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Screening of Biosurfactant-Producing Bacteria Isolated from River Rido, Kaduna, Nigeria

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ABSTRACT: Biosurfactants which are produced by microorganisms, are known to reduce the surface tension of viscous liquid. Among the roles of biosurfactants is solubilization of hydrophobic substrates especially petroleum. River Rido is a point where petroleum-rich effluents from Kaduna Refinery and Petrochemical Company are discharged. There is need to isolate and characterize bacteria capable of producing biosurfactant from River Rido, Kaduna, which will serve as a tool for bioremediation. Water samples contaminated with refinery effluents were collected from four different sites along River Rido. Biochemical tests revealed that the bacteria isolated from the water sample belong to the genera of *Bacillus* (36.36%), *Pseudomonas* (27.27%), *Corynebacterium* (18.18%) *and Streptococcus* (18.18%) species. The isolates were coded as BS1 – 11. Among the bacteria, 18.2% showed α-haemolytic activity, 54.5% showed β-haemolytic activity and 27.3% showed γ –haemolytic activity. BS1, BS2, BS4, BS6, BS7 and BS11 showed positive results for oil spreading test. BS1, BS2, BS4, BS6, BS7 and BS11 were all positive for the drop-collapse assay. Isolates BS1, BS2, BS4, BS6, BS7 and BS11 were able to emulsify crude oil. Analysis of Variance revealed that there is a significant difference in the emulsification activity of the isolates at various pH levels. Also, emulsification index of the isolates at varying temperatures are significantly different. This study revealed that *Bacillus* species, *Corynebacterium* spp. and *Pseudomonas* spp. isolated from River Rido have been confirmed biosurfactant producer with ability to produce assayable biosurfactants and can be useful tools for various environmental and industrial processes, especially in the area of bioremediation of oil spills.

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Surfactants are amphipathic molecules which reduce surface tensions of viscous liquid by forming aggregate structures such as micelles (Bodour and Miller-Maier, 2002). Due to their nature and properties, they are widely used as emulsifiers for agricultural, food, cosmetic, pharmaceutical and industrial activities (Brooijmans *et al*., 2009). Also, they can be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial regions such as organic chemicals, metallurgy, mining, petroleum, petrochemicals, biological control and management and many others (Carrillo *et al*., 1996). In recent years, biosurfactants which are considered as one of the high values of microbial products, have gained considerable interest. Biosurfactants have advantage over synthetic surfactants due to their low toxicity, specificity of action, simplicity of preparation and extensive applicability, bioavailability, structural diversity, specific activity at extreme salinity, temperatures and pH (Banat, 2005). Biosurfactants are amphipathic compounds produced in living surfaces, mostly on microbial cell surfaces or excreted extracellular hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively (Franzetti *et al*., 2010). These compounds are produced during the growth of microorganisms on water soluble and water insoluble substrates (Rosenberg and Ron, 2008). Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon, microorganisms facilitate their diffusion into the cell by producing a variety of biosurfactants. These bacteria excrete ionic surfactants which emulsify the hydrocarbon substrates in the growth medium. Many bacteria have been associated with the production of biosurfactants. Some examples of such bacteria isolated by Mulligan and Eekhari (2003) include *Pseudomonas species* and *Bacillus species.* Also, Nwilo and Badejo (2005) isolated *Corynebacterium sp.* DDV1, *Flavobacterium sp.* DDV2, *Micrococcus roseus* DDV3, *Pseudomonas aeruginosa* DDV4, *Klebsiella pneumoniae* strain IVN51 from hydrocarbon-polluted soil in Ogoniland, Nigeria. Many properties of microbial surface active compounds such as emulsification, dispersion,

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foaming, wetting and coating make them useful in physicochemical and biological remediation technologies of both organic and metal contaminants (Atlas, 2007). Biosurfactants increase the bioavailability of hydrocarbon for hydrocarbonoplastic bacteria already present in the polluted water resulting in enhanced growth and degradation of oil spills by these hydrocarbon-degrading bacteria already present in polluted water (Akpofure *et al*., 2000). In heavy-metal polluted soils, biosurfactants form complexes with metals at the soil interface, which is followed by desorption of the metal and removal from the soil surface leading to the increase of metal ions concentration and their bioavailability in the soil solution.

