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Screening and Characterization of Biosurfactant-Producing *Bacillus* Species Isolated from Contaminated Soils in Makurdi Metropolis

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Abstract
Biosurfactants synthesized by microorganisms are chemically diverse and have gained interest industrially due to their surface and interfacial tensions-reducing activities. In this study *Bacillus* species from contaminated soils were screened and characterized for biosurfactant production. The study was carried out at the Microbiology Laboratory, Federal University of Agriculture Makurdi, Nigeria. The *Bacillus* species were isolated from kerosene shops, palm oil shops, nearby restaurants, mechanic workshops and abattoir effluents-contaminated soil samples collected from Makurdi metropolis. The *Bacillus* spp. were screened for biosurfactants production potentials using various screening methods (oil spreading, beta haemolysis, drop collapse and emulsification index). Specific primers were used to amplify the *srf*AA (surfactin gene) gene in the *Bacillus* isolates and the nucleotide sequences were determined at Inqaba Biotec, South Africa. The screening results were statistically analysed using analysis of variance (ANOVA) at 95 % confidence level. Isolate RT7(4)B exhibited the ability to produce biosurfactant, as well as the highest emulsification index (E24) of 73.25 % while isolate PO7(3)C gave the highest oil displacement of 6.77 mm. The supernatant obtained from isolate RT7(4)B showed reduction in surface tension of up to 30.26 mN/m. The isolates gave positive results for biosurfactant production when subjected to drop collapse and Beta haemolytic tests. The Polymerase chain reaction (PCR) results revealed amplifications of *srf*AA gene from 7 isolates. Based on these findings, the isolates used in this study can be utilized for biosurfactant production, and can also be useful for bioremediation and industrial biotechnology applications.

Keywords: Biosurfactants; emulsification index; *Bacillus*; surface tension; Drop collapse
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Introduction

Global oil spills, generation of waste oils and increased industrial processes have led to huge environmental pollution (Parthipan et al., 2017). Several approaches have been used to address the challenges of environmental pollution , among which is microbial remediation (Ismail et al., 2013; Noparat et al., 2014). Biosurfactants which are microbial products play a major role in bioremediation (Sidkey et al., 2016). The demand for biosurfactants has witnessed an upsurge in recent years over the synthetic surfactants due to their low toxicity, biodegradability, effectiveness in bioremediation and stability at extreme pH, salinity and temperature (Desai and Banat, 1997; Akintokun et al., 2017). However, production of biosurfactants are faced with challenges arising

from their cost of production and low yield of producing strains **(**Saisa-ard et al., 2013)**.**
In order to obtain potential biosurfactant-producing microorganisms such as *Bacillus* and *Pseudonomas* species, utilization of microorganisms and understanding their heterogeneity in the soil community is pivotal (Okore et al., 2017). The use of biosurfactant-synthesizing bacteria from oil- contaminated sites can serve as an excellent tool in biosurfactant production. *Bacillus* species have been used in many industrial processes ; several studies have implicated them in producing lipopeptide type of biosurfactant (Saisa-ard et al*.,* 2013; Anitha et al., 2015; Okore et al., 2017). Molecular methods are needed to identify the genes required for the synthesis of biomolecules (Shoeb et al., 2015). One of the genes needed for surfactin biosynthesis is a large operon of 25 kb, named *srfA,* which is also vital for sporulation and competence development (Nakano et al., 1991). The presence of *srf*A operon and *sfp* gene is required for the non-ribosomal biosynthesis of surfactin. A *srf*A operon consists of four genes, *srf*AA, *srf*AB, *srf*AC and *srf*AD that make the surfactin synthetase subunits and play a vital role in the proof reading of peptide synthesis (Porob et al., 2013). The *srf*AA gene conceals phosphopantetheinyl transferase, which plays a part in non-ribosomal biosynthesis of biosurfactant (Cosmina et al., 1993). The *srf*AA gene transforms the inert protein to active protein to form surfactin synthetase (Pfeifer et al., 2001). The *srf*AA found in the *srf*A operon plays a major function in the biosynthesis of biosurfactants (Roongsawang et al., 2002).

