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Isolation and Characterization of Biosurfactant Producing *Pseudomonas aeruginosa* YLA03 and its Diesel Degradation Potentials

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Abstract

This work was aimed at isolating and characterising biosurfactant producing bacteria strain from diesel oil contamination sites. The potential of the strain to degrade diesel oil n-alkanes was further studied. Soil samples were collected from diesel rich areas, and identified strains were grown on minimal media that contained diesel oil as only carbon source. Enrichment culture technique was used to identify bacteria. Seventeen bacteria strains were identified based on their capacity to use diesel oil as carbon source. Other qualitative screening methods were employed to determine the potential to produce biosurfactants. Isolate A03 was the only candidate that shows positive signs for drop collapse, foaming, haemolytic, oil displacement and emulsification tests. The effect of various culture parameters (incubation time, concentration of diesel, nitrogen source, pH and temperature) on biodegradation of diesel was evaluated. Optimum pH was 8.0 at 35° C, while the incubation time at maximum bacteria growth was five (5) days. The favourable diesel oil concentration was 7.5% for the isolate. The isolate has shown degradative ability towards dodecane, Tridecane, tetradecane but was unable to degrade 2, 6, 10-trimethyl- dodecane. It degraded between 10.09% - 45.39% of individual diesel alkanes. The strain has exhibited potential for degrading diesel alkanes and was identified as *pseudomonas aeruginosa* strain A3 (Accession Number: MZ027605) using the 16S rRNA. Sequencing.

Keywords: Diesel, Biosurfactant, *Pseudomonas aeruginosa*, Biodegradation, n-alkanes
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Introduction

The increase in exploration and exploitation of oil results in oil spills which cause pollution of our environment. These spills pollute surface water, ground water, soil, plants, and ambient air with hydrocarbon substances like polycyclic aromatic hydrocarbons and benzene which are both capable of causing harm to human health (Zanaroli et al., 2010). Water contaminated with oil spill is hazardous to living organisms in the affected habitat. Most of the oil spills affecting the environment are mainly due to large oil tanker spills which in turn contaminate water bodies because of surface runoff, and during oil transportation, port

commotion, and illicit twaddle water discharges (Suni et al., 2007).

Diesel hydrocarbons are derivatives of crude oil (Ahmed et al., 2010) and are a complex mixture of aromatic and saturated hydrocarbon (Zanaroli et al., 2010). Diesel mostly comprises aliphatic hydrocarbons, but also contain polycyclic aromatic hydrocarbons such as naphthalene, fluorine, phenanthrene. Diesel hydrocarbons together with other components of crude oil change the characteristics of land and water, polluting the ecosystem. Bioremediation plays a major role in solving this problem, as it utilises microorganisms to decompose or degrade many types of pollutants like hydrocarbons and heavy metals (Cao et al., 2015). Many bacteria strains were reported by many researchers to possess the ability of degrading recalcitrant compounds in crude oil. Some bacteria strains can degrade spilled oil in affected locations (Zhang et al., 2014). The metabolic capacities of the degrading microorganisms play a major role in its bioremediation process (Szulc et al., 2014). The degradation ability of bacteria strains depends on other environmental factors to ensure optimal growth and high degradation rates (Das et al., 2011). Representatives of some bacterial genera, such as *Acinetobacter, Burkholderia, Gordonia, Dietzia, Brevibacterium, Aeromicrobium, Celeribacter*, *Mycobacterium* and *Sphingomonas,* isolated from petroleum-contaminated soil have proved to show potential for hydrocarbon degradation. Magdalena et al. (2014) reported that these bacteria can produce biosurfactants. The aim of this research work is to isolate, identify and characterise surfactant producing bacteria for their potential to degrade diesel oil.

Materials and Methods

Sample Collection

Samples were obtained from two auto-mobile workshops in Yola Adamawa state, Nigeria. The depth for the soil collection was 0 to 5 cm beneath the soil surface. Collected samples were kept at room temperature (4 °C) prior to analysis.