Nigeria has witnessed a lot of oil spills from the past which has made livelihood unbearable for people living in communities affected by such spills. Also, the discharge of effluents from the refineries into nearby rivers has increased the risks of locals who depend on the polluted rivers as their source of drinking water, cooking, fishing and agricultural purposes. Clean-up of such pollutants has not been achieved as previous studies on the area have shown increasing levels of hydrocarbon on surrounding plants and water body (Chen *et al.,* 2012). This study therefore becomes indispensable in studying the indigenous colonies of bacteria from River Rido, where Kaduna Refining and Petrochemical Company (KRPC) discharges its effluents, which have the potentials of secreting biosurfactants that could be used for bioremediation. The aim of the study was to isolate and screen bacteria, capable of producing biosurfactants from River Rido, Kaduna, Nigeria.

MATERIALS AND METHODS

Study Site: River Rido is located in Kaduna, Nigeria. It is a point where the discharge of effluents from Kaduna Refinery and Petrochemical Company (KRPC) is emptied. The coordinates of the study area are defined by latitude 12°24'N and longitude 08°30'E of the equator. The river is polluted with petroleum effluents from the activities of the refinery.

Sample Collection: Water samples contaminated with refinery effluents were collected from four different sites along River Rido, Kaduna. The samples were collected in sterile 15-mL universal glass bottles and taken to the Microbiology Laboratory, Nigerian Defence Academy, for physicochemical analysis and isolation of bacteria.

Isolation of biosurfactant-producing bacteria: The biosurfactant-producing bacteria were isolated using Bushnell Haas Agar (BHA) supplemented with 0.1% crude oil (Source- KPRC) which was used as a selective medium. The samples were serially diluted and 0.1ml of the 5-fold diluted effluent water was pour-plated on Bushnell Haas agar incubated at 30ºC for 7 days. After the incubation period, the colony count of the isolates was determined using plate count method. Individual colonies were selected based on the difference in colony morphology. Pure cultures of colonies obtained from BHA were inoculated into Bushnell Haas Broth (BHB) for fermentation. All the fermentations were carried out using 100ml conical flasks with 10ml working volume and incubated in a rotary incubator at 30ºC for 7days. The centrifuged supernatant of the cultured broth were used in the screening test.

Bacteria morphology: Isolated colonies of purified bacterial strains grown on solidified agar plates were observed and data regarding their morphology and texture of the colonies were recorded (Morikawa *et al*., 2000). Cells were observed with Gram staining under a microscope (oil immersion, 100×). Shape of the cells (cocci, bacilli, and coccobacilli) and arrangement of cells (scattered, bunches, and chain).

Biochemical characterization of the isolates: Biochemical tests were carried out on isolates that were capable of producing biosurfactants. Catalase test, Motility Indole Ornithine test, Oxidase test, Methyl red test, Urease test, Nitrate reduction, Citrate test, Mannitol test, Triple Sugar Iron (TSI) test and Maltose fermentation.

Screening for Biosurfactant Production: The selected isolates were screened for the production of biosurfactant using the following methods: Hemolysis Test, Oil Spreading Test and Drop collapse assay.

Haemolysis test: Each isolate was streaked on blood agar medium and incubated at 37°C for 24 - 48 hours to assay for haemolytic activity. The plates were visually inspected for zones of clearing around the colonies, indicative of biosurfactant production (Carrillo *et al*., 1996).

Oil-spreading test: For oil-spreading test, oil was layered over water in a petri plate and a drop of cell-free extract was added to the surface of oil (Morikawa *et al*., 2000). The diameter of the clear zone on the oil surface was measured in three replications for each isolate. A water drop was used as a negative control (Morikawa *et al*., 1993).

Drop Collapse Assay: The assay was carried out as described by Jain *et al*. (1991). A drop of the culture supernatant was placed carefully on an oil coated glass

slide and observed after one minute. If the drop of supernatant collapsed and spread on the oil coated surface, it signifies the presence of biosurfactant (positive). But if the drop remains after one minute, it was documented as negative. This test was simultaneously carried out on distilled water as control.

Emulsification Activity: This was done by homogenizing equal volume of crude oil and cell free supernatant by vortexing at 1000rpm for 2 minutes after which the mixture was allowed to stand for 24 hours before calculating the emulsification index using the formula given below (Cooper *et al*., 2002). Emulsification activity of the isolates was determined at different pH, temperature, Sodium chloride concentration and Carbon sources.

$$E_{24}(\%) = \frac{\text{TE}}{\text{TL}} \times 100$$

Where E_{24}= Emulsification activity after 24 hours, TE= height of emulsified layer and TL= height of liquid layer

Characterization of biosurfactants: The crude biosurfactants produced were characterized by using silica thin layer chromatography (TLC) plates. Silica plates were obtained and the crude biosurfactant were spotted on the plate along with the biosurfactants produced by us upon 7 days incubation. The biosurfactant were separated using the solvent chloroform: water: methanol in ratio 65: 24: 4. Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spots, while Anthrone reagent was sprayed on the silica plate to detect glycolipid biosurfactant as yellow spots.

