twice with 1 ml of 70% ethanol and air-dried. The dried pellet was re-suspended in 20 – 40 µl of TE buffer for further use. The obtained plasmid DNA was separated into bands based on its molecular weight using agarose gel electrophoresis. The isolate was cured and the cured strain re-screened for biosurfactant producing potential.

Bioremediation Study

The degradation study was done using a modified method of Panda *et al.* (2013). In this method, three sets of a 250 ml conical flask containing 150 ml of MSM, but supplemented with 2% crude oil, were used. Precisely 1ml of standardized culture broth of a crude oil degrading strain of *Bacillus subtilis* isolated from humic sediment (previous study (Abraham and Essien, 2016)) was then added to the first set of conical flasks. To the second set of conical flasks, 1ml of standardized culture broth of *B. subtilis* and 1ml the highest screened, biosurfactant producer were added, while the third set of flasks (un-inoculated) served as the control. The flasks were incubated at 28 °C in shaker incubator (100 rpm) for 30 days. After 30 days, the amount of the residual total petroleum hydrocarbon (TPH) and its polycyclic aromatic hydrocarbon (PAHs)

component were determined using Gas Chromatography coupled with Flame Ionization detector (GC-FID).

RESULTS AND DISCUSSION

Biosurfactants are a group of structurally diverse extracellular microbial natural products with unique biochemical properties (Lotfabad *et al.* 2009; Singh 2012). The results of the assay for biosurfactant production by the various bacterial populations (Table 1) have shown that of the thirteen culturable bacterial isolates obtained from humic sediment only 4 isolates ($EHSC_1$, $EHSC_3$, $EHSA_4$ and $EHSN_3$) exhibited the ability to produce biosurfactants. The quantity of biosurfactant produced by each of the isolates was determined based on the emulsification capacity of their culture supernatant. From the results (Table1) it was apparent that isolate $EHSA_4$ demonstrated the highest emulsification capacity. The ability of some of the bacterial isolates to produce surfactants was expected as many microorganisms, mostly bacteria and fungi, are known to produce or synthesize extra-cellular metabolites that can reduce surface tension (ST) and interfacial tensions between individual molecules on surfaces and at interfaces, respectively (Desai and Banat 1997).

The strain $EHSA_4$ was aerobic and produced bright colored colonies on TSA. Biochemically, they were gram-positive cocci that occurred in pairs and tetrads. They produced catalase, oxidase, and were positive for Methyl Red test but were unable to produce acid from glucose, sucrose, lactose, mannose, and maltose. Based on these characteristics, they showed a high similarity to *Micrococcus luteus* (Brenner *et al.* 1982). Comparative sequence analysis of the 16s rRNA (1379bp) in the GenBank database using BLAST search and manually reading through GenBank accession number, AB023371, the gene sequence comparison demonstrated 99% similarities to *M. luteus* AB023371, confirming the identification of bacterial strain $EHSA_4$.

Table 1: Biosurfactant producing potentials of the bacterial isolates

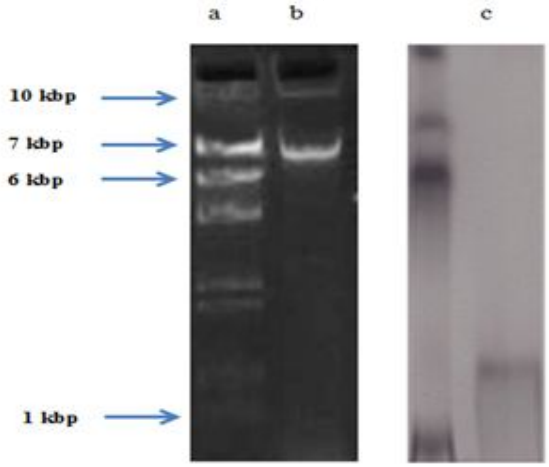
Isolates Code	Biosurfactant Producing Potentials			
	Hemolytic Activity	Drop collapse	Oil spread	Emulsification Capacity (%)
$EHSC_1$	+	+	+	7.2
$EHSC_2$	-	-	-	-
$EHSC_3$	+	+	+	11.5
$EHSC_4$	-	-	-	-
$EHSA_1$	-	-	-	-
$EHSA_2$	-	-	-	-
$EHSA_3$	-	-	-	-
$EHSA_4$	+	+	+	13.3
$EHSA_5$	-	-	-	-
$EHSN_1$	-	-	-	-
$EHSN_2$	-	-	-	-
$EHSN_3$	+	+	+	11.5
$EHSN_4$	-	-	-	-

Key: + = Positive; - = Negative

Plasmid profile analysis of the isolate (*Micrococcus luteus*) showed that the isolate possessed a 7kbp plasmid. Fig. 2 shows the uncured and cured plasmid DNA bands of *M. luteus*. This finding has shown that the biosurfactant producing potential of *M. luteus* $EHSA_4$is plasmid mediated as on curing the isolate of its 7kbp plasmid, the organism lost its potential to produce biosurfactant.