Isolation of Bacteria

The isolation was carried out according to Wuyang et al. (2019) with slight modification. Bacterial strains were isolated from the collected soil samples. 1 g of collected soil sample was added to the mineral salt medium (MSM). The medium contained (g l^{-1}): NH_4NO_3, 0.5g; K_2HPO_4, 1.5; KH_2PO_4, 0.5g; $MgSO_4 \cdot 7H_2O$, 0.2g; $FeSO_4$, 0.02g; $CaCl_2$, 0.05g; and $CuSO_4$, 0.02g. The medium was supplemented with diesel (2%) as the sole carbon source, the enriched flasks were incubated at 35 °C for 5 days in a rotary shaker with 150 rpm. Cultures (5 ml) were subsequently used from the original flasks to inoculate the second batch of flasks containing fresh medium and the flasks were maintained at the same conditions. This process was repeated three times and each time the same number of cultures was withdrawn from the older flasks and added to the new ones. Cultures from the last enriched flasks were plated on Mineral salt medium (MSM) using pour plating technique.

The morphologically different bacterial colonies were isolated from the agar plates and streaked again on MSM to get pure cultures of the isolates. The pure cultures of the isolates were maintained in MSM broth and slant bottles for further use.

Biosurfactant Assay

Strains were grown in MSM at 37 ºC with shaking at 120 rpm for 24 hrs over a period of seven (7) days. Following the incubation, the bacteria strains were subjected to biosurfactant assay. This assay includes the emulsification test, oil displacement, drop collapse test and haemolytic test. The tests were performed three times and distilled water was used as the control (María 2017).

Drop Collapse Assay

The drop collapse assay was performed according to the method described by Bodour and Maier (1998). Mineral oil (3 µL) (Sigma Aldreich) was applied onto a micro-titre plate for equilibration at 37 ºC for 1 hr. Five microliter of the supernatant was applied onto the centre of the wells, just above the mineral oil. The shape of the oil drop was assessed after a minute. Positive result for biosurfactant production gives a flattened shape, while unflattened shape is considered a negative test to biosurfactant production.

Displacement Test

Oil displacement method was performed according to the Maneerat and Phetrong method (2007). Diesel (20µL) was added to a 150 mm-diameter Petri dish containing 40 ml

of distilled water. Followed by addition of 10µL of supernatant and pellet suspension to the centre of the oil film, and after 30 s of incubation the diameter of the clear halo zone was measured.

Haemolysis Test

Each isolate was streaked on blood agar medium and incubated at 37 °C for 48 hours to assay for haemolytic activity. The plates were visually inspected for zones of clearing around the colonies, indicating the presence of biosurfactants (Carrillo et al., 1996).

Emulsification Assay

Diesel oil and culture supernatant (4 mL each) were thoroughly mixed by vortexing for 5 minutes. The resultant mixture was kept intact for 24 hrs. Emulsion layers were measured, and emulsion activity was calculated using the formula below:

$$E24\% = \frac{Height\ of\ emulsification\ layer\ (mm)}{Total\ height\ of\ the\ liquid\ column\ (mm)} \times 100\%$$

Cultural Conditions for Enhanced Biodegradation Potentials of Diesel

Determination of optimum conditions were carried out as reported by Parthipan et al., (2017). Effect of incubation time, carbon source, nitrogen source, pH, and temperature (cultural conditions) were examined. Standardisation of the inoculum (8.6×10^6 cfu/mL) was done with the McFarland standard for optimization.

Effect of Incubation Time

The time of incubation was varied for days to determine the optimum time and maximum biosurfactant production for the chosen strain. The strain was inoculated and cultured, while the absorbance was taken after a day. The absorbance indicates the growth of the bacteria.

Effect of pH

The pH (2-12) and carbon source were varied while the strain was allowed to grow on MSM with 1% diesel. Following the variation in pH, the medium was sterilised at 121 °C for 15 min. The strains were then inoculated and incubated at 37 °C for 5 days in a shaker at 150 rpm. (Guo et al., 2022).

Effect of Temperature

For temperature optimization, various temperature values that range from 25 °C to 45 °C were used. The pH of the medium was adjusted to 8.0, while the strain was cultured on MSM with diesel (1%) as the carbon source. The medium was maintained at various temperatures for five days in a shaker (150 rpm).

Variation in Carbon Source

The source of carbon for the strain was also optimised; source concentration was optimised, by choosing five concentrations (2.5, 5, 7.5, 10, 12.5 % v/v diesel). The pH was kept at 8.0, and the strain was grown on MSM. The medium was maintained at 37° C for five days in a shaker (150 rpm).