Test for biosurfactant stability: Effects of pH, heat and salinity on the activity of biosurfactant Stability studies were carried out using the cell-free broth obtained by centrifuging the cultures at 5,000 g for 20 min. The pH of the biosurfactant (4 ml) was adjusted to 2, 4, 6, 8, 10 and 12 using NaOH or HCl after which E_{24} was determined. To test the temperature stability of the biosurfactant, the broth was heated at 50°C for 15 min, cooled to room temperature and emulsification index determined. Emulsification was also determined after exposure to refrigeration 4°C for 30 mins. The effect of NaCl on the biosurfactant was also assayed at concentrations of NaCl.

RESULTS AND DISCUSSION

The bacterial count from the different sites is shown in Table 1. Site A had a bacterial count of 38×10^5 colony forming unit per milliliter of the sample. Site B had a total plate count of 57×10^5 CFU/ml of the sample. Site C had the lowest plate count of 23×10^5 CFU/ml while site D had a total of 57×10^5 CFU/ml. A total of 11 different types of colonies were isolated and were denoted as BS1 to BS11. The colonial morphology of all the isolates was observed: 81.8% were motile, while the rest of the isolates had equal proportions of regular and irregular margins with variable sizes. Furthermore, 36.4% of the isolates had convex, 18.2% concave, and 45.4% flat elevation of the colony. Most of the colonies have creamy colour and 63.6% are endospore former. Finally, 72.7% were opaque and only 27.3% produced colour pigment (Table 2). Cellular morphology which includes the arrangement, shape, and Gram reaction, was observed as it revealed that 81.8% of the isolated bacteria were rod shaped while the rest of the bacteria isolates were cocci. Furthermore, 38% had scattered cellular arrangement, 50% were in bunches, and 12% were arranged in chains. Most interesting were the Gram reactions, which revealed that isolates were 72.7% gram positive and 27.3% gram negative (Table 3).

Table1. Mean total viable heterotrophic bacteria count

Samples	(CFU/ml)
A	38×10^5
B	57×10^5
C	23×10^5
D	44×10^5

Biochemical tests shown in Table 3, identified the bacterial isolates as *Bacillus subtilis* (BS1) *Corynebacterium* species (BS2 and BS4), *Psedomonas fluorescens* (BS3), *Streptococcus* species (BS5 and BS10), *Pseudomonas* species (BS6), *Pseudomonas aeruginosa* (BS7), Bacillus species (BS8 and BS9) and *Bacillus licheniformis* (BS11). The frequency of distribution of the bacterial isolates is shown in Figure 1. The haemolytic activity was observed on blood agar and results showed that out of all the supernatants obtained after fermentation, 18.2% showed α-haemolytic activity, 54.5% showed β-haemolytic activity and 27.3% showed γ –haemolytic activity (Fig. 2).

Isolates BS3 and BS10 showed zones of α-haemolysis; isolates BS1, BS2, BS4, BS6, BS7 and BS11 showed zones of β-haemolysis; while isolates BS5, BS8 and BS9 showed zones of γ-haemolysis. Satpute *et al*. (2008) suggested that organisms must be screened for biosurfactant activities by hemolytic test, drop collapsing and emulsification index. The screening tests performed to check for the production of biosurfactants in all eleven isolates showed that only six out of the eleven isolates were able to produce

biosurfactants. A study carried out by Carter (1984) indicates that haemolytic activity is a good screening criterion in the search for biosurfactant-producing bacteria. Results obtained from haemolytic assay showed that biosurfactants produced by isolates BS1, BS2, BS4, BS6, BS7 and BS11 were able to haemolyze blood agar showing zones of β-hemolysis. The results on blood agar media were similar to the work done by Mulligan *et al*. (1984) and Mulligan *et al*. (1989) who have isolated biosurfactant overproducer mutants with blood agar method. Oil-spreading test is depicted in Table 4. From the 11 isolates, isolates BS1, BS2, BS4, BS6, BS7 and BS11 showed positive results for oil spreading test with the diameters of 12mm, 8mm, 11mm, 7mm, 5mm and 11mm respectively. Drop-collapse test indicated positive results in 54.5% of the isolates.