Materials and Methods

Collection of Samples

Soil samples were collected according to the method described by Tambekar and Gadakhl (2013) with little modifications. Briefly, soil samples were randomly collected from kerosene shops, palm oil shops, nearby restaurants, mechanic workshops and abattoir effluent- contaminated soil within Makurdi metropolis. Ten grams (10 g) of effluent-contaminated soil samples were collected from depths of 5 – 10 cm at ten (10) different locations within each of the sample collection sites. The samples were collected using sterile spatula into appropriately labelled sterile polythene bags. The temperatures of the samples were taken immediately before the samples were transferred into an ice pack and transported to the laboratory for analyses.

Isolation of Bacterial Species

Ten grams of the contaminated soil samples was suspended in 90 mL of sterile distilled water contained in 100 mL capacity Erlenmeyer flask and amended with 1% v/v fresh engine oil. The medium was incubated at ambient temperature for 48 hours on a rotary shaker (Model S150, Bartoworld, Scientific, USA) at 150 revolution per minute (rmp). After the incubation, tenfold serial dilution of the samples was carried out using sterile distilled water in test tubes. An aliquot of 1 mL was poured from the 104 diluted samples into sterile empty Petri dishes, and freshly prepared nutrient agar (Oxoid, UK) at 45-50 ^oC was poured into the inoculum. The inoculated media were incubated at 37 ^oC for 24 hours. Pure isolates obtained were stored on nutrient agar slants at 4 °C.

Identification of Bacillus Isolates

Identification of bacterial isolates was carried out according to the methods described by Onyeagba (2004). The microscopic and macroscopic evaluation of the isolates morphology (Gram reaction, spore formation and colony appearance) and presumptive biochemical tests (Catalase, Indole, Oxidase, Urease, Citrate, Nitrate, VP, H_2S and Sugars fermentation) were carried out using fresh overnight pure nutrient agar cultures of the isolates.

Preparation of Bacillus Isolates for Biosurfactant Production Screening

Screening of the *Bacillus* isolates for biosurfactant production was carried out using 30 mL nutrient broth in 100 mL flask inoculated with 3% v/v inoculum of the *Bacillus* species. The inoculum size (1.5 x 10^8 CFU/mL) was determined by comparison with readily prepared McFarland standard 0.5. The inoculated broth media were incubated on a rotary shaker at 30 °C, 150 rpm for 72 hours. The culture broths were centrifuged (Model 80-213, Germany) at 3000 rpm for 30 minutes to obtain cell-free

supernatant. The supernatants were collected and tested for emulsification stability, drop collapse and oil spread ability.

Screening of Bacillus Species for Biosurfactant Production

Emulsification Stability Test (EI24)

Emulsification stability test was measured according to the method described by Balogun and Fagade (2010). Kerosene (2 mL) was added to 2 mL supernatant, vortexed for 2 minutes using an electronic vortex machine (Model XH-B, 2012), and kept for 24 hours at ambient temperature. The EI24 index was obtained by dividing the height of the emulsified layer (mm) with the total height of the liquid (mm) and multiplied by 100. Emulsification stability test of the culture samples was measured after 24 h.

Drop Collapse Assay

Cell-free broth (10 µL) was dispensed in the centre of a drop of vegetable oil (Grand Cereal, Nigeria) on a clean glass slide. After one minute, visual observation of the drops was done. The activity of the collected supernatant was compared with the control (water) as described by Seema and Nakuleshwar (2012).

Oil Spreading Technique

Distilled water (20 mL) was dispensed into Petri dish plates. One millilitre of crude oil was dropped in the centre of each of the plates containing the distilled water. This was followed by dispensing 20 µL of the supernatant of the culture of *Bacillus* sp. in the centre of the crude oil drop. Ring formation due to displacement of crude oil was measured using a meter rule. A 20 μL distilled water was used as control (Hasham et al*.,* 2012).