Variation in Nitrogen Source

Two nitrogen sources selected were ammonium nitrate (NH_4NO_3) and urea. Preparation of MSM was accomplished with 1% diesel as a carbon source with one gram per litre of each nitrogen source. The pH was maintained at 8.0, while the temperature was 35° The culture was kept for five days in a shaker (150 rpm).

Molecular Identification of Pseodomonas aeruginosa 16S-rRNA amplification and sequencing

The most efficient isolates designated as A3 were identified using Molecular identification in which the genomic DNA of the bacteria was extracted using standard protocol. The16S rRNA was amplified using a universal primer pair, 968F (AACGCGAAGAACCTTAC) and 1541R (AAGGAGGTGATCCAGCCGCA) (White et al., 1990). Polymerase chain reaction (PCR) was performed in a 25 µl volume in thermal cycler (Master cycler Nexus gradient, Eppendorf, Germany), 1X standard buffer, 1.5 m mol l^{-1} $MgCl_2$, 0.2 µ mol l^{-1} each primer, 0.2 m mol l^{-1}dNTPs and 0.25 U Taq DNA polymerase (Sigma Aldrich, USA) and 25 ng of template DNA. The PCR reaction conditions consisted of initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45s, and a final extension at 72 °C for 7 min. PCR products and DNA marker (Bioline), were analysed on 2% agarose gel and visualised under Bio Doc-It Imaging System (ingeniuss UK). PCR products were purified with GenElute™ PCR Clean-Up Kit (Sigma Aldrich, USA). PCR products were sequenced bi-directionally using an automated sequencer by Beckman Coulter (Genome Lab GeXP, Genetic Analysis System, and USA). The 16S

rRNA consensus sequence was used for Basic Local Alignment Search Tool (BLAST) analysis against the database in the National Centre for Biotechnology Information (NCBI) Gene Bank (www.ncbi.nlm.nih.gov). Sequence data were aligned using clustal W and phylogenetic relationships among the strains were determined by the neighbour-joining method using MEGA 6 software (Thompson et al., 1994; Tamura et al., 2013).

Gas chromatography results of isolate A3 cultures

The method of Liang et al. (2018) was adopted. The composition of diesel was analysed using the GC–MS methodology. To analyse level-changes of individual molecules in diesel extracted from supernatant of cultures, 1 µL of diesel (0.2 v/v) was directly injected into the injection port of gas chromatograph (Shimadzu Co.) equipped with flame ionization detector (FID) and Rtx-5 column (30 m × 0.32 mm, id. with 0.25 µm film thickness) (Restek Co., Bellefonte, PA, USA). The sample injection was made in split mode and the split ratio was 20:1. The temperature of the injection port and detector temperature were set to 280 and 305 °C, respectively. The temperature program was started with an initial temperature at 70 °C and held for 2 min at this temperature, then 25 °C min^{-1} to 140 °C, followed by an additional increase of 3 °C min^{-1} to 240 °C, then 10 °C min^{-1} up to 300 °C, held for 15 min. The total duration of the temperature program was 59.13 min. Nitrogen was used as carrier gas, and its flow rate was 30 ml min^{-1}. Hydrogen gas flow rate and air flow rate were 40 and 400 mL min^{-1}, respectively. Level of individual compositions was estimated based on the peak area and percentage degradation rate was based on the Equation:

$$DEG\% = \frac{LEVELctl - LEVELsmp}{LEVELctl} X\ 100\%$$

where DEG% is the percentage degradation rate, LEVELctl and LEVELsmp are compound concentration in control and in sample, respectively.

Statistical Analysis

All the experiments were performed in triplicate and the results were expressed as the means ± standard error (SE), analysis was performed using SPSS software (IBM SPSS Statistics 25).

Results

Seventeen bacterial isolates were screened for biosurfactant ability among which only one isolate (A3) was identified as biosurfactant producer based on its ability to give positive result to drop collapse, foaming stability, emulsification and oil displacement as indicated in Table 1.