Table 2. Morphological characteristics of bacteria isolated from River Rido

Isolate No	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	BS10	BS11
Colony colour	Creamy	Creamy	White	Creamy	White	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
Pigment	No pigment	Pigment	No pigment	Pigment	No pigment	No pigment	No pigment	No pigment	No pigment	Pigment	No pigment
Cell shape	Rod	Rod	Rod	Rod	Cocci	Rod	Rod	Rod	Rod	Cocci	Rod
Elevation	Flat	Concave	Flat	Convex	Convex	Flat	Convex	Flat	Convex	Concave	Flat
Margin	Entire	Entire	Entire	Irregular	Entire	Entire	Irregular	Entire	Entire	Entire	Entire
Motility test	Motile	Non-motile	Motile	Non-Motile	Motile	Motile	Motile	Motile	Motile	Non-motile	Motile
Endospore staining	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	positive	Positive
Opacity	Opaque	Translucent	Opaque	Opaque	Opaque	Translucent	Opaque	Opaque	Opaque	Translucent	Opaque
Gram's staining	Positive	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Positive	positive	Positive

Table 3. Biochemical characterization of bacteria isolated from River Rido. a=*Bacillus subtilis*, b=*Corynebacterium spp*, c=*Pseudomonas fluorescens*, d=*Streptococcus spp*, e=*Pseudomonas aeruginosa*, f=*Bacillus licheniformis*

Isolate No	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	BS10	BS11
Catalase	+	+	+	+	-	+	+	+	+	-	+
Indole production	-	+	-	+	+	+	-	+	+	+	-
Oxidase	+	+	+	+	+	+	+	+	+	+	-
MR test	+	+	-	+	-	-	-	+	+	-	-
VP test	+	-	-	-	+	-	-	-	-	+	+
Urea hydrolysis	-	+	+	+	+	+	-	+	+	+	+
H_2S production	-	+	-	+	+	-	-	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	-	+	+	+	+
Glucose fermentation, A/G		A/G	-	A/G	-	-	-	A/G	A/G	A	A/G
Sucrose fermentation, A/G		A	-	A	-	-	-	A	A	A/G	A
Mannitol fermentation, A		A/G	-	A/G	A/G	-	+	A/G	A/G	A/G	A/G
Maltose fermentation, A		A	-	A	A/G	-	-	A	A	A/G	A
Lactose fermentation, A		A	+	A	A	-	-	A	A	A/G	A
Citrate test	+	+	+	-	-	-	+	+	+	A	+
Organism	a	b	c	b	d	e	e	D	d	d	f

The hydrophobic surface of the oil reduced indicates the presence of surfactant in the cell supernatants. Table 4 shows BS3, BS5, BS8, BS9 and BS10 were negative for Drop-collapse assay while BS1, BS2, BS4, BS6, BS7 and BS11 were all positive for the drop-collapse assay. These biosurfactant-producing bacteria isolates were able to displace oil on the surface of water. This suggests the production of biosurfactants as they are known to displace oil from the surface of water bodies. The use of the drop collapse method as a sensitive and easy method to test for biosurfactant production, requires no specialized equipment, and only requires a small volume of sample (Plaza *et al*., 2006). This assay relies on the destabilization of liquid droplets by surfactants produced by the isolates. Therefore, drops of cell-free supernatant on a solid microplate surface would result in either stable or spreading or even collapsing droplets depending on the presence of biosurfactant. The stability of drops is dependent on biosurfactant concentration and correlate with surface and interfacial tension (Morikawa *et al*., 2000). The drop collapse test and oil displace test are indicative of surface wetting activity (Youssef *et al*., 2004). Drop-collapse assay, performed to determine the ability of biosurfactants to reduce the hydrophobic surface of the oil, were positive for isolate BS1, BS2, BS4, BS6, BS7 and BS11.