A 30-day course degradation study showed that the degradation of crude oil, and its component by *B. subtilis*, was enhanced by addition of the culture of *M. luteus* surfactant producing strain. *Bacillus subtilis* was able to reduce the total petroleum hydrocarbon (TPH) content of crude oil from 20.3467 mg/l to 16.3082 mg/l, but higher or enhanced degradation was recorded when supported with a culture of *M. luteus* $EHSA_4$. In this case, the TPH was reduced to 10.9755 mg/l. A summary of the residual TPH content of the crude oil after degradation with monoculture of *B. subtilis* and consortium of *B. subtilis* and *M. luteus* $EHSA_4$, as revealed by GC-FID is

illustrated in Fig. 3. Analysis of the polycyclic aromatic hydrocarbons (PAHs) content of the residual crude oil revealed that enhanced degradation of crude oil also affected the PAHs content of residual oil. The combined bacterial population (*B. subtilis* and *M. luteus* EHSA$_4$) degraded more PAHs in crude oil than the single bacterial population (*B. subtilis*). The total PAH content of the residual oil degraded by *Bacillus* sp alone was 2.4547 mg/l as against 2.1833 mg/l degraded by *B. subtilis* and *M. luteus* EHSA$_4$ after 30 days.



Key: a – marker (QuantDNA1-10 kbp); b – Plasmid DNA; c – Cured Plasmid DNA

Figure 2: Uncured and cured plasmid DNA bands of *M. luteus*EHSA$_4$

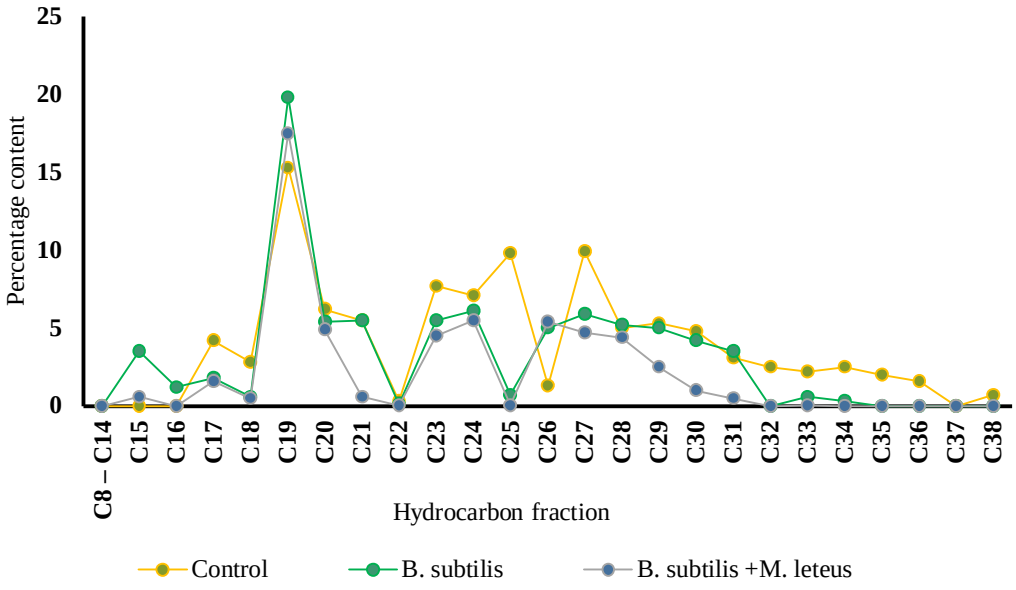


Figure 3: Crude oil degradation pattern as demonstrated by the isolates

This study has shown that although the *Micrococcus* isolated in this study utilized the crude oil weakly, its strong biosurfactant producing potentials is assumed to enhance the degradation of crude oil by *B. subtilis*. This may be ascribed to the ability of microbial surfactants to reduce the interfacial tension between the hydrophobic crude oil and bacterial cells via its amphiphilic property (Desai and Banat 1997). Previous studies have revealed that biosurfactants play a crucial role in enhancing the degradation of crude oil both in laboratory and field trials (Zhang and Miller 1995; Obayori *et al.*2009; Reddy *et al.*2010; Aparna *et al.* 2011; Matvyeyeva *et al.* 2014). These