Table 1: Screening Assay for Biosurfactant production

Isolate	Foaming	Emulsification (%)	oil Displacement (mm)	Drop Collapse	Haemolytic test
A3	+	36 ± 0.40	26 ± 0.40	+	+

+ positive; - negative

Figure 1(A-D) presented the effect of incubation time, initial pH, temperature, diesel concentration and nitrogen source for isolating A3 on the growth and degradation of diesel. The effect of incubation time was optimum at 120 hrs for the isolate. There was a gradual increase in the growth after 24 hrs and started to decline at 144 hrs as presented in figure 1A. The optimum temperature for the isolate on the growth and degradation was 35 °C as presented in figure 1B. The organism is mesophilic which indicates its effective level at moderate temperature (30-35 °C). The effect of pH on the growth and degradation of diesel for the isolate was optimum at pH 8, as presented in figure 1C. The diesel concentration was varied for the isolate; the isolate has obtained effective growth at 7.5 % (v/v) as presented in figure 1D. The effect of nitrogen on the growth and degradation of diesel for the isolate was also determined.

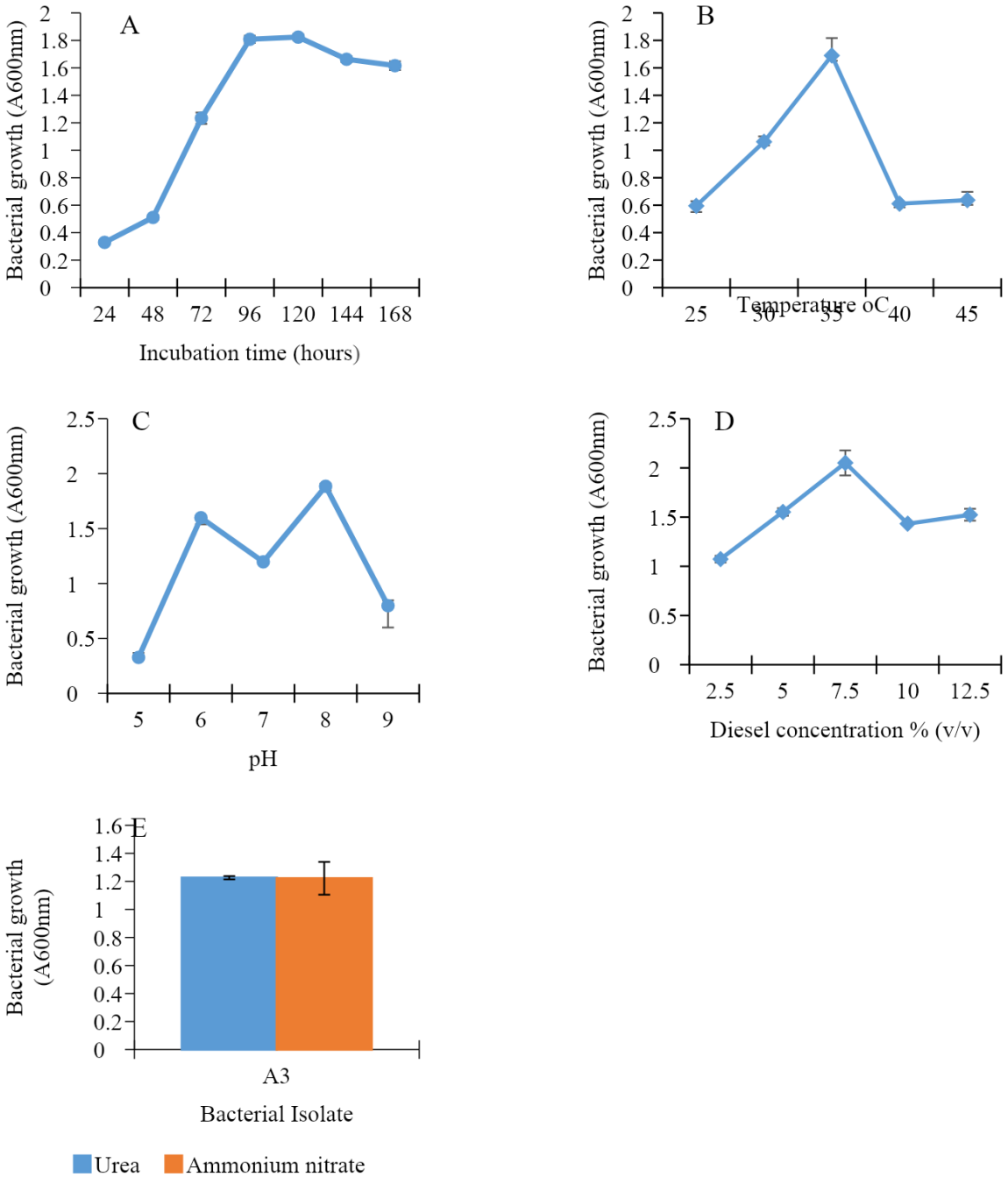

Figure 1: Effect of cultural conditions on bacterial growth and degradation of diesel (**A** effect of incubation time; **B** effect of temperature; **C** effect of pH; **D** effect of different carbon sources; **E** effect of different nitrogen sources)

Degradation Potentials of Isolate A3 on Diesel using GC-MS

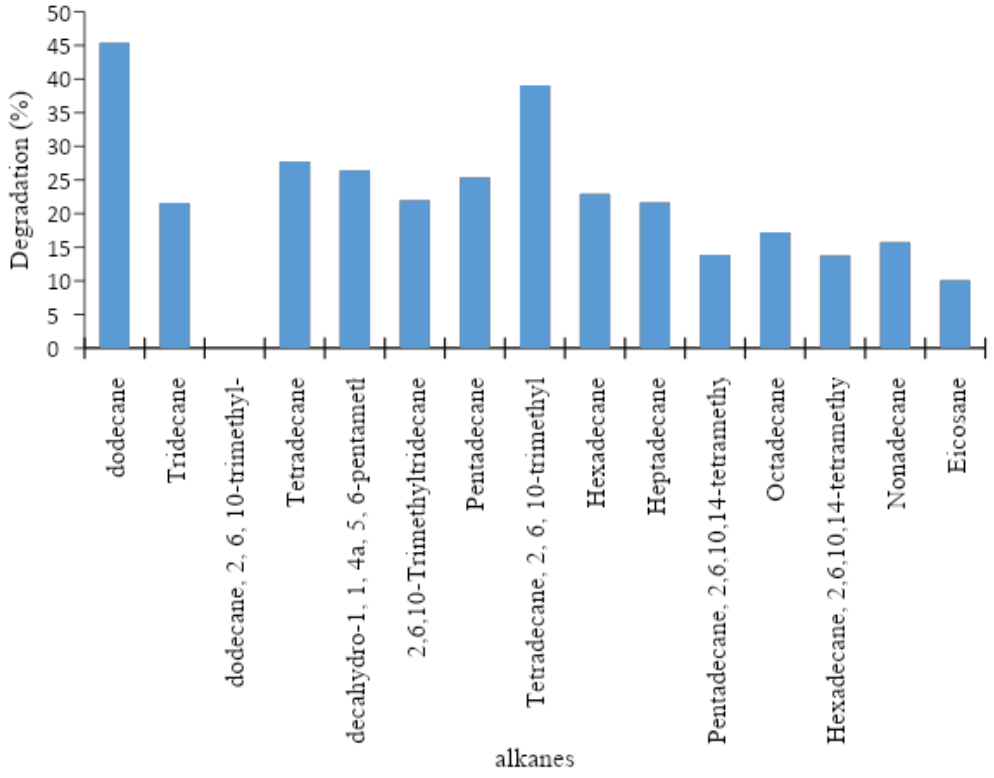

Figure 2: The isolate presented the highest degradative activity towards dodecane, 2,6, 10 Tetradecane, and Tetradecane, but was unable to degrade 2, 6, 10-trimethyl- dodecane with the best degradation percentage of 45.39% as presented.

Molecular identification of strain A3

Isolate A3 was identified using the 16S rRNA sequencing. The results of 16S rRNA sequence alignment and phylogenetic tree analysis revealed that 16S rRNA sequence of Isolate A3 was like *Pseudomonas aeruginosa* strain PAO1. The Evolutionary distance tree based on 16S rRNA sequences with their references. Bar, nucleotide difference per sequence position is shown below.