Determination of Blood Haemolysis Test

Sterilized blood agar base was allowed to cool to about 45 ºC and 20 mL aseptically collected goat blood was added, mixed gently and poured into sterile Petri dishes. About 24 h freshly grown cultures were aseptically point- inoculated using wire loops at the centre of the blood agar media. The inoculated media were incubated at 37 ºC for 24 hours. The diameters of the clear zones around the colonies were measured using a meter rule (El-Shahawy, 2014).

Surface Tension Reduction

This was carried out according to the method described by Arezoo and Salmah (2015). Culture broths were centrifuged at 5000 rpm for 30 min and supernatant collected ; the surface tension of the supernatant was determined using a Tensiometer (Model: lft-d Rs8, India).

Polymerase Chain Reaction (PCR) Amplification and Gene Sequencing

DNA extraction was conducted using a commercial kit (QIAquick® Gel Extraction kit, Qiagen, Germany), following manufacturer's instructions. The extracted DNA was used as a template for PCR amplification. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was conducted using the pairs of universal primer 8F:5 AGAGTTTGATCCTGGCTCAG-3 and 1492R:5 GGCTACCTTGTTACGACTT-3; as well as 27F:AGAGTTTGATCMTGGC and 1492R:5-GGCTACCTTGTTACGACTT-3 (Sreethar *et al.*, 2014). The nucleotide sequences were determined at Inqaba Biotec, South Africa. The closest matches of the nucleotide sequences were determined by running similarity searches with other deposited DNA sequences on the National Center for Biotechnology Information (NCBI) website using the Basic Local Alignment Search Tool (BLAST). Sequence alignment and phylogenetic analyses were carried out using MEGA version 6.0 (Sreethar et al., 2014).

Polymerase Chain Reaction (PCR) Amplification of Biosurfactant Gene (srfAA)

*The Srf*AA gene was amplified using specific primers. PCR mixture components were used according to the manufacturer's (Nippongenetic (Germany)) protocol. The forward and reverse primers: sfpF- 5′ ATGAAGATTTACGGAATTTA 3′ and sfpR- 5′ TTATAAAAGCTCTTCGTACG 3′ were used as previously demonstrated by Fateha et al. (2016).

Statistical Analysis of Data

Means were analysed statistically using ANOVA at 95 % confidence level. Means were separated using Duncan test and data presented in tables.

Results and Discussion

Among the isolates obtained from the different contaminated soil samples, the potential biosurfactant- producing isolates were confirmed as *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus cereus, Bacillus thuringiensis* and *Lysinibacillus fusiformis* by morphological and biochemical characteristics (Table 1). The phenotypic characterization indicated that the *Bacillus* species are Gram- positive, Rod-shaped, non-motile and able to ferment carbohydrates such as glucose, sucrose and galactose (Table 1). The result supported the report of Okore et al. (2017) who isolated *Bacillus* species from contaminated water samples from different contaminated soils. Previous studies by El-Sheshtawy (2013), Tambekar and Gadakh (2013) and Okore et al. (2013) had revealed the presence of different *Bacillus* species in oil and non-oil contaminated environments.

Table 1: Biochemical Identification of *Bacillus* Isolates

Reactions	*Bacillus thuringiensis*	*Bacillus subtilis*	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus licheniformis*	*Lysinibacillus fusiformus*
Gram	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Growth at 25^{0}C	+	+	+	+	+	+
Parabasal body	+	-	-	-	-	-
Urease	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Indole	-	-	-	-	-	-
H_2S	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+
VP	+	+	-	+	+	+
Gelatin	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Mannose	-	+	+	-	+	-
Xylose	-	+	-	-	+	-
Sorbitol	-	-	Nil	+	Nil	-
Rhamnose	-	-	+	+	+	-
Sucrose	-	+	+	+	+	+
Lactose	-	-	+	+	+	-
Arabinose	-	+	-	-	+	-
Raffinose	-	-	Nil	+	-	-
Salicin	-	-	-	+	Nil	-
Glycerol	+	-	-	+	-	+