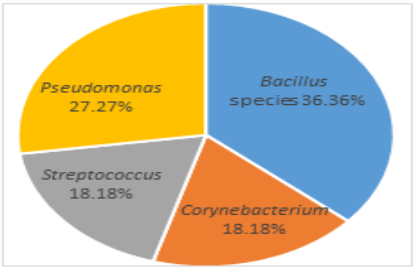

Fig.1.The frequency distribution of bacteria isolated from River Rido

Emulsification activity carried out revealed that while the supernatants obtained from isolates BS3, BS5, BS8, BS9 and BS10 showed no emulsification activity, supernatants obtained from isolates BS1, BS2, BS4, BS6, BS7 and BS11 were able to emulsify crude oil to some extent with the values of 64.5%, 53.3%, 45.6%, 61.9%, 70.5% and 42.4% respectively (Table 4). Emulsification activity is one of the criteria to determine the potential of biosurfactants. Emulsification assay is an indirect method used to screen biosurfactant production. This assumption is that if the cell-free culture broth used in this assay contains biosurfactant, then it will emulsify the hydrocarbons present in the test solution. Bonilla *et al.* (2005) had shown that emulsifying activities (E_{24}) determine the productivity of biosurfactants and that they are given as a percentage of the height of the emulsified layer divided by the total height of the liquid column. In the present study, only 45.5% of the total isolates showed negative emulsification potential, and 54.5% gave a good emulsification index with all crude.

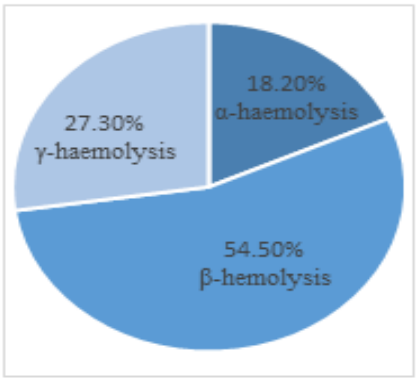

Fig. 2. Haemolytic activity of the isolates

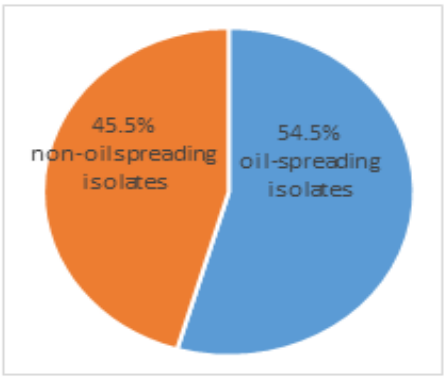

Fig. 3. Oil-spreading activity among the isolates

Results from emulsification assay carried out on crude oil showed that six of the isolates were able to emulsify crude oil as emulsification index recorded for BS1 was 64.5%, 53.3% for BS2, 45.6% for BS4, 61.9% for BS6, 70.5% for BS7 and 42.4% for BS11. Thus, biosurfactants produced by isolate B7 had the highest emulsification index against crude oil. Only the isolates exhibiting hemolytic and emulsifying activity against weathered crude oil showed positive result for drop collapsing, emulsification activity and oil-spreading test. In this study, 54.5% of isolates were found to be positive by oil-spreading method. In this study, crude oil was used as the hydrophobic substrate. Result observed in this study revealed that 54.5% of the Isolates showed positive emulsification activity. The drop-collapse test was positive in 54.5% of the isolates. An interesting finding was that isolate BS1, BS2, BS4, BS6, BS7 and BS11 gave positive results in all screening tests. They were positive for all the tests for biosurfactant production. Successful isolation of a number of biosurfactant-producing bacteria may represent the ability of microorganisms to survive in hydrocarbon-contaminated regions and their ability to produce biosurfactants (Margesin and Schinner, 2001). These isolates represent naturally occurring stress-resistant bacteria successfully surviving in heavily contaminated regions might be utilized as a productive tool for bioremediation in future (Shoeb *et al*., 2012). *Bacillus species* has proved to occur mainly in soil and because of their spore forming ability; the spores have the ability to survive harsh conditions in soil environment. Although, *Bacillus* is found on other matrices like air, its occurrence in soil samples and water were found to be relatively high when compared to other matrices. Other organisms with high incident rates develop mechanism for surviving harsh conditions of the soil. *Bacillus species*, most especially *Bacillus subtilis* produces surfactin and lipopeptides (Nitschke and Pastore, 2004), while *Pseudomonas aeruginosa* produces glycolipids which act as emulsifiers or surface-active agents; consequently reducing the surface tension of hydrophobic molecules and leading to their breakdown (Bodour and Miller-Maier, 2002; Yin *et al*., 2009).