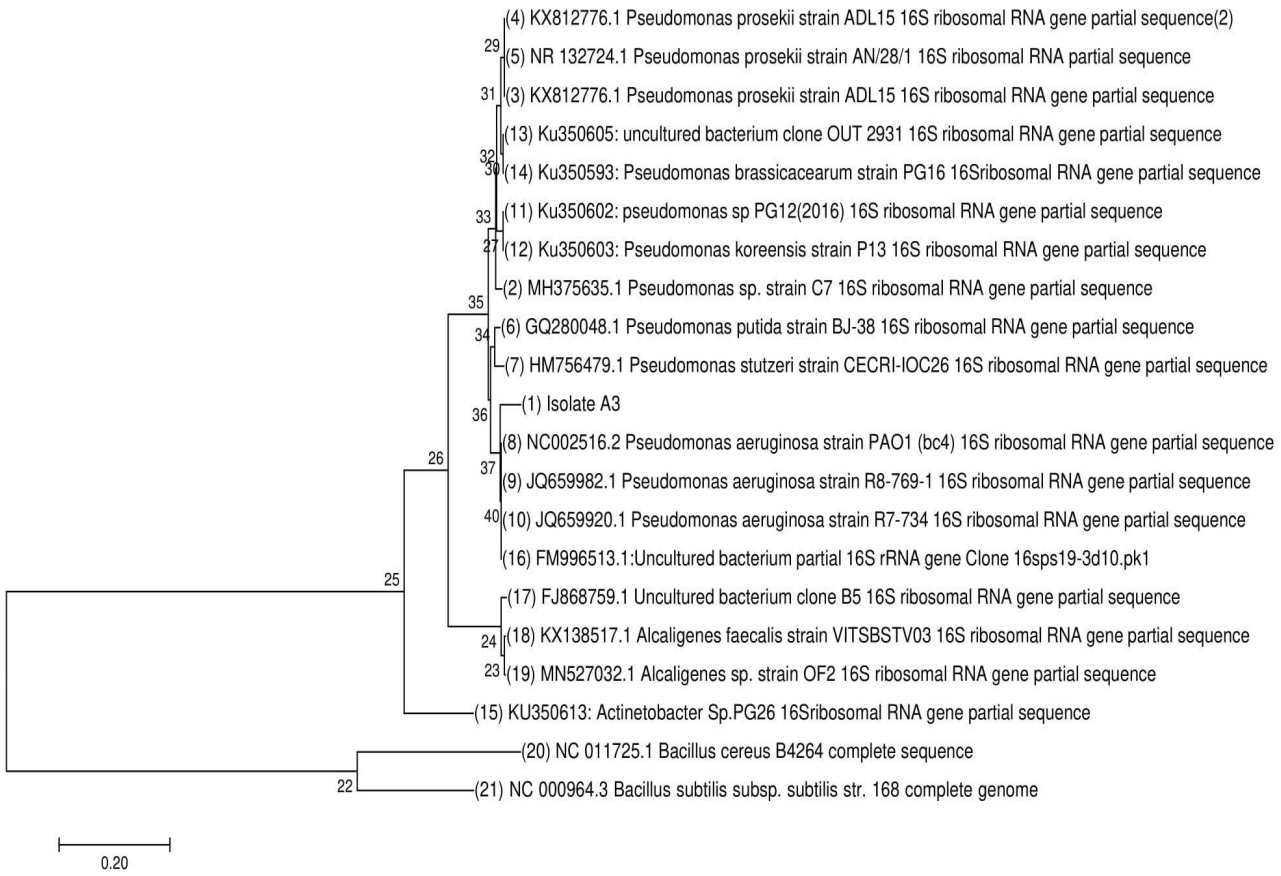

Figure 3: Comparative phylogenetic analysis of the *Pseudomonas aeruginosa* isolate A3 (MZ027605) with different *Pseudomonas* strains from GenBank using molecular evolutionary genetics analysis software version 7.0 (MEGA 7)**.**

Discussion

In this study, 17 morphological distinct bacteria strains were effectively isolated from automobile workshops in Yola. A single strain (A3) was observed to have biosurfactant and biodegradative ability towards n-alkanes present in diesel oil. The presence of a strain with both biosurfactant and biodegradative ability is not surprising as some studies conducted in hydrocarbon polluted sites have testified the presence of biosurfactant strains (Nwaguma, et al., 2016: Maria, 2017: Satpute et al., 2008). Biosurfactant strains are ubiquitously found in hydrocarbon-polluted environments (Nwaguma et al., 2016). The screening methods employed for biosurfactants were oil displacement, drop collapse, haemolytic test, emulsification and forming. The screening tests for biosurfactants were described by Maria, (2017) and Carrillo et al., (1996). The usage of this technique is comparable to the report of Ndibe et al., (2018), who used similar methods in selecting biosurfactant producers.