Where: + = Positive; - = Negative; Nil= not performed

The various *Bacillus* species identified were subjected to preliminary screening to ascertain their potential to produce biosurfactant using the following indicators: haemolytic activity, oil collapse method, emulsification index and oil spreading assays (Table 2). The Haemolysis test

results showed that sixteen (16) of the isolates exhibited beta haemolysis ability while four (4) were non- Beta haemolytic organisms. The haemolytic reaction shown by the *Bacillus* species isolated in this study is characteristic of β-haemolysis. The high level of β-haemolytic *Bacillus* species observed in this study could be attributed to the growth of the isolates on hydrocarbon contaminated soils. According to Nwaguma et al. (2016), the presence of hydrocarbons in microbial environments induces production of surface-active compounds by the microbes inhabiting the environment. Nevertheless, studies had shown that biosurfactant-producing microorganisms are naturally present not only in hydrocarbon-polluted soils but also in organic matter suitable for the growth of diverse organisms (Nwaguma et al., 2016). Anaukwu et al. (2015) and Okore et al. (2017) studied microbial diversity of biosurfactant- producing bacteria from contaminated environmental samples (water and soil) using cultural methods and indicated the presence of biosurfactants. The positive drop collapse assay result of the *Bacillus* species points to the extracellular production of biosurfactant and its surface-active nature (Das and Chandran, 2011). Said et al. (2015) had also reported that *Bacillus* species recording a negative drops-collapse test were unable to produce biosurfactant. The findings in this study is similar to the work of Shoeb et al. (2015) who showed a positive drop-collapse test in *Bacillus* species. As reported by Batiata et al. (2006), in the presence of surfactant, a drop of supernatant from a pure culture of *Bacillus* species spreads over a hydrophobic surface as the interfacial tension between the droplet and the hydrophobic surface is reduced. In contrast, the droplet remains beaded or rounded in the absence of surfactant, as the polar water molecules are repelled from the hydrophobic surface (Batiata et al., 2006).

The *Bacillus* isolates showed varying oil displacement zone formation. An oil spreading test of *Bacillus* species showed that *Bacillus thuringiensis* (PO7(3)C) produced the highest oil spreading or displacement ability of 6.60 mm. The oil spreading observed indicated that the broth used has surface activities, hence, the larger the spreading diameter, the higher the surface activity (Chandran and Das, 2010). A similar result was shown in the work of Adnan et al. (2015) which gave oil displacements zone value of 25 mm. Nur and Mohammed (2015) showed 3.6 mm oil spreading by *Bacillus thuringiensis* on crude oil. This study is in contrast with the work of Rabah and Bello (2015) which showed that *Bacillus thuringiensis* displaced vegetable oil by 18 mm and *Bacillus licheniformis* displaced the oil by 20 mm. The variation in the results of the previous studies and the present study could be attributed to the variety of oils used by the researchers in the oil spreading assay.

Emulsification activities determine productivity of bioemulsifiers (Bonilla et al., 2005). In this study, kerosene was used as the hydrophobic substrate. Emulsification activity of the kerosene oil substrate revealed biosurfactant synthesis by the *Bacillus* species. The results of emulsification index shown in Table 2 reveal variations in the emulsification potentials of the various *Bacillus* species studied . The variation implies that the different isolates possess different degrees of biosurfactant activity and biosurfactants are species- specific as has been previously reported (Aperna et al., 2012). The variation can also be attributed to differences in their habitats and physicochemical compositions from which they were isolated. The results obtained from emulsification index study in the current work is similar to the finding of Satpute *et al*. (2008) who showed emulsification index of 78 % by *Bacillus cereus* using kerosene. Ainon (2013) showed an emulsification index value of 68 % with *Pseudomonas* sp. isolated from hydrocarbon contaminated soil. One of the characteristics of biosurfactants is their potential to produce emulsion with oil. The ability of the isolates obtained in the present study to produce biosurfactant which emulsifies kerosene suggests that they can be used industrially as emulsifying agents. The ability of the *Bacillus* species to produce biosurfactant was further corroborated by reduction in surface tension. Decrease in surface tension indicates the ability of the isolates to produce biosurfactant with surface activity. Anaukwu et al. (2015) demonstrated similar results. They obtained *Bacillus* species that lowered the surface tension to below 40 mN/m (Table 2). The screening parameters selected in this study were consistent with previous works by Anaukwu et al. (2015) and Sidkey et al. (2016). Kiran et al. (2010) recommended that more than one