Table 4. Characterization of biosurfactant producing bacteria isolated from River Rido

Isolate No	Haemolysis test	Oil spreading test (mm)	Drop collapse assay	Emulsification activity (%)
BS1	β- haemolytic	12	+	64.5
BS2	β- haemolytic	8	+	53.3
BS3	α- haemolytic	No zone	-	
BS4	β- haemolytic	11	+	45.6
BS5	γ – haemolytic	No zone	-	
BS6	β- haemolytic	7	+	61.9
BS7	β- haemolytic	5	+	70.5
BS8	γ – haemolytic	No zone	-	
BS9	γ – haemolytic	No zone	-	
BS10	α- haemolytic	No zone	-	
BS11	β- hemolytic	11	+	42.4

Stability studies/emulsification assay carried out on the biosurfactants at various pH revealed that at pH 4, pH 6, pH 8 and pH 10, biosurfactants produced by BS1 showed emulsification index at the values 43%, 44.5%, 24.7% and 71% respectively; biosurfactants produced by BS2 showed emulsification index of 59%, 77.7%, 16% and 43.6% respectively; BS4 showed emulsification index of 32.5%, 52.1%, 23.3%, 55.5% respectively; BS6 showed emulsification index of 23.6%, 45.6%, 10.3% and 23.6% respectively; BS7 showed emulsification index of 66.6%, 56.7%, 17.6% and 74.2% respectively while BS11 showed emulsification index of 71.2%, 18.4%, 22.2% and 67% respectively (Fig. 4). Analysis of Variance revealed that there is a significant difference in the emulsification activity at various pH levels.

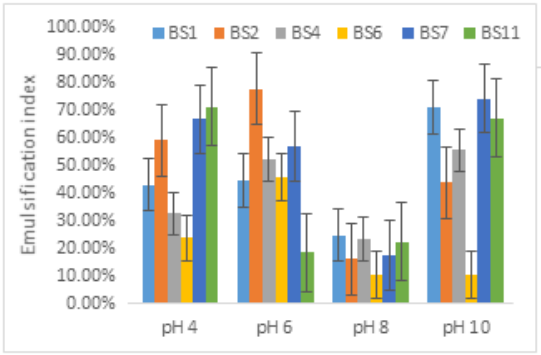

Fig. 4. Emulsification index at different pH concentrations

At 4^0C, 25^0C and 37^0C, emulsification index recorded by BS1 were 13%, 55.5% and 56.7% respectively; BS2 recorded emulsification index of 19%, 48.6% and 45% respectively; BS4 recorded emulsification index of 32.5%, 61.1%, and 25.9% respectively; BS6 recorded emulsification index of 11.8%, 33.3% and 77.3% respectively; BS7 recorded emulsification index of 28.8%, 36.7% and 19.6% respectively while BS11 recorded emulsification index of 32%, 65.4% and 56.2% respectively as shown in Fig. 5. Analysis of variance revealed that there is a significant difference in the emulsification index at varying temperatures.

High biosurfactant production is found to be characteristic under certain qualitative and quantitative environmental conditions depending on nutritional and environmental factors. This research is in agreement with the findings of Shafi and Khanna (1995) who reported that *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Pseudomonas florescens*) can produce biosurfactants when grown on carbohydrates and nitrogen source such as solely carbon, sucrose, urea and yeast extract sources. The bacteria were able to grow at different pH and temperature. The loss in emulsification was noticeable at pH 8 and 37°C. Similar result was observed by Kiran *et al*. (2009) who discovered that the optimal temperature for maximum rhamnolipid production by *P. aeruginosa* J4 strain was between 30 to 37˚C.

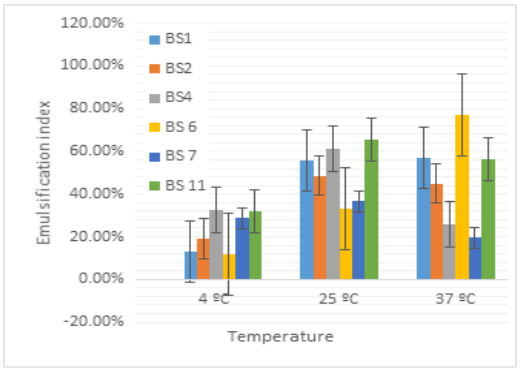

Fig. 5. Emulsification index at different temperature

Conclusion: This study revealed that *Bacillus* species, *Corynebacterium* spp. and *Pseudomonas* spp. isolated from River Rido have been confirmed biosurfactant producer with ability to produce assayable biosurfactants and can be useful tools for various environmental and industrial processes, especially in the area of bioremediation of oil spills. Bacteria isolated in this study could be a valuable source of novel environmentally friendly biosurfactants for the future replacement of synthetic surfactants. The replacement of chemical surfactants with biosurfactants should also be considered.

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