The effect of varying the incubation time on the capability of test isolate in diesel-rich media was investigated. The optimum incubation time was confirmed to be at 120 hrs (5 days) for the isolate (Fig 1A). The findings are comparable to that reported by Nwaguma et al., (2016) However, Patil et al., (2014) documented the optimum production of biosurfactant in 4 days using *Pseudomonas aeruginosa* F23.

The optimum temperature for strain A3 is 35^{o}C (Fig 1B). This is an indication that it is a mesophilic strain and can be effective at mild temperature (30-35 ^{o}C). Similar optimised condition has been described by numerous authors. Patil et al., (2014) reported the production of biosurfactants from *Pseudomonas aeruginosa* F23 at 30^{o}C. However, Nwaguma et al., (2016) reported biosurfactant production at temperature of 30^{o}C-35^{o}C for *klebsiella pneumonia* IVN51

from contaminated soil samples. Mnif et al., (2014) discovered that *Bacillus subtilis* SPB1 gave an optimum temperature of 30°C in the degradation of diesel. Most bacteria produce biosurfactants at different temperature ranges of 30-37^{o}C (Chander et al., 2012).

Strain A3 obtained maximum growth at pH 8 (Fig 1C). The pH optimization result for growth of isolates in the medium is consistent with that found by Nwaguma et al., (2016) who achieved maximum growth and production at pH 8 with K. *pneumoniae* IVN51. In addition, the result corresponds to the result obtained by Hamzah et al., (2013). The observed conditions of biosurfactant production by different strains are similar, even though the organisms and strains are not the same. An indication that emulsification and biosurfactant ability can be obtained effectively under these conditions.

The effect of diesel oil concentration was further evaluated. Results obtained showed that 7.5 % of diesel is the most favourable for the isolate (Figure 1D). The degradative ability of isolate A3 could not be compared with *Acinetobacter baumannii* where its degradative ability is most effective at 4% of diesel according to Palanisamy et al., (2014). It is important to note that degradative ability towards a particular substrate might vary among different bacteria due to differences in their genetic makeup.

The effect of the isolates to grow on different nitrogen sources was studied. The nitrogen sources used were urea and ammonium nitrate (NH_4NO_3). The result showed that isolates A3 has grown well on mineral salt medium amended with urea better than NH_4NO_3 and (Figure 1E). Shekhawat et al., (2014) reported the production and growth of *Bacillus sp*. with NH_4NO_3 as source of nitrogen. In another study by Karkera et al., (2012), optimum production of biosurfactants was observed when *Pseudomonas aeruginosa R2* was grown in NH_4NO_3 as a nitrogen source. The difference observed from other studies could be as a result of preferential need by individual strains.

In this work GC-MS analysis was conducted to determine the degradation potentials of isolate A3. The bacteria degrade between 10.09 % - 45.39 % of individual diesel oil alkanes. **(**Figure 2). The isolate showed the highest degradative activity towards dodecane, Tridecane, and 2,6,10, Tetradecane (Figure 2). A study has associated the hydrocarbon chain length with degradative ability (Wuyang et al., 2018). A number of researches have reported the degradation of short-chain components of diesel oil.

In fact, some isolates could multiply rapidly by degrading the short-chain component, which in turn increases the relative degradation rate of short chain alkanes (Nkem 2016; Verma et al., 2006; Chemla et al., 2013).

Conclusion

The isolated strain YLA03 studied in this work has demonstrated a strong biosurfactant ability. The strain has shown degradative potentials towards alkanes. The isolate A3, was identified as *Pseudomonas aeruginosa* (MZ027605) base on 16S rRNA molecular identification, at pH 8, 35^{o}C, and diesel concentration of 7.5% (v/v), the isolate was able degrade 10.09% - 45.39% of individual diesel alkanes in 5 days.

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