selection method should be used during preliminary tests to detect biosurfactant producers. The selection of these techniques was based on their user-friendliness, cost effectiveness, quick implementation and use of relatively commonly available equipments.

Table 2: Biosurfactant screening of *Bacillus* sp. isolated from contaminated soils in Makurdi

Isolates	Surface Tension (mN/m)	Oil Spreading (mm)	Emulsification Index (%)	Drop Collapse	Beta Haemolysis
PO7(3)C	47.02[cd]	6.60[a]	54.27[f]	+	+
PO4(3)A	41.41[f]	3.17[e]	57.24[d]	+	+
HC2(4)B	54.08[b]	3.03[ef]	45.22[h]	+	+
HC10(5)C	40.33[f]	5.57[b]	55.71[def]	+	+
HC3(4)C	48.47[cd]	4.97[c]	50.53[g]	+	+
HC4(4)A	43.42[ef]	5.00[c]	57.15[d]	+	+
MS1(3)C	40.59[f]	6.40[a]	55.89[def]	+	+
MS9(3)C	49.05[c]	5.67[b]	50.70[g]	+	+
MS1(3)**B**	35.42[g]	6.77[a]	61.68[c]	+	+
MS4(3)C	41.63[f]	4.37[d]	56.97[de]	+	+
MS9(3)B	42.54[ef]	4.93[c]	55.44[ef]	+	+
RT9(4)B	43.63[ef]	6.57[a]	56.65[de]	+	+
RT9(4)C	49.83[c]	4.77[cd]	51.39[g]	+	+
ABT(5)3B	34.10[g]	5.80[b]	65.48[b]	+	+
HC4(4)B	69.98[a]	3.10[ef]	20.08[j]	-	-
RT1(3)A	68.71[a]	2.57[fg]	20.80[i]	-	-
MS1(3)D	45.68[de]	5.83[b]	50.92[g]	+	+
RT6(4)D	0.00[i]	0.00[h]	0.00[l]	-	-
RTI0(3)E	0.00[i]	2.13[g]	16.44[k]	-	-
RT7(4)B	30.26[h]	5.87[b]	76.25[a]	+	+
SE	**1.06**	**0.18**	**0.53**		

Means on the same column with the same superscript do not differ significantly from each other (P = 0.05). **Where: SE = Standard error; + = Positive; - = Negative**

The molecular characterization of the obtained sequences revealed 95.6 – 100% similarity with deposited sequences in the GenBank (Table 3). This is similar to the study of Chung *et al*. (2008); Fateha et al. (2016); Plaza et al. (2017) where three different *Bacillus* species: *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus licheniformis* (KP9) were screened for the presence of *srf*AA of surfactin synthetase using PCR. The *Bacillus* isolates investigated for the *srfAA* gene showed possible relationships among the organisms based on the clustering on the Phylogenetic tree (Figure 1).