findings have shown that hydrocarbonoclastic activity of *B. subtilis* was enhanced by the activity of the biosurfactant producer (*M. luteus* EHSA$_4$). Crude oil degradation by monoculture *B. subtilis* resulted in 19.65% degradation by reducing the total petroleum hydrocarbon (TPH) content of crude oil from 20.3467 mg/l to 16.3082 mg/l within 21 days while the bacterial consortium enhanced degradation by 46.06% within the same duration by reducing the TPH to 10.9755 mg/l.

Naphthalene, anthracene, benzo(a)pyrene, acenapthylene, and benzo(a)anthracene were the most degraded PAH suites (Fig. 4). The results revealed a greater than 90% selective degrading potential of the *B. subtilis* and *M. luteus* EHSA$_4$consortium against naphthalene, anthracene, benzo(a)pyrene, acenapthylene, benzo(a)anthracene, fluorene, acenapthylene, acenapthene, benzo(b)fluoranthene and pyrene.

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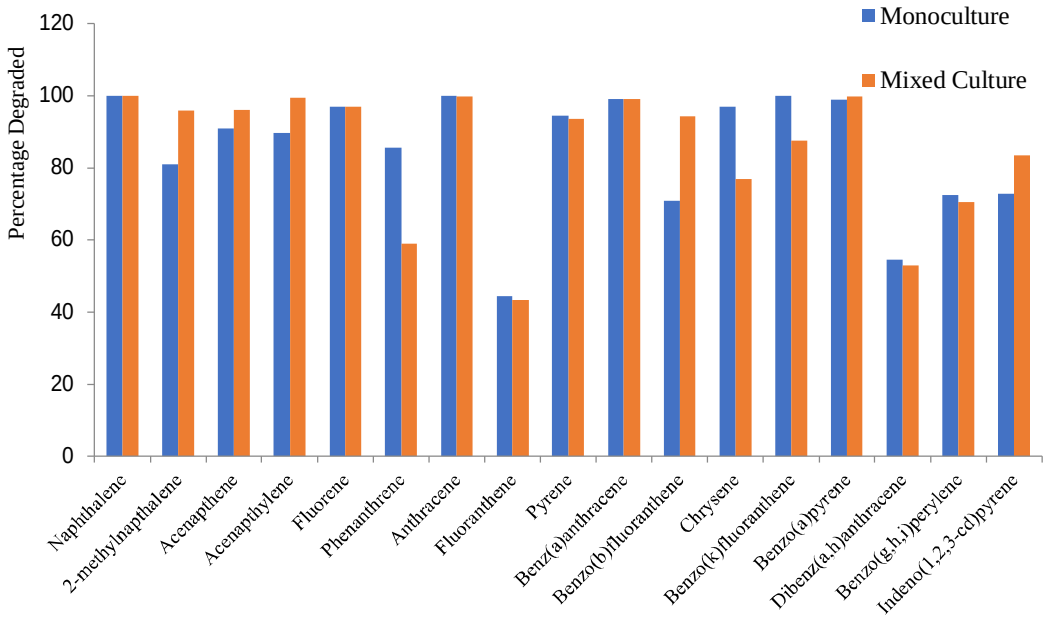

Figure 4: PAH Suites Degradation Rate

CONCLUSION AND RECOMMENDATION

This study has revealed the biosurfactant producing potential of *Micrococcus luteus*EHSA$_4$ isolated from a humic ecosystem in the Niger Delta region of Nigeria. It is suggestive that surfactants produced by the bacterium remarkably enhanced the rate of crude oil degradation by *B. subtilis* by over 26.41 %. It also enhances the ability of *B. subtilis* to degrade polycyclic aromatic hydrocarbons especially naphthalene, anthracene, benzo(a)pyrene, acenapthylene, and benzo(a)anthracene. These potentials can be explored for broader use in remediating crude oil-polluted environment and farmlands, a condition which is inherent and of high concern in the oil-producing Niger Delta region of Nigeria

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