The *Bacillus* species that showed positive results during screening were investigated for the presence of the *srfAA* gene using specific primers. The results showed amplification of *srfAA* gene fragments in seven *Bacillus* isolates. These *srfAA* -positive isolates were found to belong to the following six species of *Bacillus*: *B. subtilis*, *B. cereus*, and *B licheniformis, B thuringiensis and Lysinibacillus fusiformis.* Porob et al. (2013) and Fateha et al. (2016) also used the same set of primers in their previous works to amplify the surfactin gene**.**

Over time, contaminated soils have been proven to be a promising environment to discover microorganisms with novel capabilities. Screening of microbes from contaminated areas for the presence of *srf*AA gene revealed the

biosurfactant production activity of *Bacillus* isolates . In this study, we were successful in amplifying the genes responsible for biosurfactant production from 7 of 20 different *Bacillus* species from contaminated soil samples. Although some of the isolates showed biosurfactant production ability and have been earlier reported to possess the *srf*AA specific gene , others showed no PCR band with the PCR primers used in the present study.

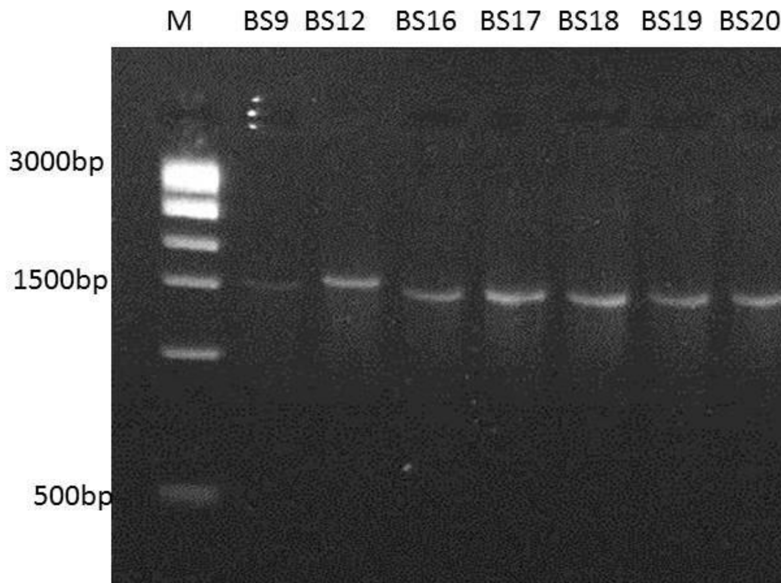

Plate I: Gel electrophoresis result of PCR amplification for different *Bacillus* species isolates. The DNA fragments form a band on the gel at a location corresponding to 1500 base pair (bp) of the DNA ladder

Table 3: Genbank Homologs of *Bacillus* Bacterial Isolates from Different Contaminated Soil Samples Used in This Study for Screening of the *Srf*AA Gene

Isolates Code	PCR Code	Identity	%Pairwise	Accession number
RT7(4)B	BS1	*Bacillus subtilis strain Hkb-1*	95.6	KP716963.1
HC2(4)B	BS2	*Bacillus thuringiensis strain C15*	99.2	CP021436
HC10(5)C	BS8	*Lysinibacillus fusiformis strain PgKB25*	99.3	MK559566
HC4(4)A	BS9	*Bacillus cereus strain A1*	97.8	CP015727
MS1(3)C	BS10	*Lysinibacillus fusiformis strain A1*	99.3	MK559526
MS1(3)B	BS12	*Bacillus cereus strain BHU1*	97.8	CP023727
MS4(3)C	BS13	*Bacillus cereus strain A1*	97.2	CP015727
PO4(3)A	BS14	*Bacillus thuringiensis strain c25*	96.5	CP022345
PO7(3)C	BS15	*Bacillus cereus strain HBL-AI*	98.1	CP023245
HC3(4)C	BS16	*Bacillus cereus* strain K3	97.5	MK530096
RT9(4)C	BS17	*Bacillus cereus* strain GX S-2	96.9	KU879246
Abt(5)3B	BS18	*Bacillus licheniformis* strain APBSWPTB159	96.8	MG733632
MS9(3)C	BS19	*Bacillus cereus* strain 35	97.2	KX058475
MS9(3)B	BS20	*Bacillus thuringiensis* strain T0139	99.7	CP035737

BS1 (*Bacillus subtilis* Hkb-

BS8 (*Lysinbacillus fusiformis* A1)

BS10 (*Lysinbacillus fusiformis*

BS19 (*Bacillus cereus* 35)

BS17 (*Bacillus cereus* GXS-2)

BS20 (*Bacillus thuringiensis*

BS2 (*Bacillus thuringiensis*

BS18(*Bacillus licheniformis*

BS12 (*Bacillus cereus*

BS15 (*Bacillus cereus* HBL-

BS13 (*Bacillus cereus* A1)

BS14 (*Bacillus thuringiensis*

BS16 (*Bacillus cereus* K3)

BS9 (*Bacillus cereus* A2)

Figure 1: Phylogenetic tree of fifteen *Bacillus* species isolated from contaminated soils. The tree was constructed using Neighbour-joining method based on evolutionary distances computed using the maximum composite likelihood method representing the relationship between the *srf*AA gene sequences. All the reference sequences used for the construction of the tree, with their corresponding accession numbers, were retrieved from GenBank.

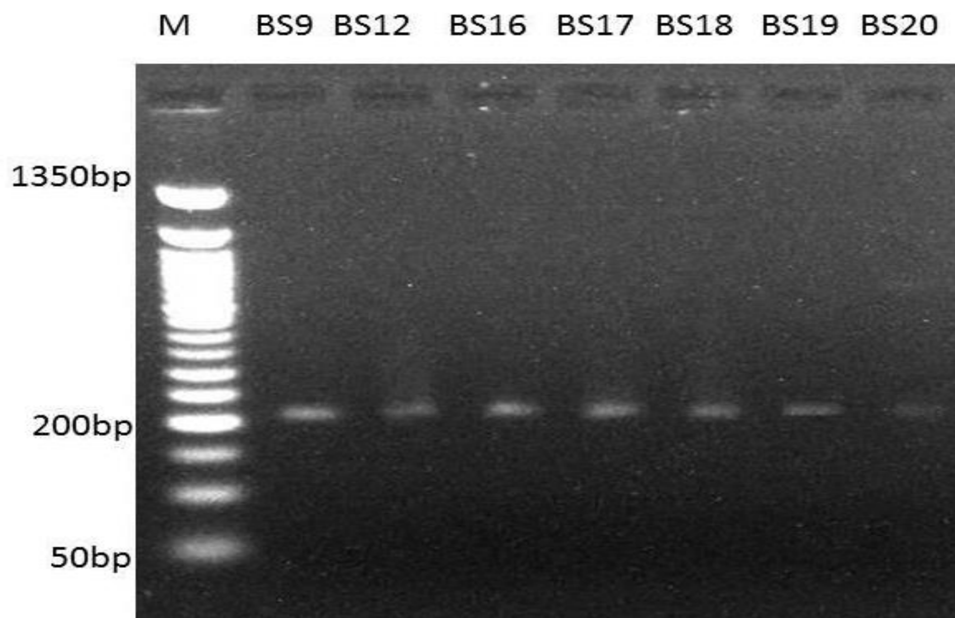

Plate II: Gel electropherogram for *srf*AA amplification from the different *Bacillus* species isolated. The result indicated 200bpDNA amplicons .

Conclusion

Based on the present study , contaminated soils in Makurdi metropolis would be an ideal source of biosurfactant- producing *Bacillus* species. This study revealed that the contaminated soil samples used harbour the following species of *Bacillus*: *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus cereus, Bacillus thuringiensis* and *Lysinibacillus fusiformis*. *Bacillus subtilis* strain Hkb-1(RT7(4)B) exhibited the highest ability to synthesize biosurfactant with 76.25 % emulsification index.

The presence of *srf*AA gene in 23.6 % of the total *Bacillus* species isolated proves that the contaminated soils are huge sources for harnessing biosurfactant- producing *Bacillus* species and hence the isolates may be vital for the biosynthesis of this bioactive compound having wide applications in different fields.

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